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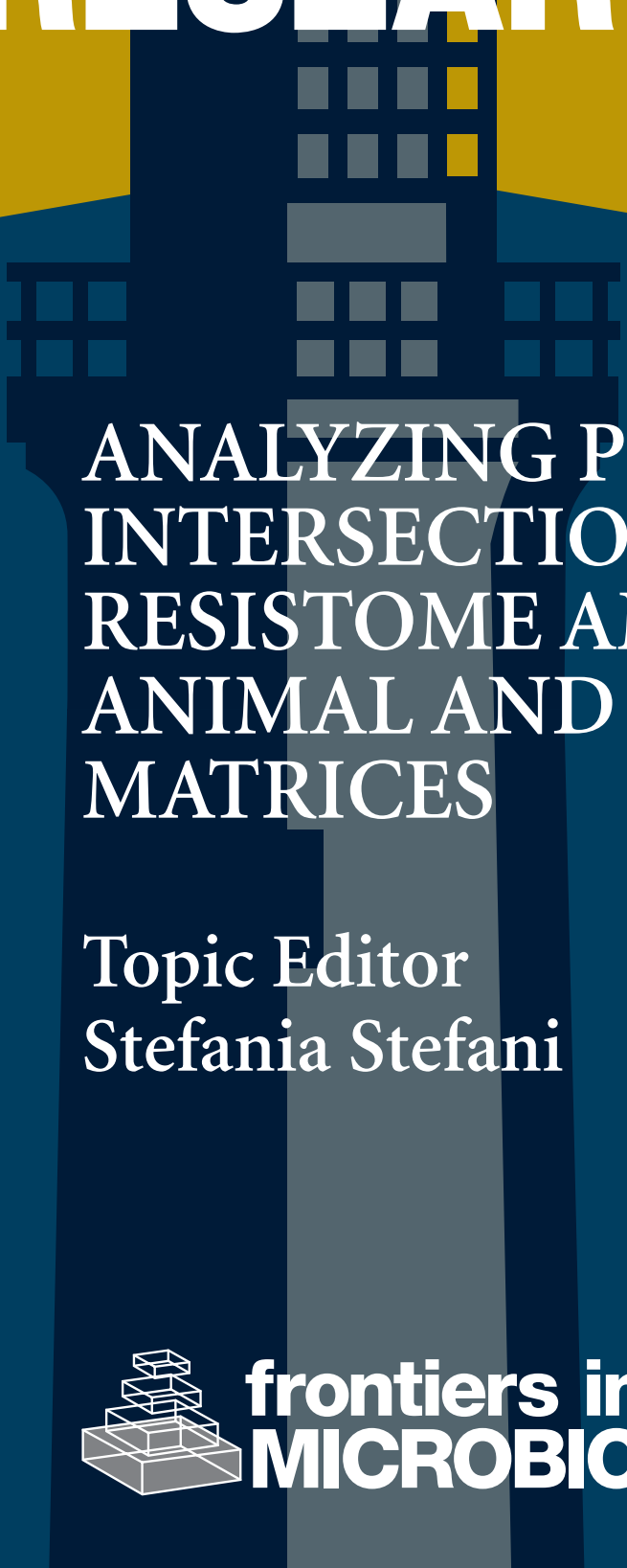

ANALYZING POSSIBLE INTERSECTIONS IN THE RESISTOME AMONG HUMAN, ANIMAL AND ENVIRONMENT MATRICES

Topic Editor
Stefania Stefani

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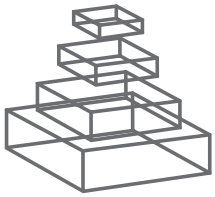

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ANALYZING POSSIBLE INTERSECTIONS IN THE RESISTOME AMONG HUMAN, ANIMAL AND ENVIRONMENT MATRICES

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Image by Stefania Stefani

The astonishing development of resistance is one of the most worrisome problems of the last 20 years. In particular, the accumulation of resistance determinants that are able to destroy different antibiotic families at the same time bringing about multi-drug resistant (MDR) or pan-drug resistant (PDR) phenotypes, is a phenomenon almost exclusively known in clinical practice, in which resistance is maintained even under a strong selective pressure. Mutations, acquisition of resistance genes by lateral gene transfer (LTG), and selection of intrinsically resistant species are at the basis of this diffused problem.

Many studies have characterized resistant bacteria, genes, mechanisms of resistance and transfer in clinical settings, as well as clarifying the role of antibiotics (both naturally produced by microorganisms and by completely synthetic processes) in driving the resistant selection, the evolution of new mechanisms and the emergence new resistant species. However, other data, until now reported in a rather sporadic way, are emerging on the possible role of animals and some specific environmental hot-spots (ground water, soil, etc) in which resistance can develop thanks to an efficient organization in clusters of genes that are then able to be selected and spread, ultimately functioning as a reservoir for further transmission to humans.

Origin of resistance genes that we now know to belong to the so-called mobilome, and their mechanism of transferability among species and in different microbiota, has to be considered a real challenge for the future evolution and antibiotic resistance in both pathogenic and opportunistic bacteria.

This Research Topic has the aim of collecting contributions from different experts in the antibiotic resistance field, covering aspects of resistance in specific microorganisms and in diverse environments.

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EDITORIAL
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Analyzing possible intersections in the resistome among human, animal, and environment matrices

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The controversial question of microbial resistance origins, i.e., if it is the result of human activity or rather a synthesis of the evolution of antibiotic biosynthetic pathways that evolved over millions of years, is still challenging, with evidence for both. However, in the last few years, a growing body of evidence suggests a role of environmental microorganisms as reservoirs of resistance genes. The concept of the antibiotic resistome predicts that resistance is the result of a dynamic process involving microbial interactions in many different environments, all these occurring before the so called "antibiotic- era." The fact that resistant microorganisms can explore a wide range of potential niches and acquire optimal adaptations for life in alternative hosts is worrisome, and could amplify their capability to acquire new determinants both in terms of virulence and resistance, with simultaneously maintaining their fitness. The variability of resistance determinants and their expression in different hosts are larger in nature than what is found in human pathogens, which implies the existence of bottlenecks modulating the transfer, spread, and stability of antibiotic resistance genes.

This research topic has collected the contributions of scientists involved in antibiotic resistance research, covering the current knowledge of single organisms and resistance determinants in various environments.

The first article, by Dr. Martínez (2012), analyzes the role of different factors that can affect the establishment of specific resistance determinants in a population of bacterial pathogens. These factors include founder effects, ecological connectivity, fitness costs, and second order selection. The Author then continues to cover housekeeping genes and human-driven contaminants.

The two papers that follow describe the possible interactions between animals and humans. The first study by Dr. Palmieri and co-authors (2011) describes the paradigmatic case of the major porcine pathogen *Streptococcus suis*, that is increasingly reported in severe infections in humans who come in contact with infected animal blood or secretions, or with pork-derived products. The available information on this microorganism, carrying many genetic elements similar to those identified in the major human pathogens *S. pyogenes* and *S. pneumoniae*, strongly suggests its role as a reservoir of resistance determinants for these important microorganisms. The second paper, by Dr. Pantosti (2012) which describes the case of livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) is different. *S. aureus* is a typical human pathogen, and the recent findings that MRSA can colonize and evolve in animals, increase our concern that this MDR and virulent microorganism is able to adapt or readapt to humans and animals without losing fitness. Dr. Pantosti (2012) describes not only the first pig-MRSA, i.e., ST398, but also other animal-adapted MRSA clones, all detected in livestock, such as ST1, ST9, and ST130, this last carrying a new *mec*A gene.

The case of ubiquitous microorganisms such as enterococci, which are incredibly successful in adapting to different hosts and environments, increases the complexity of the role of the different evolutionary forces involved. This complex problem is reviewed by Dr. Santagati and co-authors (2012). The article evaluates the host-specific traits that are characteristic of some enterococcal species and addresses the presence of common and numerous mobile genetic elements that are important forces in evolution. These are spread over diverse hosts and environments, of at least the two major species studied, i.e., *E. faecalis* and *E. faecium*.

The double life of *Acinetobacter baumannii* as commensal and extremely successful pathogen is reviewed in depth in the paper by Dr. Roca and co-authors (2012). They exhaustively go through all the different biological aspects responsible for the success of these new MDR nosocomial pathogens, today almost untreatable with all common antimicrobial agents. Next to *Acinetobacter*, Dr. Pitout (2012) describes in his contribution the incredible evolution of extra-intestinal pathogenic *E. coli* (ExPEC): virulence genes acquired by horizontal gene transfer and the acquisition of a complex array of resistance determinants have made this species, and some epidemic MDR clones, the most worrisome microorganisms among Gram-negative species.

Two papers then address the evolution of mechanisms of resistance: Dr. Cantón and co-authors (2012), take into consideration one of the most paradigmatic mechanisms of resistance to beta-lactams, i.e., the CTX-M enzymes, while Dr. Poirel and co-authors (2012) look at the possible matrix intersection of plasmid mediated quinolone resistance. CTX-M, originally detected from the environmental *Kluvyera* spp., was successfully incorporated different times to originate different CTX-M clusters by the mobilization of specialized insertion sequences associated with a multifaceted genetic structure. Selective forces, including antibiotics, have fueled diversification and evolution of all these original

clusters. Global spread was obtained after their uptake in epidemic resistance plasmids often harbored in high-risk epidemic clones. The ability to aggregate different resistance determinants (for example genes encoding different carbapenemases) makes the scenario of these pandemic MDR clones more complex.

The aquatic environment and farm animals seem to be the original source of plasmid mediated quinolone resistance, due to the action of different transferable mechanisms such as Qnr proteins, acetyltransferase AAC(6′)-Ib-cr and the efflux pumps QepA and QepB. Dr. Poirel, in the article, addresses the aquatic origin of the qnr genes.

The last two articles of this research topic are related to wildlife and bodies of water. Dr. Guenther and co-authors (2011) take the ESBL-producers *E. coli* into consideration, which was diagnosed in wild populations in Europe starting from 2006 and Dr. Lupo and co-authors (2012) review the most important mechanisms of resistance detected in water habitats and take into consideration the possible role of the bodies of water as matrices of reservoirs of resistance genes and of the spread of the mechanisms themselves.

In conclusion, MDR in all these pathogens is now widespread, resistance is pervasive and increasing in scope and impact. The antibiotic resistome has an enormous potential to provide new genes and new mechanisms; human use of antibiotics has provided the selective pressure necessary to capture, accommodate and make these complex structures functional, without affecting the bacterial fitness in diverse environments. Our knowledge on how antibiotics work and where resistance comes from is increasing: more has to be done, above all in natural environments, but for sure, resistance is natural, ancient and ineluctable.

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Bottlenecks in the transferability of antibiotic resistance from natural ecosystems to human bacterial pathogens

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It is generally accepted that resistance genes acquired by human pathogens through horizontal gene transfer originated in environmental, non-pathogenic bacteria. As a consequence, there is increasing concern on the roles that natural, non-clinical ecosystems, may play in the evolution of resistance. Recent studies have shown that the variability of determinants that can provide antibiotic resistance on their expression in a heterologous host is much larger than what is actually found in human pathogens, which implies the existence of bottlenecks modulating the transfer, spread, and stability of antibiotic resistance genes. In this review, the role that different factors such as founder effects, ecological connectivity, fitness costs, or second-order selection may have on the establishment of a specific resistance determinant in a population of bacterial pathogens is analyzed.

Keywords: horizontal gene transfer, antibiotic resistance, founder effect, fitness costs, genetic exchange community, second-order selection

INTRODUCTION

Bacterial pathogens can develop antibiotic resistance either by mutations, or by the acquisition of antibiotic resistance genes from other microorganisms through horizontal gene transfer (HGT). Since bacterial pathogens were (presumably) susceptible to antibiotics at the time of the development of these compounds, it is reasonable to think that resistance genes have been acquired from non-pathogenic microorganisms. Indeed, the analysis of *Escherichia coli* plasmids from bacterial strains isolated before and after the use of antibiotics for therapy demonstrated that the plasmid families were similar, but incorporated resistance genes after the antibiotic era (Datta and Hughes, 1983).

Since the resistance genes did not originate in bacterial pathogens, the sources for these genes would be environmental microorganisms (Martinez et al., 2009a; Davies and Davies, 2010). Indeed, the fact that most antibiotics currently used in clinics originated in environmental microorganisms (Waksman and Woodruff, 1940) led to the proposal that the origin of resistance genes are the antibiotic-producing organisms, where resistance genes may play an auto-protective role (Benveniste and Davies, 1973; Davies, 1997). Recent work indicates that indeed antibiotic-producing environmental microorganisms harbor a large number of resistance genes that could be potentially transferred to human pathogens (D'Costa et al., 2006). Nevertheless, the presence of resistance genes in the environment is not confined to antibiotic producers (Aminov, 2009). For instance, the quinolone resistance gene *qnrA* originated in the water-borne bacteria *Shewanella algae*, which is not known to produce an antibiotic (Poirel et al., 2005). Given that quinolones are synthetic drugs, the existence of these determinants indicate the antibiotic resistance genes can have disparate functions in their original hosts, in such a way that the universe of potential resistance genes that can be incorporated into mobile genetic elements is even larger than predicted from the analysis of antibiotic producers. Support for this statement is the finding of genes that contribute to intrinsic resistance in different bacterial species (Fajardo et al., 2008; Tamae et al., 2008; Girgis et al., 2009; Alvarez-Ortega et al., 2010; Liu et al., 2010) and functional metagenomic analyses indicating that the wide dissemination of a large number of resistance genes (D'Costa et al., 2006; Sommer et al., 2009) in all analyzed ecosystems (including the human gut) whether or not contaminated by human activities.

It would be expected that this diversity of resistance genes in microorganisms, that can confer an antibiotic resistant phenotype on their transfer to a new host might be mirrored by a large variability of resistance genes, acquired by HGT, in human pathogens. However, the number of different resistance determinants found among human bacterial pathogens is low in comparison to those present in the different metagenomes. This indicates that the transfer of a resistance gene from its original host to a human pathogen might be constrained by different bottlenecks, as discussed in this review.

THE TWO FACES OF ANTIBIOTIC RESISTANCE DETERMINANTS: HOUSEKEEPING GENES AND HUMAN-DRIVEN CONTAMINANTS

The existence of resistance genes in natural ecosystems, even those without any record of pollution by antibiotics was reported more than four decades ago (Gardner et al., 1969). However, detailed studies on this topic are more recent. In the last years an increasing number of studies of the presence of resistance genes in non-clinical ecosystems have been published. Several are based on non-culture methods for detecting resistance. Briefly, two different methodologies are applied; one is the search for any potential gene that confers resistance on expression in a heterologous host by using functional genomic techniques (D'Costa et al., 2006, 2011; Sommer et al., 2009). The other is the search for resistance

genes already present in human pathogens, usually by PCR, in metagenomic DNA (Koike et al., 2007). Whereas, in the first analysis the purpose is to characterize any gene that can cause resistance if transferred and hence study the potential natural resistome of the studied ecosystem, the second type of study analyses contamination by resistance genes already acquired by human pathogen.

These studies thus provide different information. Functional metagenomics serves to define novel mechanisms of resistance (potentiality, see Martinez et al., 2007), but predicting whether such mechanisms will be transferred to human pathogens is not obvious (see below). Indeed, the fact that the origin of the antibiotic resistance genes currently present in human pathogens is known in only a few cases indicates that defining the environmental resistome is a needed but not sufficient condition for predicting the emergence of resistance. It is important to note however that the finding of novel mechanisms of resistance can be a valuable tool for the design of antibiotic modifications before resistance arises (Wright, 2007; Martinez et al., 2011).

The analysis of the presence in different ecosystems (contaminated and pristine) of genes that have been already acquired by human pathogens would provide information on the stability of these elements, the reservoirs and the factors that enrich their presence in nature. These studies can be used to evaluate the risks for human health from pollution of natural ecosystems by antibiotic resistance determinants, together with antibiotics that serve as selectors of resistance themselves (Martinez, 2008, 2009). This knowledge might serve for the identification of intervention strategies to reduce the impact of anthropogenic activities on the enrichment of resistance elements, already present in mobile genetic elements (MGEs), in natural (non-clinical) ecosystems (Baquero et al., 2008). The relevance that farming and transport of food-borne animals or pets (Guardabassi et al., 2004; Aarestrup, 2005; Moreno et al., 2008), as well as the transport of goods (Ruiz et al., 2000), or human migration (Kumarasamy et al., 2010) may have for the dissemination or resistance is well known. For these processes, procedures for tracking the presence and dissemination of resistance genes might be implemented. More difficult will be the implementation of such studies for analyzing the role of wild animals in the spread of resistance (Gilliver et al., 1999; Livermore et al., 2001; Allen et al., 2010). Important in this respect is the finding of resistance in migratory birds that can disseminate both antibiotic resistance determinants and infective resistant bacteria all over the world (Middleton and Ambrose, 2005; Steele et al., 2005; Simoes et al., 2010).

Between these types of studies are functional analyses on the resistance mobilome, those resistance genes that are already present on mobile elements, irrespective of whether or not they have been acquired by human pathogens. The transfer of a potential resistance gene from the chromosome of an environmental bacterium to a human pathogen requires it to be mobilizable after its capture by a translocative element and its integration in an MGE. This means that once the resistance element has been incorporated in a mobile element, the possibility of its acquisition by a human pathogen can be high, especially if this element is present in the human bacterial population. Unfortunately, studies on the environmental resistance mobilome are difficult and still rare (Szczepanowski et al., 2008, 2009; Moura et al., 2010; Parsley et al., 2010).

ECOLOGICAL CONNECTIVITY

The first requirement for the transfer of a resistance gene is that both the donor and the receptor share the same habitat. In the case of pathogenic bacteria, the pathogens need not co-exist with the donor, because a chain of microorganisms may link the donor and the recipient. However, since acquiring resistance genes might confer a fitness cost (see below), the establishment of a successful gene-transfer chain is possible only with positive selection for the resistance determinant. In other words, unless resistance is selected (mainly by antibiotics), it is unlikely that MGEs containing resistance genes will be fixed in the populations of environmental microorganisms en route to human pathogens. Since the natural concentrations of antibiotics in non-clinical ecosystems are much lower than at hospitals (Davies, 2006), only in the case of pollution by antibiotics (aquaculture, waste disposal from cities, farms, or industries) a positive selection for MGEs containing resistance genes can be envisaged. Following this reasoning, it has been proposed that the possibility of a given resistance gene being transferred to a human pathogen will largely depend on whether the habitat where the donor micro-organism is present close to human-linked ecosystems (Baquero et al., 2009). For instance, it would be rare for resistance genes found in deep soil allocations (Brown and Balkwill, 2009) or at a glacial ice core (Miteva et al., 2004) would be transferred to human pathogens. In contrast, it has been suggested that ecosystems such as waste-water treatment plants or farms, where human pathogens and environmental bacteria co-exist in the presence of contaminating antibiotic residues, might be hot-spots for the acquisition of resistance genes by bacterial pathogens (Baquero et al., 2008; Aminov, 2011).

Ecological connectivity is not restricted to the spatial distribution of microorganisms. For those microorganisms sharing the same ecosystem, some of them are more prone to exchange genetic material than others. The organisms that can share genes, have been named as genetic exchange communities (Jain et al., 2003). As stated in (Skippington and Ragan, 2011), "gene exchange communities can vary widely in spatial extent, taxonomic diversity, density of internal connectivity, and involvement of vector types." These communities usually share some plasmid (or transposon) types and do not possess strong restriction/modification systems that would impede the interchange of DNA. As a consequence, the entrance of a resistance gene, located in a proficient MGE into a well established gene exchange community might allow its spread among different organisms and consequently fixation in populations of bacterial pathogens. This spread will be modulated by specific fitness costs that preclude the stability of the gene in some bacterial species.

FOUNDER EFFECT

By founder effect, we refer to the situation in which the first gene to arrive is the one to win (Baquero et al., 2009). When there are several resistance determinants with a similar substrate profile, usually one prevails once transferred to human bacterial pathogens. As we will discuss later, this situation can be the consequence of differential fitness costs, nevertheless, a certain

degree of serendipity might be the basis of the successful transfer, spread and fixation of a given resistance determinant. One example of this situation is the TEM-1 beta-lactamase, which, followed by SHV-1 and OXA enzymes, has been the predominant plasmid-encoded beta-lactamase in *Enterobacteriaceae* for many years (Simpson et al., 1980; Medeiros, 1997). The TEM-1 beta-lactamase was acquired soon after the introduction of the first generation of beta-lactams for therapy, and plasmids coding this beta-lactamase spread rapidly among bacterial pathogens. The study of several different ecosystems has shown that there exist a large number of beta-lactamases nearly everywhere, which can confer resistance to the same antibiotics as TEM-1. Why then, has TEM-1 prevailed in these pathogen populations? Antibiotic resistance genes are acquired and maintained because of the strong selective pressure of antibiotics. Once bacteria have acquired a determinant that allow them to resist antibiotics, there is not a selection pressure for replacement of the determinant already present in bacterial populations. This situation can change if the selective pressure is altered, for example when new antibiotics are launched into clinical use (Livermore, 2009; Salverda et al., 2010). Indeed, the introduction of beta-lactamase inhibitors and novel beta-lactams for which TEM-1 presented low activity generated two different processes: (i) evolution of the TEM-enzyme that most likely occurred in clinical settings when bacterial pathogens were exposed to the novel selective pressure (ii) acquisition of novel beta-lactamase coding genes by human pathogens with novel substrate profiles.

FITNESS COSTS

It is generally assumed that the acquisition of an antibiotic resistance determinant confers a fitness cost (Andersson and Levin, 1999), meaning that in the absence of selection, resistant bacteria will be outcompeted by the susceptible ones. In the case of genes acquired by HGT, these costs might be the consequence of the metabolic load imposed by the replication, transcription, and translation of the novel genetic elements. If this was the unique cause of fitness costs, the disadvantage of carrying one or another resistance gene will be similar and the fitness cost would not constitute a relevant bottleneck in selecting one resistance determinant over another. However, different studies have shown that, at least on occasion, the introduction of a given resistance gene does not impose a non-specific metabolic burden but leads to specific changes in bacterial physiology. This may be the case for AmpC beta-lactamase genes, which are infrequently found on *Salmonella* plasmids unless the plasmid also harbors the repressor of their expression (Verdet et al., 2000) or elements that compensate the biological costs associated to AmpC expression (Hossain et al., 2004). It has been found that AmpC alters the physiology of *Salmonella*, decreasing its virulence and hence a differential fitness cost that decreases the probability of dissemination of specific gene among *Salmonella* strains (Morosini et al., 2000). This example indicates that the fitness costs can be gene-specific and do not necessarily derive from a general metabolic burden. In this context, those resistance determinants conferring high fitness costs are unlikely to be fixed in bacterial populations because they would be outcompeted by other resistance determinants which lower fitness costs (Martinez et al., 2011).

This reasoning must be however modified by the chances of acquiring compensatory mutations (Andersson and Hughes, 2011; Martinez et al., 2011). If a resistance determinant confers high fitness costs, but compensatory mutations are easily selected, the probability of being maintained in bacterial pathogens is high. In such cases, the location of the mutations is a relevant issue. If the compensatory mutations occur in the chromosome, not in the MGE, the acquisition of the resistance gene by a new host implies a new fitness cost, and as a consequence the spread of the resistance determinant will be compromised. However, it the mutation occurs in the MGE, the chances for spread will be enhanced.

SECOND-ORDER SELECTION AND MAINTENANCE OF RESISTANCE GENES IN THE ABSENCE OF SELECTION

If the acquisition of resistance confers fitness costs it is logical to suppose that resistant organisms will be outcompeted by their susceptible, fitter counterparts in the absence of selection. However, some resistant strains present no-cost (Rozen et al., 2007; Balsalobre and De La Campa, 2008) and even some resistance determinants can be beneficial under certain conditions (Alonso et al., 2004; Maughan et al., 2004; Luo et al., 2005; Perkins and Nicholson, 2008; Michon et al., 2011). Finally, some fitness costs can be compensated by mutations that do not impede to keep resistance (Bjorkman et al., 1998; Maisnier-Patin et al., 2002; Paulander et al., 2007; Lind et al., 2010; Shcherbakov et al., 2010). This indicates that reversing resistance once established can be a difficult task (Andersson and Hughes, 2010).

Maintenance of resistance genes in habitats without a strong antibiotic pressure is favored as well by second-order selection processes. Antibiotic resistance genes on MGEs are usually in association to other resistance determinants. This means that the selection for one antibiotic will select for the whole array of resistance genes present in this specific MGE. Furthermore, MGEs besides resistance genes may carry other elements such as heavy-metal resistance determinants (Baker-Austin et al., 2006), or genes coding for production of siderophores, toxins, or bacteriocins (De Lorenzo and Martinez, 1988; Clewell, 1990; Herrero et al., 2008); these can confer an ecological advantage in some ecosystems and thus co-select resistance in the absence of antibiotics. Cross-selection might also be a relevant second-order process that allows maintenance of resistance in the absence of selection. Certain resistance determinants, such as multidrug (MDR) efflux pumps confer resistance to different compounds (antibiotics, biocides, or heavy metals; Martinez et al., 2009b). This means that selection with the biocide or the heavy metal might result in cross-resistance to the antibiotic (Hernandez et al., 1998; Sanchez et al., 2005).

A final mechanism for the maintenance of resistance is based on the inherent systems for plasmid stability. Plasmids frequently encode toxin/antitoxin systems, which provoke death of bacteria that lose the plasmid (Hayes, 2003; Hayes and Van Melderen, 2011). If one such plasmid incorporates an antibiotic resistance determinant, the probabilities for its maintenance will be high.

Taking these considerations into account, resistance genes might evade elimination (Andersson and Hughes, 2011) in the absence of antibiotics; indeed, resistance determinants present in human pathogens have been found on identical MGEs, in

antibiotic-pristine habitats (Pallecchi et al., 2008) such as wild animals (Gilliver et al., 1999; Livermore et al., 2001) and primitive human populations without any known exposure to antibiotics (Grenet et al., 2004; Bartoloni et al., 2009). These observations indicate that anthropogenic activity has enriched for a small number of resistance genes in natural ecosystems and that this type of pollution will be difficult to eradicate (Salyers and Amabile-Cuevas, 1997; Martinez, 2009). Supporting this notion, analyses of soils sampled in The Netherlands from 1940 to 2008, reveal that the abundance of different antibiotic resistance genes increased during this period. This, in spite of the fact that restrictions on non-therapeutic use of antibiotics in agriculture and in waste management procedures have been strongly enforced (Knapp et al., 2010).

CONCLUSION

Research on antibiotic resistance has been mainly focused on bacterial pathogens isolated from infections or in clinical settings. However, the fact that HGT-acquired genes originated in natural, non-clinically relevant microorganisms and that the first step in the transfer of resistance likely occurs in natural ecosystems emphasizes the need to analyze resistance in non-clinical ecosystems. Furthermore, the constant release of antibiotic resistance determinants already present in MGEs located in human pathogens, and in some circumstances associated with selective concentrations of antibiotics, may disrupt natural microbiota, which then serve as reservoirs for resistance genes. Non-culture based methods have demonstrated their value for the analysis of resistance in natural ecosystems. Among them, functional metagenomics provides the means to identify novel mechanisms of resistance independently of whether they will be acquired by bacterial pathogens. On the other hand, PCR analyses for specific genes serve to define reservoirs and to study elements like pollution in the dissemination and maintenance of resistance. Functional metagenomic studies indicate that very few among the resistance genes present in nature have been transferred to human pathogens. Whereas the founder effect can provide stochasticity to these acquisitions, other factors such as fitness costs, ecological connectivity, which includes the formation of gene exchange communities, are relevant bottlenecks that serve to modulate the acquisition of resistance genes by animal or human pathogens.

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Streptococcus suis, an emerging drug-resistant animal and human pathogen

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Streptococcus suis, a major porcine pathogen, has been receiving growing attention not only for its role in severe and increasingly reported infections in humans, but also for its involvement in drug resistance. Recent studies and the analysis of sequenced genomes have been providing important insights into the *S. suis* resistome, and have resulted in the identification of resistance determinants for tetracyclines, macrolides, aminoglycosides, chloramphenicol, antifolate drugs, streptothricin, and cadmium salts. Resistance gene-carrying genetic elements described so far include integrative and conjugative elements, transposons, genomic islands, phages, and chimeric elements. Some of these elements are similar to those reported in major streptococcal pathogens such as *Streptococcus pyogenes, Streptococcus pneumoniae,* and *Streptococcus agalactiae* and share the same chromosomal insertion sites. The available information strongly suggests that *S. suis* is an important antibiotic resistance reservoir that can contribute to the spread of resistance genes to the above-mentioned streptococci. *S. suis* is thus a paradigmatic example of possible intersections between animal and human resistomes.

Keywords: *Streptococcus suis*, zoonotic pathogen, resistome, integrative and conjugative element, transposon, genomic island, phage, chimeric element

The massive use of antibiotics by the livestock industry, either for growth promotion (a practice recently banned in Europe) or for prophylaxis and therapy, contributed to the emergence, and spread of antibiotic resistance (McEwen and Fedorka-Cray, 2002). Antimicrobials for veterinary use are the same or belong to the same classes as those for human use and exert a constant pressure on the animal microflora, selecting for resistance (Witte, 1997). *Streptococcus suis*, an emerging zoonotic pathogen, has been receiving growing attention for its involvement not only in severe and increasingly reported human infections, but also in drug resistance. Here we outline the current knowledge about the *S. suis* resistome and assess its role as a possible reservoir of antibiotic resistance determinants for streptococcal pathogens.

S. SUIS AND *S. SUIS* INFECTIONS

Streptococcus suis is a major porcine pathogen worldwide, endemic in all countries where intensive pig farming is practiced. Moreover, it is increasingly being isolated from mammalian species other than pigs, from birds, and from the environment (Gottschalk et al., 2010). In humans, *S. suis* is now considered as an emerging zoonotic pathogen, causing systemic infection: meningitis with possible residual deafness or vestibular dysfunctions is the most frequent clinical presentation; endocarditis, cellulitis, peritonitis, rhabdomyolysis, arthritis, spondylodiscitis, pneumonia, uveitis, and endophthalmitis have also been reported (Lun et al., 2007; Wertheim et al., 2009a). The human infection is mainly an occupational disease that may affect those who come into contact with animal infected blood or secretions or with pork-derived products. Thirty-three capsular serotypes are currently recognized (Staats et al., 1997; Hill et al., 2005). Serotype 2 is the most virulent and is responsible for severe infections in both swine and humans worldwide.

After the first reported human case of *S. suis* infection in Denmark in 1968 (Perch et al., 1968), sporadic cases (mainly of meningitis) have been reported in Europe and South-East Asia in the following decades (Lun et al., 2007; Wertheim et al., 2009a). In 2005, a severe epidemic caused by *S. suis* serotype 2 broke out in China's Sichuan Province, preceded by a small outbreak in 1998 (Yu et al., 2006). From 2005 onward, an increasing number of *S. suis* human infections have been reported worldwide, also in countries where the infection had been rarely or never reported before (Gottschalk et al., 2010). Currently, the majority of cases occur in South-East Asia where *S. suis* is a leading cause of adult meningitis, in particular in Vietnam (Mai et al., 2008; Wertheim et al., 2009b). A multilocus sequence typing scheme (King et al., 2002) disclosed a high genetic diversity of *S. suis* isolates with over 250 sequence types (ST) identified (http://ssuis.mlst.net). Four major ST clonal complexes (CC; CC–ST1, CC–ST16, CC–ST25, and CC–ST27) dominate the population; CC–ST1, the most virulent, includes ST1, found throughout the world, and ST7, responsible for the Chinese epidemic (Ye et al., 2006). It has been suggested that the increase in human cases may also reflect the recent awareness of *S. suis* as an emerging agent of meningitis and the improvement of microbiological diagnostic techniques (Gottschalk et al., 2010). Actually, the fact that *S. suis* does share several characteristics with other bacteria causing meningitis can result in misdiagnosis. Indeed, many isolates that had originally been identified as *Streptococcus pneumoniae,* enterococci, *Streptococcus bovis,* viridans streptococci, or even *Listeria* spp. were re-identified as *S. suis* in retrospective studies. In addition, recent

serologic data suggest that human infection occurs more frequently than previously believed (Smith et al., 2008). Altogether these findings suggest that *S. suis* disease has been under diagnosed in the past. The growing interest in this pathogen is reflected by recent (2007–2011) whole-genome sequencing studies. So far, eight strains have been sequenced (Chen et al., 2007; Holden et al., 2009; Ye et al., 2009; Hu et al., 2011a,b), showing that ∼40% of the ∼2 Mb genome is unique. This suggests that *S. suis* is phylogenetically distinct from other *Streptococcus* species whose genome sequences are currently available. Intraspecies genomic comparisons have shown high levels of sequence conservation. However, these data may be influenced by the clonal relatedness of the sequenced strains, six of eight being serotype 2 strains belonging to the CC–ST1.

CURRENT KNOWLEDGE OF THE *S. SUIS* RESISTOME

High rates of *S. suis* resistance to tetracyclines (up to >90%) and macrolides (up to >70%) have been reported in pig isolates worldwide (Wisselink et al., 2006; Hendriksen et al., 2008; Zhang et al., 2008; Princivalli et al., 2009). In the 1990s, a retrospective study of historic pig isolates in Denmark demonstrated that the increase in tetracycline and macrolide resistance had begun in the early 1980s (Aarestrup et al., 1998). Resistances to tetracyclines and macrolides in human strains were first reported in the second half of the first decade of 2000, but retrospective studies showed that they were already widespread in previous decades (Ye et al., 2008; Chu et al., 2009; Hoa et al., 2011). The genetic basis of tetracycline and macrolide resistance in *S. suis* has been extensively investigated.

Tetracycline resistance in streptococci is mainly due to ribosomal protection genes *tet*(M) and *tet*(O), and less frequently *tet*(Q), *tet*(T), and *tet*(W), and to efflux genes *tet*(K) and *tet*(L) (Chopra and Roberts, 2001; Roberts, 2005). Until a few years ago, *tet*(M) and *tet*(O) were the only tetracycline resistance determinants reported in *S. suis*; further determinants have lately been detected, such as ribosomal protection genes *tet*(W) and mosaic *tet*(O/W/32/O) and efflux genes *tet*(L), *tet*(B), and *tet*(40). Of these, *tet*(O/W/32/O), *tet*(B), and *tet*(40) had never been described in the genus *Streptococcus* before. *tet*(W) is an emerging tetracycline resistance determinant whose host range, including Gram-positive and Gram-negative, aerobic and anaerobic bacteria, is second only to that of *tet*(M) among ribosomal protection *tet* genes (Roberts, 2005). In *S. suis, tet*(W) was first detected in 2008, in an isolate from a case of meningitis in Italy (ST1 strain SsCA) (Manzin et al., 2008); it was subsequently described in other Italian strains (ST1 human strain SSUD and three pig isolates) (Princivalli et al., 2009), in the sequenced genome of the Chinese ST1 human strain GZ1 (Ye et al., 2009), and in two Vietnamese human isolates (Hoa et al., 2011). *tet*(O/W/32/O) is a new mosaic gene reported in 2009 in clonally unrelated pig isolates of *S. suis* (Princivalli et al., 2009). Mosaic *tet* genes are a recently discovered class of hybrids of ribosomal protection genes (Thaker et al., 2010). Mosaic derivatives of *tet*(O) and *tet*(W) were first detected in 2003 in anaerobic Gram-negative *Megasphaera elsdenii* from swine intestine (Stanton and Humphrey, 2003). Other mosaic genes, also including portions of *tet*(32), were later detected in *Clostridium difficile* (Patterson et al., 2007) and *Clostridium saccharolyticum* (Kazimierczak et al., 2008). *tet*(L), commonly carried in streptococci by small transmissible plasmids (Chopra and Roberts, 2001), has recently been detected in a sequenced genome of *S. suis*, where it is carried by a Tn*916*-like element (Holden et al., 2009), and lately, still outside plasmids, in Vietnamese human isolates (Hoa et al., 2011). *tet*(B), which had never been described in Gram-positive bacteria, was detected in 2010 in Chinese pig isolates of *S. suis* (Chander et al., 2011). *tet*(40) is a novel efflux gene recently detected in *C. saccharolyticum* in tandem with a mosaic *tet* gene [*tet*(O/32/O)] (Kazimierczak et al., 2008).

Macrolide resistance in streptococci is mainly due to methylase-mediated target site modification by *erm* genes and to active efflux by *mef* genes. *erm*(B), the old-established *erm* determinant in streptococci, can be expressed either constitutively or inducibly and is usually associated with high-level resistance; *erm*(TR), an *erm*(A) subclass, is normally inducible and is widely distributed in *S. pyogenes* isolates; *erm*(T) was detected in inducibly erythromycin-resistant isolates of group D streptococci and in *S. pyogenes* (Varaldo et al., 2009), and more recently in *Streptococcus dysgalactiae* subsp. *equisimilis* (Palmieri et al., 2011b). *mef*-class genes, which include some variants, are associated to a low-level resistance pattern affecting, among MLS antibiotics, only 14- and 15-membered macrolides (M phenotype) (Sutcliffe et al., 1996). *mef*(A) and *mef*(E) are widespread in *S. pyogenes* and *S. pneumoniae,* respectively, but they are also common in other streptococcal species (Varaldo et al., 2009). In *S. suis*, while *mef*(A) (Martel et al., 2003; Chu et al., 2009) and *mef*(E) (Hu et al., 2011a,c) have only occasionally been reported, *erm*(B) is found in >90% of macrolide-resistant pig isolates (Martel et al., 2001, 2003; Princivalli et al., 2009; Hoa et al., 2011); it has recently been reported also in human isolates (Manzin et al., 2008; Holden et al., 2009; Princivalli et al., 2009; Hoa et al., 2011). Although no further macrolide resistance determinants have been documented, the isolation of macrolide-resistant strains negative for the above genes (Martel et al., 2003; Princivalli et al., 2009; Hoa et al., 2011) suggests that other genetic determinants or target site mutations may occur.

Strains resistant to other antibiotics, such as β-lactams, aminoglycosides, trimethoprim–sulfamethoxazole, chloramphenicol, and fluoroquinolones, have been reported. The genetic basis of resistance to these antibiotics has only occasionally been investigated. Penicillin resistance was first reported in a human isolate in UK in 1980 (Shneerson et al., 1980) and more recently among pig isolates (Marie et al., 2002; Higgins and Gottschalk, 2005; Huang et al., 2005; Zhang et al., 2008). Penicillin-binding protein modifications (altered molecular weight and/or decreased affinity for penicillin) are involved in the resistance mechanism (Cain et al., 1995). Quite recently, resistance also to third-generation cephalosporins has been reported (Hu et al., 2011c). Aminoglycoside resistance has frequently been reported in *S. suis* (Touil et al., 1988; Wasteson et al., 1994; Marie et al., 2002; Tian et al., 2004; Wisselink et al., 2006; Hendriksen et al., 2008). Recently, genes coding for resistance to kanamycin [aminoglycoside-3′-phosphotransferase (*aphA*)] and to streptomycin [aminoglycoside-6′-adenyltransferase (*aadE*)] have been detected in multiresistant strains (Chen et al., 2007; Holden

et al., 2009; Hu et al., 2011a; Palmieri et al., 2011a). Resistance to antifolate drugs has frequently been reported (Mengelers et al., 1989; Wisselink et al., 2006; Zhang et al., 2008). A dihydrofolate reductase (*dhfr*) resistance gene has been detected in the sequenced strain BM407 (Holden et al., 2009). Chloramphenicol resistance has rarely been reported (Takamatsu et al., 2003). However, a recent increase in chloramphenicol-resistant strains has been noted among human isolates in Vietnam (Hoa et al., 2011). Fluoroquinolone resistance has occasionally been described (Aarestrup et al., 1998; Escudero et al., 2007, 2011; Hendriksen et al., 2008; Hu et al., 2011c). Resistance is associated with single point mutations in the quinolone resistance-determining regions of ParC and GyrA (Escudero et al., 2007; Hu et al., 2011c), but a novel efflux pump has recently been described (Escudero et al., 2011).

CONTRIBUTION OF EXOGENOUS GENETIC ELEMENTS TO THE *S. SUIS* RESISTOME

Recent studies (Li et al., 2011; Palmieri et al., 2011a,c) and the analysis of sequenced genomes (Chen et al., 2007; Holden et al., 2009; Ye et al., 2009; Hu et al., 2011a,b,c) have provided significant insights into the *S. suis* resistome, leading to the identification of several genetic elements carrying resistance determinants for tetracyclines, macrolides, aminoglycosides, chloramphenicol, antifolate drugs, streptothricin, and cadmium salts. These elements, that include integrative and conjugative elements (ICEs), transposons, genomic islands (GIs), phages, and chimeric elements, are illustrated in **Figure 1**. A scheme of their integration sites into the *S. suis* core chromosome is shown in **Figure 2**.

Five ICEs have been described in the sequenced genomes of ST7 (98HAH33, 05ZYH33, and SC84) and ST1 (BM407) human strains (Chen et al., 2007; Holden et al., 2009). Of these, four – ICE$_{Ssu98HAH33}$, ICE$_{Ssu05ZYH33}$, and ICE$_{SsuSC84}$ (~90 kb, three virtually identical elements), and ICE$_{SsuBM407.2}$ (~80 kb) – are closely related, except for cargo genes, to ICE$_{Sde3396}$ of *S. dysgalactiae* subsp. *equisimilis* (Davies et al., 2009): they are integrated immediately downstream of the 50S ribosomal gene L7/L12 (*rplL*), they harbor a tyrosine family integrase, and share an almost identical set of genes for the conjugative machinery. ICE$_{Ssu98HAH33}$, ICE$_{Ssu05ZYH33}$, and ICE$_{SsuSC84}$ bear three distinct cargo regions: (i) a putative bacteriocin biosynthesis cluster that is disrupted by a putative integron containing the *aadE* gene; (ii) a Tn*916* transposon, regularly carrying *tet*(M); and (iii) a cluster of genes associated with lantibiotic export/resistance. ICE$_{SsuBM407.2}$ contains three distinct cargo regions: (i) a Tn*916*-like element carrying,

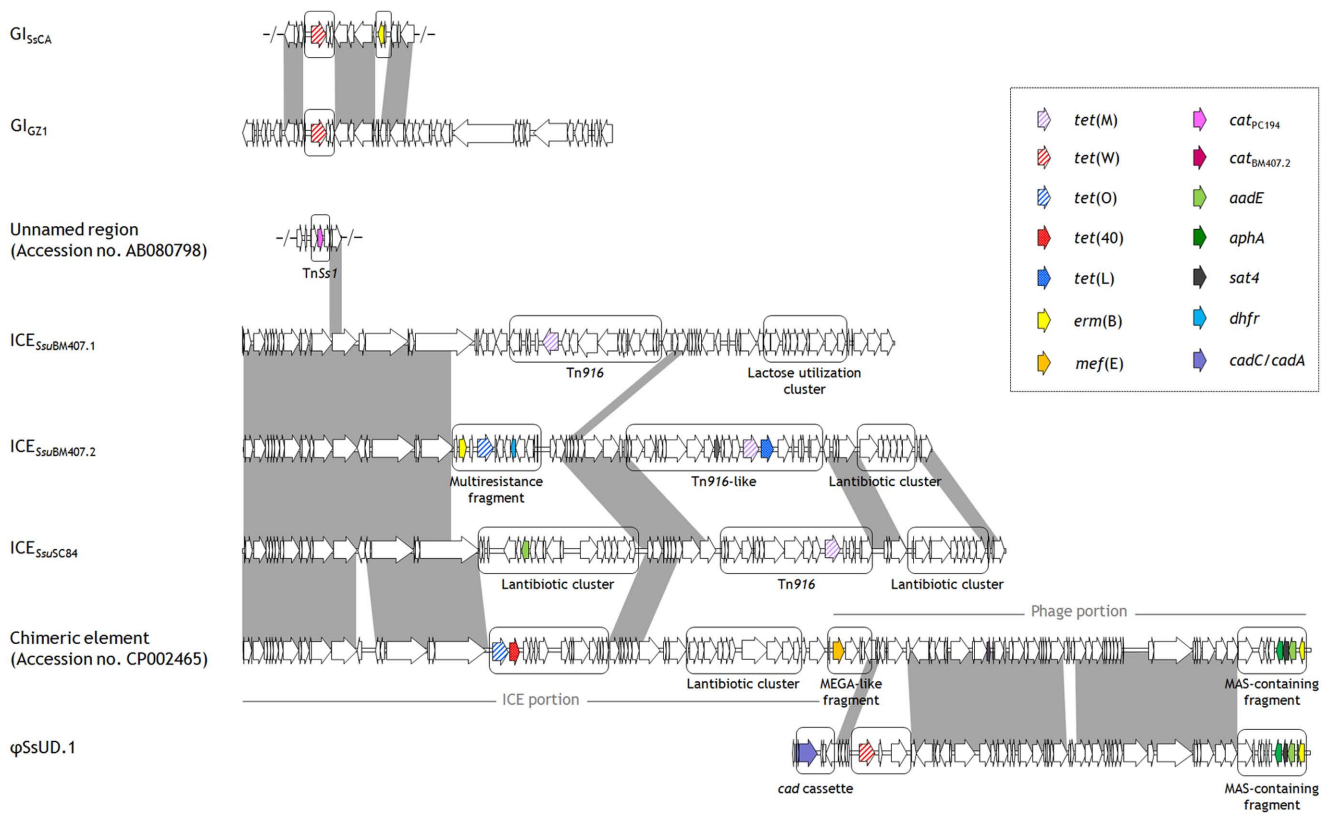

FIGURE 1 | ORF maps and genome organization of recognized *S. suis* genetic elements carrying resistance genes. ICE$_{SsuSC84}$ was chosen as a representative of the ICEs of ST7 strains. The ORFs, indicated as'arrows pointing in the direction of transcription, are depicted as white arrows, except for resistance genes which are specified in the box. Cargo regions are enclosed in rounded rectangles. Gray areas between ORF maps denote >90% DNA identity (identities between cargo regions are not shown).

besides *tet*(M), *tet*(L) and a novel chloramphenicol acetyltransferase (*cat*) gene; (ii) a multiresistance fragment bearing *tet*(O), *erm*(B), and a *dhfr* gene; and (iii) a lantibiotic cluster similar to the one mentioned above. The fifth ICE ($ICE_{SsuBM407.1}$) displays a similar scaffold but contains a different integrase, belonging to serine recombinase family, which directs the insertion of this element into a luciferase-like monooxygenase gene (Holden et al., 2009). $ICE_{SsuBM407.1}$ contains two cargo regions: (i) a Tn*916* transposon, regularly carrying *tet*(M), and (ii) a lactose utilization cluster. Structural and compositional analysis strongly suggests that all five ICEs have the potential to undergo excision and transfer. In particular, $ICE_{Ssu05ZYH33}$ was transferred at high frequency to *S. suis* recipients in conjugation assays (Li et al., 2011). Another ICE ($ICE_{Ssu32457}$, ∼56 kb), recently identified in a pig isolate in Italy and shown to carry *tet*(O/W/32/O), is the first reported genetic support for a mosaic *tet* gene in streptococci (Palmieri et al., 2011c). $ICE_{Ssu32457}$ is transferable to *S. suis, S. pyogenes, S. agalactiae,* and *S. pneumoniae*, and similar to $ICE_{Ssu98HAH33}$, $ICE_{Ssu05ZYH33}$, $ICE_{SsuSC84}$, and $ICE_{SsuBM407.2}$ mentioned above, it is closely related to $ICE_{Sde3396}$ of *S. dysgalactiae* subsp. *equisimilis* except for cargo genes (Palmieri et al., submitted).

A *tet*(W)-carrying ∼47 kb GI (here designated GI_{GZ1}), located immediately downstream of the lysyl-tRNA synthetase chromosomal gene (*lysS*), has been identified in the sequenced genome of the Chinese strain GZ1 (Ye et al., 2009). An almost identical, non-transferable, *tet*(W)-carrying GI (GI_{SsCA}), differing from GI_{GZ1} only for the presence of an *erm*(B)-containing insertion, has recently been detected in an Italian human strain (Palmieri et al., 2011a).

In another Italian human strain, the presence of *tet*(W) has been reported in a phage (φSsUD.1, ∼61 kb) (Palmieri et al., 2011a). φSsUD.1 carries a unique combination of antibiotic and heavy metal resistance genes resulting from the presence, besides *tet*(W), of an *erm*(B)-containing MAS (macrolide–aminoglycoside–streptothricin) – like fragment and a *cadC/cadA* cadmium efflux cassette. The MAS-like fragment closely resembles the one recently described in the pneumococcal transposons Tn*6003* and Tn*1545* (Cochetti et al., 2007, 2008). The resistance genes fitting in the φSsUD.1 phage scaffold differ from, but are in the same position as, the cargo genes carried by *S. pyogenes* phages such as φ10394.4 (Banks et al., 2003) and φm46.1 (Brenciani et al., 2010). φSsUD.1 is integrated at the 3′ end of a conserved RNA uracil methyltransferase (*rum*) gene and is transferable to *S. pyogenes.*

A ∼124 kb chimeric element constituted of two portions, an ICE (∼69 kb) and a phage (∼55 kb), has recently been detected in the sequenced genome of JS14 (Hu et al., 2011a), integrated at the 3′ end of the *rum* gene. The ICE portion harbors a site-specific integrase, it displays a scaffold similar to the *S. suis* ICEs mentioned above, and contains two cargo regions, one bearing *tet*(O) in tandem with *tet*(40) and the other a bacteriocin gene cluster. The phage portion displays the typical modular organization of tailed phages, and closely resembles φSsUD.1 (Palmieri et al., 2011a). The right end of the phage portion bears an *erm*(B)-containing MAS-like fragment, like φSsUD.1; the left end bears a mega-like genetic structure, similar to the *mef*(E)-carrying mega element originally described in *S. pneumoniae* (Gay and Stephens, 2001), in the same position as the *cadC/cadA* cassette in φSsUD.1.

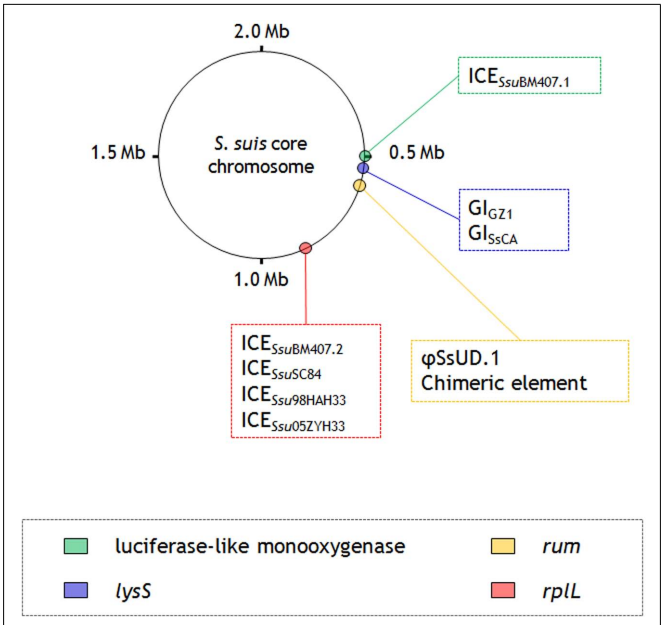

FIGURE 2 | Schematic illustration showing the *S. suis* core chromosome with the insertion sites of recognized genetic elements carrying resistance genes.

Beside the Tn*916*-family elements, only another transposon carrying resistance genes, Tn*Ss1*, has been described in *S. suis* (Takamatsu et al., 2003). It contains a *cat* gene showing 97% identity with cat_{PC194} (Widdowson et al., 2000) flanked by direct repeats of an IS6-family element.

CONCLUDING REMARKS

Although the emergence of *S. suis* as a human pathogen has caused a flurry of research, current knowledge on the *S. suis* resistome is still fairly sketchy. This is mainly due to the so far limited number of studies of the genetic basis of resistance and to the redundancy of data from the strains sequenced, many of which are clonally related. Next-generation DNA sequencing will probably provide a wealth of new data in the next few years. The available information suggests that *S. suis* may contribute to the spread of antibiotic resistance genes to streptococcal human pathogens such as *S. pyogenes, S. pneumoniae,* and *S. agalactiae*, acting as a resistance reservoir. The notion is supported by studies demonstrating that *S. suis* harbors mobile resistance genetic elements, similar to those of the above-mentioned streptococci, that share the same conserved chromosomal insertion sites. Thus, *S. suis* is a paradigmatic example of possible intersections between animal and human resistomes.

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Methicillin-resistant *Staphylococcus aureus* associated with animals and its relevance to human health

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Staphylococcus aureus is a typical human pathogen. Some animal *S. aureus* lineages have derived from human strains following profound genetic adaptation determining a change in host specificity. Due to the close relationship of animals with the environmental microbiome and resistome, animal staphylococcal strains also represent a source of resistance determinants. Methicillin-resistant *S. aureus* (MRSA) emerged 50 years ago as a nosocomial pathogen but in the last decade it has also become a frequent cause of infections in the community. The recent finding that MRSA frequently colonizes animals, especially livestock, has been a reason for concern, as it has revealed an expanded reservoir of MRSA. While MRSA strains recovered from companion animals are generally similar to human nosocomial MRSA, MRSA strains recovered from food animals appear to be specific animal-adapted clones. Since 2005, MRSA belonging to ST398 was recognized as a colonizer of pigs and human subjects professionally exposed to pig farming. The "pig" MRSA was also found to colonize other species of farmed animals, including horses, cattle, and poultry and was therefore designated livestock-associated (LA)-MRSA. LA-MRSA ST398 can cause infections in humans in contact with animals, and can infect hospitalized people, although at the moment this occurrence is relatively rare. Other animal-adapted MRSA clones have been detected in livestock, such as ST1 and ST9. Recently, ST130 MRSA isolated from bovine mastitis has been found to carry a novel *mecA* gene that eludes detection by conventional PCR tests. Similar ST130 strains have been isolated from human infections in UK, Denmark, and Germany at low frequency. It is plausible that the increased attention to animal MRSA will reveal other strains with peculiar characteristics that can pose a risk to human health.

Keywords: *Staphylococcus aureus*, MRSA, ST398, animals, *spa* type

INFECTIONS AND ANIMALS

Infectious diseases have accompanied humankind since its dawn. However, it is not until the end of the last ice age, approximately 10,000 years ago when the agricultural revolution began, that humans became more exposed to the pathogens of infectious disease that have since shaped human history. In the previous era when humans were hunter–gatherers, they moved nomadically over vast territories. Then humans started breeding wild weeds and domesticating animals –sheep, goats, cattle, pigs, poultry, and horses– for food and labor. Stable settlements and proximity to animals caused pathogens to thrive and spread between animals and humans, and infectious diseases become prominent in influencing life and death (Porter, 1997). There began also the sharing of bacterial species between animals and humans, and co-evolution of pathogens with their hosts.

The heritage of the Neolithic Age continues today. It is estimated that over 60% of the emerging human pathogens come from animals (Cutler et al., 2010). To feed the growing human population, there is an increasing demand for intensive animal farming involving large numbers of animals, different species in the same area, and the use of growth promoters and antibiotics. These practices can facilitate the emergence of new pathogens, including antibiotic-resistant organisms, and their transmission to humans. In addition, food of animal origin can be a major vehicle for animal pathogens and their spread can be amplified by the market globalization.

FROM *STAPHYLOCOCCUS AUREUS* TO METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

Staphylococcal infections are of major importance in both human and veterinary medicine. *Staphylococcus aureus* is a major resident or transient colonizer of the skin and the mucosa of humans and primates. It occasionally lives on domestic animals, although these are usually colonized by other species of staphylococci. When *S. aureus* gains entry into the host, it is able to cause a variety of infections, from mild skin infection to life threatening invasive infections.

Staphylococcus aureus has the characteristic ability to rapidly develop resistance to virtually any antibiotic drug coming into clinical use (Pantosti et al., 2007). Resistance to methicillin – that indicates resistance to all beta-lactam agents – was first reported in 1961, the date that marks the appearance of Methicillin-resistant *S. aureus* (MRSA). Methicillin resistance is due to the acquisition of the *mecA* gene, that encodes a new protein designated

PBP2a, belonging to a family of enzymes necessary in building the bacterial cell wall. PBP2a has a very low affinity for β-lactam antibiotics and confers resistance to methicillin and the other beta-lactams (Pantosti et al., 2007). The *mecA* gene is located on a mobile genetic element, named staphylococcal cassette chromosome *mec* (SCC*mec*) inserted in the *S. aureus* chromosome upstream *orf* X (Katayama et al., 2000). Different types of SCC*mec* can be distinguished on the basis of different key elements present, that are the *mec* gene complex, comprising *mecA* and its regulatory genes *mecI* and *mecR1*, and the *ccr* genes complex comprising two different *ccr* recombinases that are responsible for the mobility of the element (Ito et al., 2001, 2004; Ma et al., 2002). To date, the website of the International Working Group on the Staphylococcal Cassette Chromosome elements (http://www.sccmec.org/Pages/SCC_TypesEN.html, accessed on February 2, 2012) lists 11 types of SCC*mec* elements, originating from the combination of eight different *ccr* genes complex and five different *mec* gene complex.

As MRSA is generated by the introduction of a *mecA*-carrying element in a methicillin-susceptible *S. aureus* (Enright et al., 2002), the origin of *mecA* has long been searched for. Couto et al. (1996) found a *mecA* homolog with 80% identity to the *S. aureus* gene in *S. sciuri*, a methicillin-susceptible *Staphylococcus* of rodents, and other primitive mammals. Another *mecA* homolog with 91% identity with *S. aureus mecA* was found in staphylococci recovered from horses, and specifically in *S. vitulinus* (Schnellmann et al., 2006). According to recent data, *S. fleurettii*, belonging to *S. sciuri* group, could be the origin of *mecA* as this species contains *mecA* and the chromosomal locus surrounding *mecA* that are almost identical to the corresponding sequence of SCC*mec* (Tsubakishita et al., 2010). Taken together, these studies indicate that animal staphylococci are the likely origin and reservoir of *mecA*.

DIFFERENT TYPES OF MRSA

Methicillin-resistant *S. aureus* has long been considered the prototype of multidrug-resistant nosocomial pathogens, causing infections in hospitals and healthcare facilities. The proportion of MRSA infections has increased greatly in the last three decades and strains defined healthcare-associated MRSA (HA-MRSA) have become endemic in industrialized countries as causes of serious infections such as septicemia, pneumonia, ventilator-associated pneumonia, and surgical site infections (Diekema et al., 2001). In the US, the National Nosocomial Infections Surveillance System (NNISS) reported that in 2003, over 60% of *S. aureus* isolates in ICUs were MRSA (Klevens et al., 2006). In Europe according to data collected by EARS-Net, the rate of MRSA in bloodstream infections ranges from below 1% to over 50% in different countries, with lower rates in North European countries and a higher rate in Mediterranean countries (Kock et al., 2010). At the end of the 1990s, a new generation of MRSA appeared suddenly in a different setting, the community. The new MRSA strain, designated community-acquired (CA)-MRSA, is able to cause infections in young and otherwise healthy individuals, showing an unusual virulence and capacity to spread (Deleo et al., 2010).

CA-MRSA commonly causes SSTIs with different severity degrees, from mild (furuncles or impetigo), to serious infections (necrotizing fasciitis; Miller et al., 2005), and rarely can cause a severe form of pneumonia (necrotizing pneumonia) associated with high mortality (Francis et al., 2005). CA-MRSA possesses some distinctive features with regard to HA-MRSA: such as presence of SCC*mec* type IV or type V and rare multi-resistance (Naimi et al., 2003). Characteristically, the large majority of CA-MRSA harbors the genes for the Panton–Valentine leukocidin (PVL), a toxin that is absent in HA-MRSA (Vandenesch et al., 2003). PVL has been considered the principal virulence determinant (Lina et al., 1999; Boyle-Vavra and Daum, 2007) or a simple marker of CA-MRSA strains (Voyich et al., 2006). In the USA, the increase in the prevalence of CA-MRSA took the shape of an epidemic, largely due to the expansion of a single clone, designated USA300 (Moran et al., 2006; Kennedy et al., 2008; Tenover and Goering, 2009). In Europe, where the prevalence of CA-MRSA infections is lower than in the USA (Del Giudice et al., 2006), the most common clone is the European ST80 clone, (Urth et al., 2005), alongside a variety of different CA-MRSA clones (Tristan et al., 2007; Sanchini et al., 2011).

Recently, MRSA emerged as a frequent colonizer of animal populations, possibly favored by the large antibiotic use in animals. In food animals, primarily in pigs, a new MRSA strain with zoonotic potential has been recognized and designated livestock-associated (LA)-MRSA. The following chapters illustrate the association between MRSA and animals and the implications for human health.

STAPHYLOCOCCUS AUREUS AS AN ANIMAL-ADAPTED PATHOGEN

The ability to colonize and produce infections in different hosts, including humans, is an important characteristic of some *S. aureus* lineages. In early studies, the available methods to type *S. aureus* were exclusively phenotypic (phage typing, biotyping, and typing of enzymes or toxins). Nevertheless, they allowed the differentiation of *S. aureus* strains into host specific subsets or ecovars, typically associated with man, poultry, sheep, goats, and cattle (Devriese, 1984), while a minority of *S. aureus* strains belonged to non-host specific types and were shared among different hosts. The application of molecular biology techniques confirmed these early findings, revealing that particular genetic backgrounds (clones or lineages) are associated with specific mammalian hosts (Sung et al., 2008; Cuny et al., 2010). These studies also showed that some lineages are not restricted and can be found to colonize or cause infections in a broad variety of animal species, including humans. One notable example is represented by ST398, originally defined the "pig" clone that has been recovered also from other food animals and from humans (Voss et al., 2005; Witte et al., 2007). Some primarily "human" clones are often found in animals: ST1, a frequent clone in human infections (Grundmann et al., 2010) can cause bovine mastitis (Juhasz-Kaszanyitzky et al., 2007) and is carried by pigs in Italy and other European countries (European Food Safety Authority, 2009; Franco et al., 2011).

Staphylococcus aureus adapts specifically to different animal hosts by genetically determined mechanisms that are just starting to be understood. In some cases, host specificity is determined by a selective affinity of certain bacterial receptors to proteins of the preferred host. *S. aureus* acquires iron, an essential bacterial nutrient, from hemoglobin, following lysis of blood red cells

produced by *S. aureus* hemolysin. Hemoglobin is released and bound to a surface *S. aureus* receptor, the iron surface determinant B (IsdB), before being degraded. This receptor has a higher affinity for human hemoglobin than for the hemoglobin of other mammals. This explains, at least in part, why *S. aureus* thrives in the presence of human blood and why humans are generally the preferential host for *S. aureus* (Pishchany et al., 2010).

Recent phylogenetic studies revealed that some animal *S. aureus* lineages derived from human lineages. In this host-jump, *S. aureus* underwent genetic adaptation losing virulence factors that were useless in the new host and acquiring new characteristics. Some examples are depicted in **Table 1**.

The poultry ST5 lineage which spread in poultry farms worldwide (Lowder et al., 2009) has only recently diverged from the human ST5 lineage. This host switch was associated with loss of expression of several proteins that are relevant to human, but not to avian pathogenesis, including the immune evasion cluster and the staphylococcal protein A (SpA). SpA plays a major role in the pathogenesis of human staphylococcal infections; among several functions it binds the FC fragment of human IgG impairing antibody functions, but it does not bind the FC fragment of IgY, the avian analog of IgG. On the other hand, poultry ST5 has acquired novel mobile genetic elements that encode for advantageous traits for survival in an avian host, including resistance to avian phagocytes killing. Similarly, the pathogenic lineage CC133 established in small ruminants (sheep and goats) derived from human *S. aureus* through a combination of allelic diversification, gene decay, and acquisition of mobile genetic elements (Guinane et al., 2010). In particular, through horizontal gene transfer, this clone acquired a pathogenicity island (SaPI) encoding a novel variant of the von Willebrand factor-binding protein that has ruminant-specific coagulase activity (Viana et al., 2010).

In line with these findings, a microarray study (Sung et al., 2008) revealed differences in the frequency of specific genes carried by animal *S. aureus* and those carried by human isolates. In animal isolates, there was a low prevalence of a cluster of genes encoding proteins relevant for host immune evasion that target specifically human immune responses (van Wamel et al., 2006). One such gene is *sak*, coding for staphylokinase, a fibrin-specific plasminogen activator that is present in over 80% of human isolates, but is absent from some cattle and pig lineages (Monecke et al., 2007; Sung et al., 2008; Franco et al., 2011).

MRSA IN COMPANION ANIMALS

In industrialized countries, companion animals have become an integral part of the household. More than 50% of households in the USA have pets and 25% of households in the United Kingdom have dogs (Chomel and Sun, 2011). In general, MRSA strains recovered from companion animals (cats, dogs, or horses) are different from those recovered from food animals. In the first case strains are usually similar to human HA-MRSA, while in the second case they appear to belong to specific animal-adapted clones, unrelated to the most common HA-MRSA (**Table 2**).

Other staphylococcal species that share with *S. aureus* the ability to acquire methicillin resistance, specifically *S. intermedius*, *S. pseudintermedius*, and *S. schleiferi*, are more common in pets (Hanselman et al., 2008, 2009). Reports of MRSA isolated from pets were sporadic until the late 1990s and were mostly related to

Table 1 | Distribution of some *S. aureus* genes among human and animal strains.

Gene	Function	Effect	Presence/absence in isolates from		Reference
			Humans	Animals (species), clone	
spa	FC binding	Impairment of antibody function	P	A* (poultry), ST5 A* (cattle), ST151	Lowder et al. (2009), Herron-Olson et al. (2007)
chp	Chemotaxis inhibitor	Impairment of host native immunity	P	A (poultry), ST5 A (cattle), ST151	Lowder et al. (2009), Monecke et al. (2007), Sung et al. (2008)
scn	Complement inhibitor	Impairment of host native immunity	P	A (poultry), ST5 A (cattle), ST151	Lowder et al. (2009), Monecke et al. (2007), Sung et al. (2008)
sak	Plasminogen activator	Coagulation of plasma	P	A (cattle), ST151 A (poultry, pigs), ST1	Lowder et al. (2009), Monecke et al. (2007), Sung et al. (2008), Franco et al. (2011)
vWbp^{Sov2}	von Willebrand factor-binding (coagulase)^	Coagulation of plasma	A	P (ruminants), CC133	Guinane et al. (2010)

P, present; A, absent.

**Gene disrupted or pseudogene.*

^ Ruminant variant.

Table 2 | Principal MRSA clones shared between animals and humans.

Lineage	Clone*	Companion animals	Horses	Pigs	Poultry	Cattle	Humans	Reference
CC1	ST1			●		●	●	Juhasz-Kaszanyitzky et al. (2007), Cuny et al. (2008), Franco et al. (2011)
CC5	ST5 (USA100)	●		●	●	●	●	Khanna et al. (2008), Hata et al. (2010), Hasman et al. (2010), McDougal et al. (2003)
CC8	ST8 (USA500)		●				●	Morgan (2008), McDougal et al. (2003)
	ST254		●					Cuny et al. (2008)
CC9	ST9		●	●				European Food Safety Authority (2009), Cuny et al. (2008), Battisti et al. (2010)
CC22	ST22 (EMRSA-15)	●					●	Loeffler et al. (2005), Ellington et al. (2010)
	ST36 (EMRSA-16)	●					●	Loeffler et al. (2005), Ellington et al. (2010)
CC97	ST97			●			●	Battisti et al. (2010), European Food Safety Authority (2009), Gomez-Sanz et al. (2010), Monecke et al. (2011)
CC130	ST130					●	●	Cuny et al. (2011), Garcia-Alvarez et al. (2011), Shore et al. (2011)
CC398	ST398*	●	●	●	●	●	●	Loeffler et al. (2009), Cuny et al. (2008), de Neeling et al. (2007), Nemati et al. (2008), Vanderhaeghen et al. (2010a), Kock et al. (2011)

**Principal ST encountered, including also single and double locus variants. The dot means present.*

clinical infections. In addition, before the identification of *S. intermedius* and *S. pseudintermedius*, some misclassification may have occurred (Devriese et al., 2005). The emergence of CA-MRSA in the last decade and the importance of tracing antibiotic-resistant organisms also in the community, have prompted many studies on MRSA in pets and its possible transmission to pet owners.

According to studies performed in various countries, especially in the UK and Australia, MRSA colonization is rare in healthy pets. No MRSA was found in healthy cats in several studies (Baptiste et al., 2005; Loeffler et al., 2005; Hanselman et al., 2009), although in a recent study 2.1% of cats presented to veterinary clinics in Greater London area were colonized by MRSA (Loeffler et al., 2011). In dogs in a household or at admission to a veterinary hospital, colonization rates varied from 0 to 2.1% (Bagcigil et al., 2007; Boost et al., 2008; Hanselman et al., 2008, 2009; Loeffler et al., 2011). In some particular settings, e.g., dogs in a rescue shelter (Loeffler et al., 2010) or in a veterinary hospital (Loeffler et al., 2005), a high MRSA colonization rate, up to 9%, was found.

Methicillin-resistant *S. aureus* infections in pets are mainly represented by skin and soft tissue infections and are sometimes associated with veterinary surgeries. In a large study in the UK, MRSA was recovered from 1.5% of samples from infected animals (Rich and Roberts, 2004). In several studies, dogs appear to have more MRSA infections than cats (Morgan, 2008), but as there are no direct comparative studies, any implication of different susceptibility to MRSA infections between these animal species should be verified. The MRSA types recovered from cats and dogs are similar to those affecting humans, with a similar regional distribution (**Table 2**). For instance, in the USA the most common MRSA type in pets is the clone identified as USA100 (ST5), which is also the most common HA-MRSA clone in humans (McDougal et al., 2003). In the United Kingdom the most common clones are those identified as EMRSA-15 (ST22) and EMRSA-16 (ST36) that are prevalent in UK hospitals (Ellington et al., 2010). Recently, dogs have been found to be colonized by the livestock-associated (LA)-MRSA clone characteristic of food animals and identified as ST398 (Loeffler et al., 2009).

Horses are colonized by MRSA in a percentage ranging from 0 to 11% (Vengust et al., 2006; Bagcigil et al., 2007; Loeffler et al., 2011). In a large study of colonization in Canada involving over 2,000 horses, 2.7% were found to be colonized by MRSA, mainly intranasally (Weese et al., 2005) while studies performed at equine hospitals showed a higher rate (Baptiste et al., 2005). Sporadic cases and outbreak of MRSA infections in horses were reported in large establishments or post-operatively in veterinary hospitals (Weese et al., 2005; Morgan, 2008).

Horses, differently from pets, are colonized or infected by MRSA types quite different from the lineages predominant in humans in the corresponding areas (Loeffler and Lloyd, 2010). Horse MRSA commonly belong to ST8 and related STs within clonal complex (CC) 8. A typical horse clone is USA500 (Morgan, 2008), that emerged as an HA-MRSA clone in the USA but is now infrequently recovered in human patients (McDougal et al., 2003). More recently, studies from Europe and Canada reported horses to be colonized by LA-MRSA ST398 (Cuny et al., 2008; Tokateloff et al., 2009; **Table 2**).

TRANSMISSION OF MRSA BETWEEN HUMANS AND COMPANION ANIMALS

Transmission of bacterial strains between companion animals and their owners has been demonstrated in several instances. Molecular analyses have shown the presence of indistinguishable MRSA strains in pets and humans living in the same household, and have suggested, but not definitely proved, the direction of transmission

(Weese, 2010). As the isolates from cats and dogs resemble nosocomial MRSA, it is usually assumed that companion animals acquire MRSA from humans. Both humans and animals are more often colonized than infected and both can act as reservoirs of MRSA for recirculation of strains inside the household (Morgan, 2008). According to a study performed in Canada and in the US, the owners of companion animals have a MRSA colonization rate (18%) significantly higher than the general population (1–2%) although they do not appear to be at an increased risk for MRSA infections (Faires et al., 2009). In a nursing home for the elderly in UK patients, staff, and the resident cat were all colonized by the same MRSA strain. The cat harbored the MRSA strain on the fur and paws and was the most probable vehicle of MRSA transmission in the nursing home (Scott et al., 1988). In a veterinary hospital the transmission of an EMRSA-15 (ST22) strain from infected dogs to staff members has been demonstrated by molecular typing methods (Baptiste et al., 2005). Pets can be also colonized by CA-MRSA, as the spread of a PVL-positive CA-MRSA strain in a household in the Netherlands was shown to involve also the family dog (van Duijkeren et al., 2005).

Methicillin-resistant *S. aureus* transmission from horses to humans (and sometimes vice-versa) is also well documented, especially with regard to professionals caring for infected animals in veterinary hospitals. Staff caring for an MRSA-infected foal experienced skin infections due to the characteristic equine strain USA500, the same carried by the foal (Weese et al., 2006).

MRSA IN FOOD ANIMALS: LA-MRSA ST398

In 2004, a baby and her parents who lived on a pig farm in the Netherlands were unexpectedly found to be colonized by MRSA. Other pig farmers and their close contacts, as well as one pig were found colonized (Voss et al., 2005). The colonization rate in pig farmers was found to be 23%, a figure 100 times higher than the normal colonization rate in the Netherlands. All MRSA isolates were found to share similar characteristics, such as non-typeability by PFGE and identical *spa* type. Similar findings in pigs and pig farmers were reported from France (Armand-Lefevre et al., 2005). The "pig MRSA" was found to belong to a unique MRSA clone, ST398, and to a group of related *spa* types.

Subsequent studies demonstrated that the presence of the "pig MRSA" in the Netherlands and France was not an exceptional occurrence, as this clone was recovered from pigs in several European countries (Guardabassi et al., 2007; Vanderhaeghen et al., 2010b). In addition, MRSA ST398 was shown to colonize other species of livestock besides swine (**Table 2**); it was thus more appropriately designated LA-MRSA. The reported carriage rates of LA-MRSA ST398 in pigs varied according to the geographical area and the type of study: from 1% in Denmark (Guardabassi et al., 2007), to 40% in Belgium and the Netherlands (de Neeling et al., 2007; Vanderhaeghen et al., 2010b).

In a study funded by the European Food Safety Agency (EFSA), conducted in 2008, 24 European countries were involved in an active survey of MRSA in the dust of the environment of pig holdings as an indication of pig colonization. Overall, MRSA were found in pig holdings of 17 countries, with a prevalence varying between countries from 1 to 50% of the holdings sampled. Generally, countries with a higher density of pig farming were also countries with a higher recovery rate of MRSA (European Food Safety Authority, 2009). In this survey, *spa* types related to ST398 were, as expected, largely dominant; however, different clones, such as ST1, ST9, and ST97, were also found associated with pig carriage, although at a much lower frequency (**Figure 1**).

The presence of LA-MRSA ST398 in pigs is not exclusive to Europe, as a high rate of ST398 colonization was also found in pigs in Canada (Khanna et al., 2008) and in the US (Smith et al., 2009).

Other MRSA clones besides ST398 have been found to colonize pigs, generally with a regional distribution (**Table 2**). Studies performed in China reported the presence of ST9 colonizing pigs and workers (Wagenaar et al., 2009). In other areas, common human clones were identified in pigs, such as USA100 (ST5) in Canada (Khanna et al., 2008) and recently, ST22 in Ireland (Horgan et al., 2011). In Italy, pigs at the slaughterhouse were found to be colonized by different clones of MRSA besides ST398, including t127/ST1, ST9, and ST97 (Battisti et al., 2010). ST1 is a common human clone, although it is frequently recovered in Italian hospitals in recent years (Monaco et al., 2010). It is possible that the recovery of human clones in pigs represents human-to-pig contamination, but it is also possible that some strains, such as the t127/ST1 clone are in fact animal-adapted strains of ancient human origin.

Despite the high rate of colonization in pigs, LA-MRSA ST398 has only sporadically been reported to cause clinical infections in this animal species (Weese, 2010): in pigs infections are rarely caused by *S. aureus*, the most common staphylococcal pathogen being *S. hyicus*.

The contamination of food products by animal MRSA is a big threat, as it has a potential for wide dissemination in the general population (Kluytmans, 2010). A study performed in the Netherlands found that 11% of raw meat samples from the retail market (including pork, beef, veal, lamb, and chicken) was contaminated by MRSA, represented mainly by MRSA ST398 (de Boer et al., 2009). A study from South Italy reported the presence of MRSA of *spa* types corresponding to ST398 in mozzarella cheese products (Crisetti et al., 2011).

LA-MRSA ST398 has been isolated not only from pigs, but also from other animal species, as already mentioned, including dogs, horses, calves, and poultry (Cuny et al., 2008; Hasman et al., 2010; Huber et al., 2010; Loeffler and Lloyd, 2010). In a study from Belgium, 12% of the isolates obtained in 2006 from healthy chickens were MRSA that belonged to *spa* types associated with ST398 (Nemati et al., 2008; **Table 2**).

MICROBIOLOGICAL, MOLECULAR, AND VIRULENCE CHARACTERISTICS OF LA-MRSA ST398

LA-MRSA strains were initially distinguished by their peculiar resistance to digestion by *Sma*I, the restriction enzyme most frequently used for PFGE typing of *S. aureus* (Chung et al., 2000), due to the presence of a new methylation enzyme protecting restriction sites (Bens et al., 2006). Otherwise, LA-MRSA were perfectly typeable by current molecular methods: they were associated with a specific group of *spa* types with related repeat sequences, including t011, t034, t108, and t899 (Vanderhaeghen et al., 2010b; **Figure 1**) and belonged to ST398, a clone historically quite rare

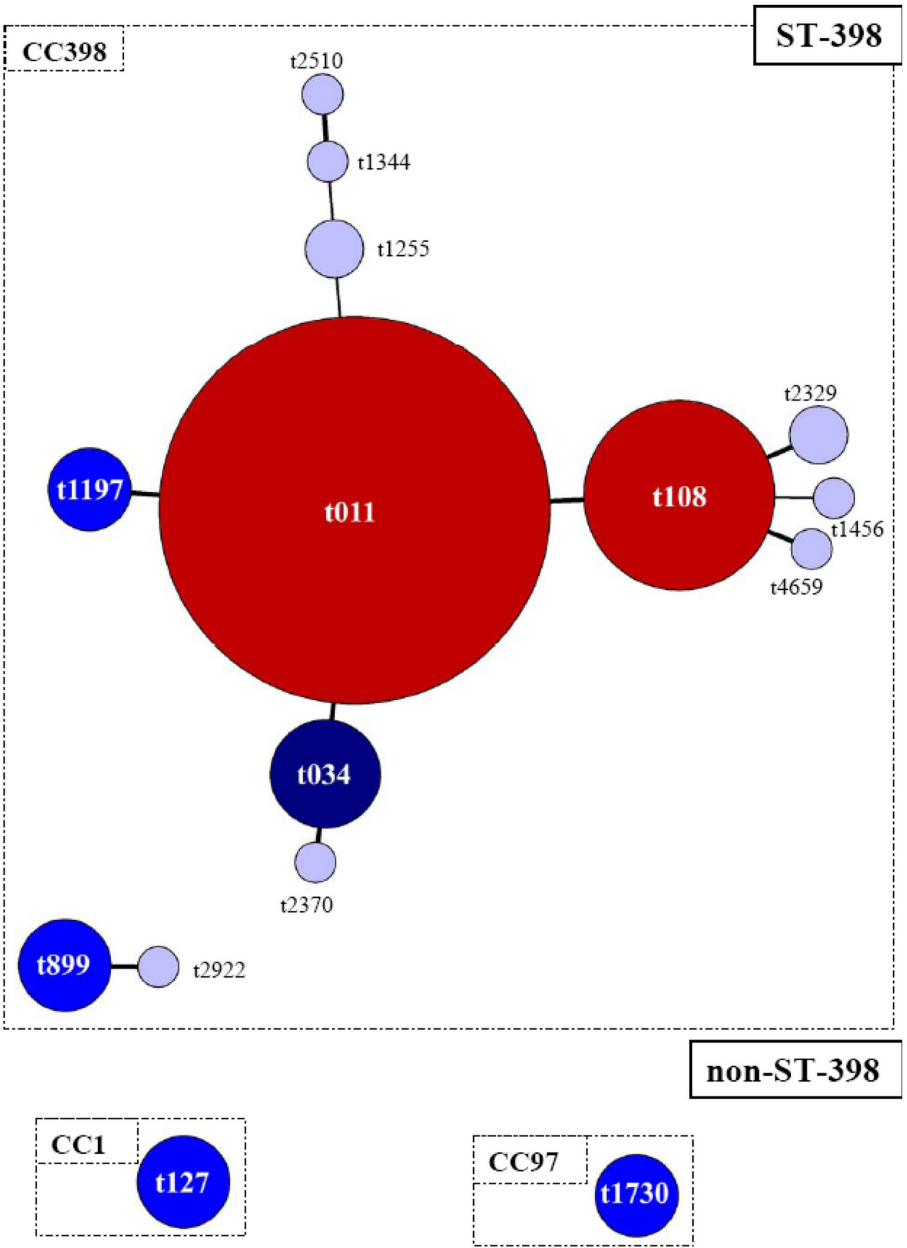

FIGURE 1 | *spa* types of MRSA isolated from pig production holdings in Europe, their genetic relationship, and association with ST398 and with less frequent CCs. Size and color of each circle correspond to the prevalence of the *spa* type indicated, from red (most frequent) to pale blue (less frequent). Reproduced from the EFSA J. with permission (European Food Safety Authority, 2009).

in humans that did not derive from the most common MRSA lineages. Characteristically, LA-MRSA ST398 strains carry SCC*mec* type type IV or, more frequently, type V (Monecke et al., 2011). The presence of these SCC*mec* types in LA-MRSA is shared with CA-MRSA and suggests a transmission of genetic elements between these two MRSA groups. However, differently from CA-MRSA, ST398 generally does not possess the phage-encoded PVL-genes, that contribute to the virulence of the former. Exceptions involve strains of human infections that may have acquired PVL-genes from human MRSA: one patient from the Netherlands and two patients from Sweden had infections associated with PVL-positive ST398 (van Belkum et al., 2008; Welinder-Olsson et al., 2008). Also sporadic ST398 MRSA from China has been found to be PVL-positive (Yu et al., 2008; Stegger et al., 2010). No specific virulence factor characteristic of ST398 has been identified so far (Monecke et al., 2011).

LA-MRSA ST398 is generally susceptible to antibiotics other than beta-lactams, but it is characteristically resistant to tetracycline, which suggests that heavy tetracycline use in the pig industry may have favored the emergence of this clone. A recent study

Table 3 | Prevalence of MRSA and ST398 MRSA carriage in different healthy populations, in patients, and among clinical isolates.

Population	Country	No. of subjects	MRSA	ST398°	Reference
CARRIAGE					
Pig farmers	Netherlands	26	23%	23%	Voss et al. (2005)
Veterinarians	International (mainly from USA)	345	7.0%	0	Hanselman et al. (2006)
Pig veterinarians	International (mainly from Europe)	235	14.0%	13.1%	Wulf et al. (2008b)
Veterinarians and veterinary personnel	Czech Republic	280	0.7%	0	Zemlickova et al. (2009)
Veterinarians	Belgium	146	9.5%	7.5%	Garcia-Graells et al. (2012)
	Denmark	143	1.4%	1.4%	
Pig farmers	Belgium	127	37.8%	37.8%	Denis et al. (2009)
Pig farmers*	Germany	113		86%	Cuny et al. (2009)
Family members		116		4.3%	
Pig veterinarians		49		45%	
Pupils (10–16 years)		462		<1%	
Pig workers	USA	20	45%	45%	Smith et al. (2009)
Pig farmers	Ireland	100	2%	0	Horgan et al. (2011)
Patients at admission	Germany	834	71%	25%	Kock et al. (2011)
CLINICAL SAMPLES					
Clinical MRSA isolates	Europe	8,262		1.7%	van Cleef et al. (2011)

°MRSA resistant to SmaI digestion or belonging to specific spa types are included under ST398.

**Farmers exposed to MRSA-positive pigs.*

showed that the addition of tetracycline or zinc in animal feed increased the number of ST398 bacterial cells in pigs' nostrils although it had no effect on MRSA transmission (Moodley et al., 2011). LA-MRSA ST398 strains have been found to carry previously unidentified resistance genes, such as a novel trimethoprim resistance gene, *dfrK*, (Kadlec and Schwarz, 2010) and a novel gene, *vga*(C), encoding an ABC efflux pumps and conferring resistance to streptogramins and lincosamides (Kadlec and Schwarz, 2009). In addition, an isolate from the nose of a pig was found to carry the multidrug resistance gene *cfr* (Kehrenberg et al., 2009) that is able to confer resistance to five different antibiotic classes, including linezolid, a "last-resource" antibiotic for serious infections due to multidrug-resistant Gram-positive bacteria. Due to their characteristic multi-host specificity, ST398 strains can represent an efficient vehicle of these resistant determinants that are plasmid-encoded, favoring their transmission and spread.

LA-MRSA ST398 CARRIAGE AND INFECTIONS IN HUMANS

Before the "epidemic" of pig colonization, ST398 isolates were rarely reported from human colonization or infection. After the first report from the Netherlands, many studies from Europe and other areas indicated that living or working on a farm was a risk factor for acquiring MRSA (**Table 3**). A case-control study performed in the Netherlands showed that carriers of non-typeable MRSA were more often pig or cattle farmers and that the density of non-typeable MRSA isolates corresponded to the density of pig farming (van Loo et al., 2007). In Belgium 37.8% of pig farmers were found colonized by ST398 (Denis et al., 2009); in Germany 86% of pig farmers and 45% of veterinarians caring for pigs were colonized (Cuny et al., 2009). Family members that were not directly exposed to pigs were colonized in a lower percentage (Cuny et al., 2009), indicating that inter-human transmission can occur but at low frequency. Veterinarians and other attendees of an international conference on pig health in Denmark were sampled for MRSA carriage and 12.5% were found to carry MRSA that mostly belonged to *spa* types corresponding to ST398 (Wulf et al., 2008a).

In Ontario (Canada), 20% of pig farmers were colonized by MRSA and a correlation was noted between the presence of MRSA in pigs and humans on farms (Khanna et al., 2008).

Infection can follow colonization and LA-MRSA ST398 has been found to be associated with human infections (Krziwanek et al., 2009), including serious forms such as deep abscesses, cellulitis, necrotizing fasciitis (Pan et al., 2009; Soavi et al., 2010), and bacteremia (van der Mee-Marquet et al., 2011). A survey carried out in 17 European countries in 2007 on the prevalence of ST398 among MRSA isolates from human infections, revealed that the highest proportion of ST398 was found in the Netherlands and in Belgium, while in the other countries the proportion was <2% (van Cleef et al., 2011; **Table 3**). A small hospital outbreak due to ST398 occurred in Denmark in 2007 (Wulf et al., 2008a), indicating that this clone is capable of inter-human spread outside the farming setting, although this phenomenon is currently limited. In a recent study, 25% of patients admitted to a University Hospital in 2008–2009 in a German region with high a density of pig farming carried MRSA of *spa* types associated with ST398 and a number of clinical infections due to this clone were detected (Kock et al., 2011). According to a Dutch study, ST398 seems to be less transmittable inside hospitals than non-ST398 MRSA (Wassenberg et al., 2011). It could be speculated that the scarce propensity of LA-MRSA to spread in hospitals may be due to its susceptibility to antibiotics other than beta-lactams compared to typical HA-MRSA. However, the possibility that LA-MRSA may acquire other resistant traits and pose a greater risk to the human population cannot be dismissed.

MRSA IN BOVINE MASTITIS

Staphylococcus aureus is one of the most important bacterial pathogens in bovine mastitis, a disease that causes significant economical losses in the milk industry; thus, *S. aureus* in general and MRSA in particular have been the focus of several studies in dairy cattle. The first report of MRSA in bovine mastitis milk comes from Belgium where in 1972 Devriese and Hommez isolated strains that, using biotyping methods, appeared to be of human origin (Devriese and Hommez, 1975). After this report, and until recently, MRSA was only sporadically isolated from cow milk. In the Republic of Korea a small proportion (0.4%) of MRSA was found among 3047 bacterial isolates from bovine mastitis milk samples obtained between 1997 and 2004 (Moon et al., 2007). In Switzerland MRSA were found in 1.4% of mastitis milk samples (Huber et al., 2010) and in Hungary MRSA characterized by *spa* type t127 and ST1, of likely human origin, was recovered in 4% of samples from cows with subclinical mastitis. In this last study, transmission of MRSA to a farmer working in close contact with bovines was also demonstrated (Juhasz-Kaszanyitzky et al., 2007). A study from Japan examined 363 *S. aureus* from bovine milk: of these, only four were MRSA, all belonging to CC5 (Hata et al., 2010). In Belgium MRSA was found in nearly 10% of the farms where *S. aureus* mastitis was present. This comparatively high prevalence was associated with the finding that all isolates were LA-MRSA ST398 (Vanderhaeghen et al., 2010a; **Table 2**).

Few studies dealt with colonization of healthy cattle: a study from Switzerland only found MRSA in 1% of nasal swabs from calves and in 0.25% of nasal swabs from cattle (Huber et al., 2010). In this study the MRSA isolates from calves were ST398, providing evidence that LA-MRSA ST398 can colonize also cattle, although the colonization rate is much lower than in pigs (**Table 2**).

A NEW *mecA* GENE IN BOVINE ISOLATES

A recently published article (Garcia-Alvarez et al., 2011) reported the presence of MRSA strains with unusual features in bovine milk samples from the UK. These strains belonged mainly to CC130 (more rarely to CC425) and carried a novel *mecA* gene that was only 70% identical at the nucleotide level to the classical *mecA* gene, escaping detection by routine PCR assays. Similarly, the encoded PBP2a protein was only 63% identical to the classical PBP2a protein and could not be detected by the commercially available antibody-based assays. The novel *mecA* was named $mecA_{LGA251}$, after the designation of the bovine strain where it was identified for the first time. In turn $mecA_{LGA251}$ was part of a novel SCC*mec* element, identified as type XI. Other distinguished features of the SCC*mec* type XI are the presence of a *mec* complex that has a different organization from other SCC*mec* elements including also the beta-lactamase resistance gene *blaZ*, and the presence of an arsenic resistance operon.

Although previously CC130 had been associated only with methicillin-susceptible *S. aureus* from animals, isolates with the same novel $mecA_{LGA251}$ were recovered from human clinical infections in the UK and Denmark. The ability of these strains to cause infections in humans was confirmed by a concurrent report describing two patients infected with similar strains in Ireland (Shore et al., 2011) and by another study from Germany where 11 CC130 strains carrying SCC*mec* type XI and $mecA_{LGA251}$ were identified out of a collection of 12,691 MRSA obtained in 2006–2011 (Cuny et al., 2011).

It is possible that these strains had been circulating for some time among cattle: for instance in 2003 a report from Korea described some MRSA strains from dairy cattle with low-level resistance to oxacillin that were *mecA*-negative (Lee, 2003). Studies to investigate these findings further were not performed, but it is plausible to speculate that these strains carried the $mecA_{LGA251}$ gene or a similar variant.

The strains with the novel *mecA* are a new example of LA-MRSA. At the moment they do not seem to pose a great risk, but their evolution is unpredictable: they could become better able to colonize, more resistant, or more pathogenic (Fluit, 2011). Continuous surveillance is necessary to promptly detect new types of MRSA from animals and to evaluate their impact on human health.

CONCLUSION

Methicillin-resistant *S. aureus* made its first appearance as the resistant variant of *S. aureus* 50 years ago and has since established as a typical multi-resistant nosocomial pathogen (Jevons, 1961). In the last two decades, new generations of MRSA have emerged, with the distinctive ability to occupy new niches, specifically the human community and food-producing animals. The risk of the presence of MRSA in food animals is clearly associated with the transfer of these microorganisms to humans. If these new MRSA enter hospitals, they will find frail patients who will be easily infected.

Phylogenetic studies revealed that some lineages of animal-adapted staphylococci originated from human strains following profound genetic modifications, and that today these animal-adapted strains can be transmitted back to humans. Both wild and food animals are in contact with the large microbiome and resistome pools of the environment and can therefore carry new strains and become their vehicle to the human community. Different staphylococcal species present in animals and the environment represent a large reservoir of resistance genes, from which MRSA has already picked not only the *mec* gene, but also other resistance determinants. The new MRSA from animals may be able to convey to humans novel resistant determinants and novel virulence factors.

While in hospitals the proportion of MRSA is clearly associated with patterns of high antibiotic consumption, it is unclear if the emergence of animal strains has been driven by high or inappropriate antibiotic consumption in the animal farming setting. However, the control of antibiotic use appears to be one of the few possible interventions today to reduce the spread and transmission of MRSA. As for MRSA colonizing food animals, good farming practices also appear to be essential in the control of the spread of MRSA.

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Genomic diversification of enterococci in hosts: the role of the mobilome

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Enterococci are ubiquitous lactic acid bacteria, possessing a flexible nature that allows them to colonize various environments and hosts but also to be opportunistic pathogens. Many papers have contributed to a better understanding of: (i) the taxonomy of this complex group of microorganisms; (ii) intra-species variability; (iii) the role of different pathogenicity traits; and (iv) some markers related to the character of host-specificity, but the reasons of such incredible success of adaptability is still far from being fully explained. Recently, genomic-based studies have improved our understanding of the genome diversity of the most studied species, i.e., *E. faecalis* and *E. faecium*. From these studies, what is becoming evident is the role of the mobilome in adding new abilities to colonize new hosts and environments, and eventually in driving their evolution: specific clones associated with human infections or specific hosts can exist, but probably the consideration of these populations as strictly clonal groups is only partially correct. The variable presence of mobile genetic elements may, indeed, be one of the factors involved in the evolution of one specific group in a specific host and/or environment. Certainly more extensive studies using new high throughput technologies are mandatory to fully understand the evolution of predominant clones and species in different hosts and environments.

Keywords: enterococci, population analysis, antibiotic resistance, genomic, mobile genetics elements, host specificity, *E. faecalis*, *E. faecium*

THE GENUS *ENTEROCOCCUS*

The genus *Enterococcus* contains bacterial species that are ecologically diverse. They are Gram-positive lactic acid bacteria that are found in the gastrointestinal consortia of humans, other mammals, reptiles, amphibians, birds, and insects and are used in the production of fermented foods and probiotics (Benno et al., 1992; Aarestrup et al., 2002; Tannock and Cook, 2002). To date, only species from humans and domestic animals have been studied in some detail.

Enterococci have gained notoriety over the past few decades as frequent causes of hospital-acquired infection at extra-intestinal sites, including surgical site wounds, urinary tract, and heart. The ability of these microorganisms to cause infections has been linked to the intrinsic ruggedness of these species, which allows the organism to persist in the hospital environment and survive many host defenses compounded by the acquisition of a variety of variable virulence and resistance traits by horizontal transfer from other organisms: being rugged and genetically flexible is an important feature of these microorganisms (Fisher and Phillips, 2009; Palmer et al., 2010b; Laverde Gomez et al., 2011a).

Enterococci are among the most antibiotic-resistant bacterial pathogens known. For reasons not well understood, they appear to have served as a key collection point for a wide variety of antibiotic-resistance determinants. It is well known that enterococci possess the intrinsic low-level resistance to cephalosporins, some beta-lactam antibiotics and about 83% of clinical isolates of *E. faecium* show the high levels of resistance due to the presence of an alternative penicillin-binding protein (PBP5), and aminoglycosides. In addition, the acquired high-level resistance to beta-lactams, aminoglycosides, and glycopeptides is associated with the acquisition of foreign DNA mediated by lateral gene transfer (LTG; Shepard and Gilmore, 2002). It was recently shown that enterococci have transferred vancomycin resistance to methicillin-resistant *Staphylococcus aureus*, and the opposite transfer from *Staphylococcus* to *Enterococcus* clinical strains has also been documented (Weigel et al., 2003; Perichon and Courvalin, 2004; Sarti et al., 2012).

The genus *Enterococcus*, after different taxonomical allocations that have identified more than 40 different species (http://www.bacterio.cict.fr/enterococcus.html), has retained 17 species: formal infra-species division has not been made in the genus, though some ecovar-related variability has become apparent in *E. faecium*. These ecovars pertain to biochemical reaction types (biotypes) and, in some cases, they have been found to be host associated: example are raffinose-positive *E. faecium* in poultry and sorbitol-positive *E. faecium* found in dogs; but more convincingly, some genotypes have been associated with certain animal host species (Devriese et al., 1994; Quednau et al., 1998). Association of some genogroups with different hosts has been recently found in a group of vancomycin-resistant *E. faecium* (VRE) isolated from hospitalized, non-hospitalized patients, and different animal sources, by using AFLP analysis (Willems et al., 2000). The Authors, in this subgroup of strains, also demonstrated that various pig strains were indistinguishable from human strains.

Among these 17 species, *E. faecalis* and *E. faecium* are mainly isolated from human infections: a few years ago, the proportion

between the two species was 80–90% for *E. faecalis* and 5–12% for *E. faecium* (Cetinkaya et al., 2000). In recent years, the emergence of enterococci has been associated with a gradual replacement of *E. faecalis* (responsible for approximately 40% of enterococcal infections) with *E. faecium* (more than 60% of these infections) probably because of the rapid accumulation of antibiotic-resistance determinants in this latter species (Iwen et al., 1997; Treitman et al., 2005; Top et al., 2007; Hidron et al., 2008).

HABITAT

The best known, though not the only habitat of the enterococci, is the gut of mammals and birds; they may be a significant component of other animal groups as well. Most enterococcal species known to date are typically associated with the intestine of humans and domestic animals, and when found outside the gut, they are interpreted as indicators of fecal pollution or, in the case of the human body, as possible pathogens. Some species, i.e., *E. casseliflavus*, *E. mundtii*, and *E. sulfureus* appear to have adapted to vegetative life in environmental habitats and can colonize plants (Klein, 2003). A recent study demonstrated single *E. casseliflavus* populations in submerged aquatic vegetation and the Authors concluded that this species represents a naturalized reproducing indicator bacteria, not directly related to pollution events (Badgley et al., 2010).

As stated before, *E. faecalis* and *E. faecium* are the most common isolates in the human gastrointestinal tract: the number of *E. faecalis* in human feces range from 10^5 to 10^7 per gram and those of *E. faecium* from 10^4 to 10^5 per gram (Tannock and Cook, 2002; Fisher and Phillips, 2009).

Taking into consideration different species, there are certain variations depending on different factors: host, age, and feeding behavior. For example, *E. faecalis* and *E. faecium* occur predominantly in humans; *E. cecorum* is a member of the enterococcal flora of pigs and poultry; *E. hirae* is a frequent inhabitant of the porcine gut and may occur in poultry, cattle, dogs, and cats, *E. asini* that is specific for donkeys (Aarestrup et al., 2002). Furthermore, enterococcal colonization takes place more during the very first period of life, and varies depending on intestinal compartment, and on type of feeding (Vaughn et al., 1979; Collins et al., 1986).

E. faecalis and *E. faecium* are also regularly isolated from cheese, fish, sausages, minced beef, and pork (Fontana et al., 2009; Nieto-Arribas et al., 2011). In some cases these species are involved in food spoilage and fermentation; in others (above all when they are isolated from food of animal origins) they are often associated with contamination due to their ability to survive the heating process (Fisher and Phillips, 2009).

Some *E. faecium* and *E. faecalis* strains are used as probiotics and are then ingested at high inocula. Such probiotics are used to treat various dysbiosis (antibiotic-associated diarrhea or irritable bowel syndrome), to lower cholesterol or to improve host immunity (Franz et al., 2011). All these benefits were assessed and confirmed by practical use and, recently, in an animal model (Tarasova et al., 2010), but in view of the emergence of problematic enterococcal lineages and the potential for gene transfer in the gastrointestinal tract of both human and animals, their use needs to be carefully monitored (Franz et al., 2011).

In conclusion, it is evident that enterococci are able to colonize a variety of niches due to their ability to survive in a wide range of environmental conditions.

NOSOCOMIAL PATHOGENS AND MULTI-DRUG RESISTANCE

Enterococci are known to be causes of endocarditis and rare cases of meningitis. However, this picture has changed dramatically over the last 20 years, in which enterococci have become one of the leading causes of nosocomial infections and – according to the recent National Nosocomial Infection Surveillance (NNIS) surveys (NNIS, 2004; Rosenthal et al., 2008) – they remain in the top three most common pathogens responsible for urinary tract, intra-abdominal, pelvic, surgical, wound, and central venous catheter (CVC) associated infections and bacteremia, which may seed to more distant sites. For example, genitourinary tract infections or instrumentation use often precedes the onset of enterococcal endocarditis. Pleural space infections, as well as skin and soft tissue infections, have also been reported (Rice et al., 2004; Deshpande et al., 2007).

Hospital-associated enterococcal infections emerged differently in the USA with respect to Europe (around 1990) and concurrently with the acquisition of vancomycin resistance. Even if with a different percentage of isolation, vancomycin resistance in Europe has so far not spread to hospitals at the same levels as in the USA (there are, in any case, variations among European countries with some, such as Greece and Ireland, having rates exceeding 30%, while Italian and Spanish prevalence is less than 5%, Germany less than 10%, and the UK with approximately 13%; Werner et al., 2008). In general, most *E. faecium* isolates recovered from hospitalized patients are more resistant to antimicrobial agents than community-derived isolates. In particular ampicillin and fluoroquinolone resistance are important markers that distinguish hospital from community-derived isolates (Coque et al., 2005; Willems et al., 2005; Willems and van Schaik, 2009).

PATHOGENIC ENTEROCOCCI

In general, the virulence of enterococci is lower than that of other organisms such as *S. aureus*. However, enterococcal infections often occur in debilitated patients and as a part of polymicrobial infections: these factors limit the ability of investigators to determine the independent contribution of enterococcal infections to mortality and morbidity.

Perturbation in the dynamics of the host/commensal relationship is related to different causes: (i) the access to extra-intestinal sites can be promoted by antibiotic-treatment, host injury, or diminished host immunity; and (ii) the transition from a commensal behavior to a pathogen happens through the acquisition of new traits. The latter has been gaining ground after the identification of pathogenicity islands (PAI) in *E. faecalis* (Shankar et al., 2002). These elements encode several genetic determinants involved in colonization and virulence, and possess modular structures able to adapt their genetic content, with the acquisition or loss of pathogenicity factors. Diverse PAI variants are widely distributed among enterococcal strains belonging to various clonal complexes (CC_s), origins, and hosts, which enrich their accessory genomes with new traits able to enhance their pathogenicity in hospitalized patients (McBride et al., 2009). The spread of

these more infective clones is mainly due to the presence of factors involved in colonization and ability to form biofilm, the first crucial steps in clinical infection and nosocomial spread of antibiotic-resistant strains.

In *E. faecalis*, some of the most prominent virulence determinants and factors involved in colonization and biofilm formation are: a secreted toxin cytolysin; a collagen-binding adhesin of the microbial surface component recognizing adhesive matrix molecules (MSCRAMM-ACE); an adhesin expressed on the surface of the bacteria designated Esp (proteins associated with virulence, initially found only in hospital-derived strains, and now variably present in some animal isolates); an autolysin (Atn formerly AtlA); a sugar-binding transcriptional regulator (BopD); a secreted proteases gelatinase (GelE); a cell-anchored protein (Bee); and a sortase associated to surface pili formation (Ebp; Singh et al., 1998, 2005; Rich et al., 1999; Eaton and Gasson, 2002; Hufnagel et al., 2004; Mohamed et al., 2004; Shankar et al., 2004; Tendolkar et al., 2006; Schluter et al., 2009; Heikens et al., 2011; Nallapareddy et al., 2011; Pinkston et al., 2011).

It has been ascertained that *E. faecalis* is more virulent than *E. faecium*. Even if in the past *E. faecium* have been less studied, recently various aspects regarding its virulence and pathogenicity have examined. A glycosyl hydrolase, encoded by the hyl_{Efm} gene, has been hypothesized to be involved in infections of hospital-associated *E. faecium*, but a recent study performed on a murine peritonitis *E. faecium* model, did not show any *in vivo* effect on virulence (Willems et al., 2001; Woodford et al., 2001; Coque et al., 2002; Rice et al., 2003; Leavis et al., 2004; Panesso et al., 2011). Recently, the group of Murray demonstrated the involvement of two *gls*-loci in the adaptation to the intestinal environment and virulence, in response to the *in vitro* bile salts stress, and the presence of the $ebpABC_{fm}$ locus encoding pili, in *E. faecium* TX82, confirming their role in pathogenicity and biofilm formation (Sillanpää et al., 2010; Choudhury et al., 2011). As in *E. faecalis*, the role of Esp in forming biofilm has been demonstrated also in *E. faecium* (Sava et al., 2010).

It is clear that the presence of a specific virulence trait can not be always considered predictive of pathogenicity in itself: complex interactions between these and other traits, as well as host and environmental conditions, can influence microbial behavior; in this context, the study of key regulators of gene expression is of great importance, such as the recent identification of small RNAs (sRNA) as mediators of virulence and stress inducible gene expression, in *E. faecalis* V583 (Shioya et al., 2011).

KNOWLEDGE ON POPULATION BIOLOGY OF DRUG-RESISTANT ENTEROCOCCI FROM HOSPITAL AND NON-HOSPITAL ORIGINS

Many studies published in recent years have indicated that hospital-derived strains have acquired traits involved in resistance and pathogenesis (Baldassarri et al., 2001; Willems et al., 2001; Woodford et al., 2001). These studies have recently been supported by more global experimental designs able to give a more complete perspective.

We report here results of different studies aiming to improve our understanding on the population biology diversity of enterococci isolated from different hosts.

A recent comparative genomic hybridization study using a mixed whole genomic array (Leavis et al., 2007) on strains of *E. faecium* isolated from various genetic and ecological backgrounds, demonstrated that: (i) hospital-derived isolates were grouped together; and (ii) IS elements together with resistance genes, genes encoding novel metabolic pathways, genes encoding membrane proteins and regulatory genes, were more that 80% specifically associated. Furthermore, in MLST-based studies, antibiotic-resistant strains that cause infections clustered into distinct groups with respect to strains colonizing the gastrointestinal tract of healthy individuals in the community (Willems and van Schaik, 2009). As stated before, in addition to possessing resistances to multiple antibiotics such as vancomycin, enterococcal strains often possess a set of genes that contribute to virulence (van Schaik and Willems, 2010). Potential virulent strains can also arise in the same clonal complex (CC), due to the acquisition of virulence factors carried by PAI elements, that could contribute to change commensal *E. faecalis* strains into pathogenic ones, to confer and increase their ability to colonize different gastrointestinal tract niches (Coburn et al., 2007; Willems and van Schaik, 2009).

Even if only few lineages/clonal complexes (CC_s) of *E. faecium* and *E. faecalis* have been currently associated with hospital outbreaks, the large number of resistance and colonization traits harbored in hospital isolates suggests consecutive cumulative gene acquisition, integration and successful adaptation to these new conditions (Baquero, 2004; Leavis et al., 2007; McBride et al., 2007).

Several other recent studies have demonstrated that hospital-acquired isolates clustered in few clonal complexes – CC2 and CC9 in *E. faecalis* and CC17 in *E. faecium* – these have also been recovered from farm animals and pets; moreover, strains belonging to CCs commonly found among animals have also been isolated from humans (*E. faecium* CC5, *E. faecalis* CC16 or CC21; Leavis et al., 2006a; Biavasco et al., 2007; Damborg et al., 2009; Freitas et al., 2009a,b; Willems and van Schaik, 2009; Larsen et al., 2010).

Many enterococcal strains from human and swine hosts – all vancomycin-resistant (VRE) – showed different STs (clustering mainly in *E. faecium* CC17 and CC5, and *E. faecalis* CC2), harbored Tn*1546* on indistinguishable plasmids (Freitas et al., 2011). In surveillance studies performed in Portugal, Denmark, Spain, Switzerland, and the United States from 1995 to 2008, a sample of VRE isolates from pigs and healthy people was compared with outbreak/prevalent VRE clinical strains (isolated from 23 countries in the same period). This study demonstrated intra- and inter-national diffusion of *E. faecium* and *E. faecalis* strains showing the same CCs and plasmids among swine and humans (Freitas et al., 2011).

In another MLST-based study in which ampicillin-resistant *E. faecium* isolates from dogs and humans were compared, the widespread occurrence of hospital-associated lineages in dogs was demonstrated (Damborg et al., 2009) and two of them, i.e., ST78 and ST192 are among the most common lineages causing infections in European and Asian hospitals (Ko et al., 2005; Bonora et al., 2007; Werner et al., 2007; Top et al., 2008). The knowledge of the host-specificity of *E. faecium* and *E. faecalis* genetic backgrounds that cluster according to the species of origin was not confirmed here, indicating that dogs may play a role in the

spread of this nosocomial pathogen (Willems et al., 1999, 2000; Leavis et al., 2006a; Damborg et al., 2009). Damborg et al. (2009) demonstrated that what distinguished canine from human isolates were the virulence and antimicrobial resistance profiles observed: those strains causing human infections were MDR and virulent bacterial populations, despite the genetic similarities observed.

The above mentioned ST78 and ST192, together with ST19, ST117, 202 and 18 – all included in the hospital-associated CC17 – were also the first beta-lactamase producing *E. faecium* recently isolated in Italy (Sarti et al., 2011; Sarti et al., 2012. Analyzing MLST data from deposited *E. faecium* sequences and making a comparison with beta-lactamase producing strains, belonging to different PFGE, a clear ST clustering of hospital isolates together with isolates from dogs and cats and, less frequently, with non-hospital strains, was found (data not shown; Sarti et al., 2011).

As reported before, dogs can be frequent carriers of CC17-related lineages, in particular ST78 and ST192 and the human microbiota can indeed be an excellent hot-spot of recombination for the transfer of resistance mechanisms, including beta-lactams (Damborg et al., 2009). Even if mechanisms of the ecological dominance of these CC17 hospital-acquired *E. faecium* strains are not well understood, there are hypotheses that the acquisition of antibiotic-resistance traits, together with cell-surface proteins, may have contributed to their success (Leavis et al., 2006b; Heikens et al., 2008).

ENTEROCOCCAL GENOMES AND GENOME-BASED STUDIES

Enterococcal genome sequences still remain relatively limited, especially for *E. faecium* strains, making difficult the understanding of their fundamental biology and virulence-associated traits, when compared to *E. faecalis*.

The sequencing of the *E. faecalis* V583 genome was undertaken in the late 1990s and completed in 2002, and revealed a large content of PAI, mobile genetic elements (MGE) and plasmids carrying antibiotic-resistance determinants, but lacked the *esp* and *cyl* genes because a 17-kb DNA fragment carrying these genes had been excised from the PAI itself (Paulsen et al., 2003). The sequencing of the V583 genome appeared to provide new insight into enterococcal genomes, into their genetic makeup and biology. Unfortunately, since then only two other *E. faecalis* genome sequences have been published (OG1RF and EF62), for which the publicly available genome sequence is not completely annotated, reducing their usefulness as a starting-point for genome-wide studies (Bourgogne et al., 2008). With regard to *E. faecalis* EF62, this strain was isolated in a healthy Norwegian infant in 2006 and belonged to CC6, which had never been associated with nosocomial infections. In this genome, the presence of genomic islands (GIs) carrying genes involved in lactose and other carbohydrate metabolisms instead of virulence determinants, emphasized its adaptation to its commensal existence (Solheim et al., 2011).

In 2007, a partial genome analysis of the commercial probiotic strain *E. faecalis* Symbioflor (Symbiopharm, Herborn, Germany) was made; this strain does not possess any virulence determinants, and for this reason was proposed as a probiotic, but no information was available due to the absence of sequence data for this strain (Domann et al., 2007).

Even less sequence information is available for *E. faecium*, making it the only major nosocomial pathogen for which no complete genome sequence is publicly available. In fact, the *E. faecium* strain TX0016 genome sequence (Acc. No. ACIY00000000); (formerly *E. faecium* DO strain, isolated in 1992 from a case of endocarditis), already announced in 2000, has not yet been finished (van Schaik and Willems, 2010). Furthermore, annotations regarding genes encoding essential products – such as ribosomal proteins – are missing, indicating an incomplete assembly.

Recently, van Schaik et al. (2010) have undertaken a genome sequencing project of seven *E. faecium* strains, isolated from different ecological niches in different periods, using pyrosequencing technology, to partially resolve the current lack of genomic information on this species. Briefly, their conclusions can be summarized in three important messages: (i) hospital-associated isolates accumulate genomic differences related to antibiotic resistance and colonization genes; (ii) strains belonging to the same CC, i.e., CC17, are closely related in the core genome, but still have a large difference in the gene content; and (iii) the pan-genome analysis of *E. faecium* indicated that the total available gene pool within this species is essentially unlimited, depending on the ecological niches that this species can colonize. The gain and/or loss of MGEs, rather than evolutionary descent, is the most important driving force in enterococci.

In addition to this, an interesting report was published in 2010, in which the draft genome sequences for 28 enterococcal strains of diverse origin, including the species *E. faecalis, E. faecium, Enterococcus casseliflavus,* and *Enterococcus gallinarum*, were analyzed. These new data could possibly fill the gap in enterococcal genome data and provide new insights into basic enterococcal physiology (Palmer et al., 2010a).

All these published genome-based studies of enterococci have contributed to our understanding of genomic diversity, especially in *E. faecalis* and *E. faecium*, confirming the affirmation of specific sub-populations associated with humans, which possess large differences in their accessory genes, including MGEs, making them an important factor in phenotypic characteristics.

Comparative and genome hybridization studies published so far are going in the same direction as previous studies, that enterococcal diversity depends on a considerable inter-strain genomic diversity due to genetic exchange, which is mainly linked to the variable presence of phages, plasmids, PAI, and conjugative elements. A recently described mechanism of PAI movement by plasmid integration, due to a pheromone-responsive plasmid as mediator of genome plasticity, was described in *E. faecalis*. The Authors observed that the amount of transferred chromosome varied considerably, mainly when the V583 genome was used as donor chromosome from which the largest transfer (over 25%) was obtained. Traits that were mobilized into the *E. faecalis* OG1RF recipient included a capsule locus, a vancomycin-resistance transposon, the PAI, and even MLST markers, creating a double locus variant of the parental strain in a single event (Manson et al., 2010).

In a recent study, the differences and identities among 16 *E. faecalis* draft genome sequences were correlated to the location and content of "Clustered, regularly interspaced short palindromic repeats" (CRISPR) loci (Palmer and Gilmore, 2010). CRISPR loci have been shown in Bacteria and Archaea to confer resistance

to plasmid and phage entry, in a manner analogous to acquired immunity. This immunity depends on the presence of specific target-derived spacer sequences, the intervening repeat palindromes, and nuclease activity encoded by the *cas* genes (Barrangou et al., 2007; Marraffini and Sontheimer, 2008, 2010; Horvath and Barrangou, 2010). The comparison of the genomic sequence of *E. faecalis* OG1RF and *E. faecalis* V583, revealed that the former possesses two CRISPR loci – a CRISPR locus carrying their *cas* genes (CRISPR1-*cas*), and an orphan locus lacking *cas* genes (CRISPR2) – differing from the latter, which showed only the orphan CRISPR2 locus, and lacking CRISPR1-*cas*. (Barrangou et al., 2007; Marraffini and Sontheimer, 2008, 2010; Horvath and Barrangou, 2010).

In *E. faecalis* V583, the absence of CRISPR-*cas* may have reduced the barrier to entry of foreign elements, resulting in the convergence and accumulation of 6 plasmids or plasmid remnants, 7 phage or phage remnants, and over 40 IS elements, while OG1RF natively lacks plasmids (McBride et al., 2007; Bourgogne et al., 2008). The same Authors also found a highly significant inverse relationship between the presence of a CRISPR-*cas* locus and acquired antibiotic resistance in *E. faecalis* and similarly in additional 8 genomes, suggesting that antibiotic use inadvertently selects for enterococcal strains with compromised genome defense (Palmer and Gilmore, 2010). It is interesting that no CRISPR spacers have yet been identified with sequence identity to conjugative transposons such as Tn*916* and, in a similar manner, spacers targeting the Inc18 plasmid family, with the "crucial" role in the dissemination of vancomycin-resistance genes from enterococci to MRSA are also absent (Sieradzki et al., 1999; Tenover et al., 2001; Srinivasan et al., 2002; Chang et al., 2003; Malachowa and DeLeo, 2010; de Niederhausern et al., 2011). In all these cases it has been possible to hypothesize that these elements may evade the CRISPR-*cas* system defense.

A recent paper of van Schaik et al. (2010) has demonstrated the lack of the CRISPR-*cas* in 7 pyrosequenced-based *E. faecium* genome analyses.

DOES THE MOBILOME DRIVE THE CHANGE?

The mobilome, defined as all MGEs able to move around within or between genomes, contributes to genome plasticity as well as dissemination of antibiotic-resistance genes and pathogenicity. For our purposes here, for example in *E. faecalis V587*, mobile elements, that constitute one-quarter of its genome, include three independently replicating plasmids, three chromosomally integrated plasmid remnants, seven prophages, and a PAI (Shankar et al., 2002; Paulsen et al., 2003). It has been also reported that the acquisition of exogenous DNA could be involved in the conversion from a commensal to a pathogenic behavior in *E. faecium* (Willems and van Schaik, 2009).

The *E. faecium* strains belonging to CC17, are similarly characterized by an abundance of exogenously acquired genes, including insertion sequences, phages, and antibiotic-resistance genes carried on transposons (Leavis et al., 2007).

In this respect, the first CTn (conjugative transposon), Tn*916*, was originally discovered in the late 1970s in *E. faecalis* when tetracycline resistance was demonstrated to be transferable from *E. faecalis* DS16 to *E. faecalis* JH2-2. Tn*916* belongs to the Tn*916*/Tn*1545* family and contains 24 ORFs involved in conjugal transfer, excision, integration, and antibiotic resistance. This genetic element has an extraordinary ability to acquire accessory genes such as resistance genes to various antibiotics or lantibiotic immunity, and it is able to transfer onto over 35 different genera of bacteria. For all these reasons, Tn*916*-like elements assume a pivotal role as vectors in the dissemination of various traits among environmental, commensal, and pathogenic bacteria (Roberts and Mullany, 2009).

After the emergence of enterococcal antibiotic resistance to beta-lactams and aminoglycosides in the 1980s, the first reports on vancomycin resistance in hospital isolates in Europe (Uttley et al., 1988) were very disturbing; but more disturbing was the detection of this resistance outside health-care settings, and precisely in the feces of pigs, poultry, and pets in Europe, for the first time, in 1993, inducing the European Union to ban glycopeptide use as a growth promoter in animals (Bates et al., 1994; Klare et al., 1995; Bates, 1997; van den Bogaard et al., 2000).

Resistance to glycopeptides in enterococci is mediated by nine different vancomycin-resistance determinants, but major vancomycin-resistance phenotypes are VanA and VanB (Courvalin, 2006; Boyd et al., 2008; Lebreton et al., 2011). The former is associated with Tn*1546* carrying the *van*A gene, often located on a plasmid belonging to the broad host range Inc18 family, involved in the vanA transfer from enterococci to MRSA; while the *van*B operon, carried by the Tn*1549* conjugative transposon, can be frequently part of large conjugative chromosomal elements or integrated in conjugative plasmids. More recently, the first description of a *van*B2-Tn*1549*-like element in pheromone-responsive (pCF10-like) plasmids in *E. faecalis* strains has been reported. This transfer was mediated by a single event, resulting in the contemporary acquisition of: (i) the conjugative transposon Tn*1549* carrying the *van*B2-type gene; (ii) genes involved in the pheromone-response of self-transferable plasmids; and (iii) the origin of plasmid transfer (oriT; Zheng et al., 2009; Hegstad et al., 2010). In addition, Tn*1546* has undergone a large number of changes in VRE and a total of 22 different Tn*1546-like* elements have been identified: they can contain mutations, deletions or insertions of IS (IS*1216V*, IS*1251*, IS*1216V-IS3*-like, IS*Ef1*; Novais et al., 2008; Werner et al., 2008).

Composite multi-resistance elements have also been described: among them, Tn*5385* is a 65-kb element integrated into the chromosome of a clinical *E. faecalis,* carrying genes involved in erythromycin, streptomycin, tetracycline/minocycline, penicillin, and mercury resistance. This composite element contains regions previously found in staphylococcal and enterococcal transposons: Tn*5381* and Tn*5385* from enterococci and Tn*4001* and Tn*552* from staphylococcal origin, carrying respectively aminoglycosides (*aac*A-*aph*D) and beta-lactams (*bla*I-*bla*R1-*blaZ*; Rice and Carias, 1998).

Plasmids are abundant in enterococci and they comprise a substantial part of the auxiliary genome: they are responsible for much of the horizontal gene transfer that has allowed antibiotic and virulence traits to converge in hospital adapted lineages (Palmer et al., 2010b; Rosvoll et al., 2010). The pheromone-responsive plasmids have been described mainly in *E. faecalis* (Palmer et al., 2010b;

Laverde Gomez et al., 2011a,b). pAD1, and subsequently, pCF10 were the first plasmids to be described with pheromone-mediated transfer, even between different species (Dunny et al., 1978, 1981; An and Clewell, 1997).

Recent studies described the location of hyl_{Efm} gene in association with other resistance determinants such as the *vanA* operon, the *ermB* gene and the *tcrYAZB* operon (heavy metal resistance) in a large conjugative plasmids, pLG1 (281.02 kb) in *E. faecium* CC17. The hyl_{Efm} gene, encoding a putative hyaluronidase, an important factor involved in colonization and adhesion, is also described as a part of a genomic island (GI). The diffusion of a multi-resistant megaplasmid pLG1 carrying hyl_{Efm} could explain the diffusion of the so frequent hospital-associated *E. faecium* CC17 genotype (Freitas et al., 2010; Kim et al., 2010; Laverde Gomez et al., 2011b; Panesso et al., 2011).

CONCLUSION

In conclusion, population biology and genome sequence-based studies have greatly improved our understanding on enterococci, at least with respect to the most diffused and studies species, i.e., *E. faecalis* and *E. faecium*.

Even if not conclusive and not valid for all species, from the numerous studies involving strains isolated from different origins (humans, animals, various environments), it is becoming more evident the role of the mobilome in driving the colonization of new niches and hosts, eventually influencing their evolution.

Mobile genetic elements are important forces of evolution in many bacterial species: the discovery that up to 25% of the *E. faecalis* V583 genome is made up of exogenous mobile genes, opens the question if this is a limited characteristic or an enterococcal genome character. Important contributions will come from the complete genome sequence comparisons, now more easily obtainable by using next generation sequencing technologies, and probably all these studies will resolve many questions related to the ability of these microorganisms to be, at the same time, host-specific and host-variable, to be harmless and opportunistic pathogens. Furthermore, studies on gene regulation in different hosts and environments, involving, for example, global regulators such as sRNA, will probably give further insight into this flexible group of microorganisms.

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The *Acinetobacter baumannii* oxymoron: commensal hospital dweller turned pan-drug-resistant menace

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During the past few decades *Acinetobacter baumannii* has evolved from being a commensal dweller of health-care facilities to constitute one of the most annoying pathogens responsible for hospitalary outbreaks and it is currently considered one of the most important nosocomial pathogens. In a prevalence study of infections in intensive care units conducted among 75 countries of the five continents, this microorganism was found to be the fifth most common pathogen. Two main features contribute to the success of *A. baumannii*: (i) *A. baumannii* exhibits an outstanding ability to accumulate a great variety of resistance mechanisms acquired by different mechanisms, either mutations or acquisition of genetic elements such as plasmids, integrons, transposons, or resistant islands, making this microorganism multi- or pan-drug-resistant and (ii) The ability to survive in the environment during prolonged periods of time which, combined with its innate resistance to desiccation and disinfectants, makes *A. baumannii* almost impossible to eradicate from the clinical setting. In addition, its ability to produce biofilm greatly contributes to both persistence and resistance. In this review, the pathogenesis of the infections caused by this microorganism as well as the molecular bases of antibacterial resistance and clinical aspects such as treatment and potential future therapeutic strategies are discussed in depth.

Keywords: ***Acinetobacter baumannii*, antimicrobial resistance, pathogenesis, biofilm, antimicrobial peptides**

INTRODUCTION

The *Acinetobacter* genus comprises Gram-negative non-fermenting coccobacilli with 25 validly named species and 9 genomic species defined by genomic DNA–DNA hybridization (Espinal et al., 2011b). Although *Acinetobacter pittii* and *Acinetobacter nosocomialis* (formerly *Acinetobacter* genomic species 3 and gen. sp. 13TU, respectively (Nemec et al., 2011) are emerging as important pathogens and have been involved in a number of outbreaks in intensive care units, *Acinetobacter baumannii* is, undoubtedly the species showing the highest clinical relevance, mainly in the nosocomial setting. In fact, the three clinically important members of this group, also known as the *A. baumannii* (Ab) group (Peleg et al., 2008), are phenotypically related and cannot be differentiated by currently available identification systems. Indeed, *A. pittii* and *A. nosocomialis* are often erroneously identified as *A. baumannii* by routine commercial systems (Bernards et al., 1996). In a recent report by Espinal et al. (2011c) the use of MALDI–TOF mass spectrometry to differentiate the three species was analyzed, showing that *A. nosocomialis* was misidentified as *A. baumannii*. Inclusion of specific signature profiles for *A. nosocomialis* within the Bruker database allowed the correct identification of this genomic species, thus, MALDI–TOF MS spectra can be used as a fast, simple, and reliable method to identify members of the Ab group. Two main features contribute to the success of *A. baumannii*: (i) They normally exhibit multidrug resistance (MDR), acquired by different mechanisms, either mutations or acquisition of genetic elements such as plasmids, transposons, or resistant islands, and (ii) The ability to survive in the environment, in which, the production of biofilm plays an important role (discussed below). Several reviews dealing with the taxonomy, epidemiology, and infection of *A. baumannii* have been published on recent years (Dijkshoorn et al., 2007; Peleg et al., 2008; Towner, 2009; Visca et al., 2011). The review presented here provides an updated overview of the clinical and treatment aspects as well as the pathogenesis of antimicrobial resistance in *A. baumannii*. In addition, future potential therapeutic alternatives are discussed.

PATHOGENESIS AND VIRULENCE FACTORS OF *ACINETOBACTER BAUMANNII*

Multiple bacterial virulence factors are required for the pathogenesis of infections caused by *A. baumannii*. These factors enable microorganisms to colonize/infect the host efficiently. However, very little information is known about the virulence factors in *A. baumannii* and host responses to infection (Cerqueira and Peleg, 2011). Considering that *A. baumannii* is a multidrug-resistant microorganism, identification of the virulence factors, and the pathogenicity mechanisms could contribute to the development of novel therapeutic alternatives for the control of *Acinetobacter* infections.

MOTILITY AND ADHERENCE

Acinetobacter baumannii, has been described as non-motile (Tomaras et al., 2003). Its name is derived from the Greek "akineto," which means motionless or non-motile and was given due to

the lack of flagella (McBride, 2010). Analysis of new *A. baumannii* genome sequences has revealed an absence of flagellar genes, therefore swarming, which is mediated by flagella (Clemmer et al., 2011), is unlikely in this microorganism. However, *A. baumannii* spreads rapidly over surfaces probably as the result of twitching motility, a form of surface translocation previously described in *Acinetobacter calcoaceticus* (Henrichsen and Blom, 1975). Twitching motility mediated by the extension and retraction of type IV pili is controlled by a large number of genes, some involved in the assembly of type IV pili (*pilA, pilB, pilC, pilF, pilM, pilN, pilO, pilP, pilQ, pilZ, pilW*), twitching (*pilR, pilS, pilT, pilU*), and the pilin filament (*pilA*; Mattick, 2002; Jarrell and McBride, 2008). Although there is no current experimental evidence of the actual involvement of type IV pili in *A. baumannii* motility, a recent publication by Eijkelkamp et al. (2011) has shown the presence of several genes associated with the synthesis of type IV pili in the genomes of fully sequenced *A. baumannii* strains. The authors have also demonstrated a positive correlation between the degree of sequence conservation of the gene encoding the pilin subunit PilA and the twitching phenotype exhibited. Moreover, type IV pili has also been related to twitching motility in other non-flagellated gamma-proteobacteria (De La Fuente et al., 2007).

Twitching motility is also controlled by a range of signal transduction systems, including two-component sensor-regulators and a complex chemosensory system (Mattick, 2002). In a recent study, Clemmer et al. (2011) found that the motility exhibited by *A. baumannii* was partially dependent on a functional *pilT* gene. The loss of this gene in the *A. baumannii* M2 strain resulted in a 54% reduction in motility, suggesting that twitching represents a significant component of the overall motility in *A. baumannii*.

BIOFILM

Adherence to host cells represents the initial step of colonization or infection. During colonization, bacteria may form microcolonies which result in a highly structured microbial community, called biofilm. Biofilm constitutes a structural community of multiple bacterial cells associated with a biotic or abiotic surface, enclosed in a polymeric matrix (comprised of carbohydrates, nucleic acids, proteins, and other macromolecules; Costerton, 1995), constituting a protective mechanism to survive in harsh environments and during host infection. These bacteria become more resistant to antimicrobial stressors, antibiotics, or cleaning than their planktonic counterparts and therefore the ability to generate biofilms represents an important virulence factor (Donlan, 2002; Wroblewska et al., 2008; de Breij et al., 2009; Gaddy and Actis, 2009).

Biofilm can be influenced by common factors such as nutrient availability, bacterial appendages, bacterial surface components, quorum sensing (QS), macromolecular secretions (Irie and Parsek, 2008; Gaddy and Actis, 2009; Bhargava et al., 2010), and complex regulatory networks including two-component regulatory systems and transcriptional regulators which are related to the expression of biofilm-associated gene products in response to environmental signals (Stanley and Lazazzera, 2004). Tomaras et al. (2003) demonstrated that the ability of *A. baumannii* strain ATCC 19606^T to form pili and to adhere and form biofilm on abiotic surfaces depends on the expression of the *csuE* gene, which is a component of the CsuA/BABCDE chaperone-usher complex important for the assembly and production of pili involved in adhesion to surfaces. Inactivation of *csuE* results in the abolition of pili production and biofilm formation suggesting that CsuA/BABCDE-mediated pili play a role in the initial steps of biofilm formation. The expression of this operon is controlled by a two-component regulatory system including a sensor kinase encoded by *bfmS* and a response regulator encoded by *bfmR*. Inactivation of *bfmR* results in a loss of expression of the *csu* operon and, therefore, abolition of pili production and biofilm formation on abiotic surfaces. Additionally, the coupling of pili to host cell receptors may induce the production of inflammatory mediators such as chemokines and cytokines (Sauer et al., 2000).

In addition to the CsuA/BABCDE-mediated pili, de Breij et al. (2009) found that *A. baumannii* ATCC19606^T produces a CsuA/BABCDE-independent short pilus, which may be involved in the adherence of the bacteria to biotic surfaces, such as human respiratory cells.

For the development of mature biofilm structures, an ortholog of a staphylococcal biofilm-associated protein (Bap) was found in *A. baumannii* strain 307-0294. Transposon inactivation of this protein involved in cell–cell interactions resulted in destabilization of the mature biofilm on abiotic or biotic surfaces (Loehfelm et al., 2008).

Several studies have shown that some *A. baumannii* strains can adhere to human cells and form biofilm on abiotic surfaces. *A. baumannii* survives on fingertips and inanimate objects such as glass, plastic, and other environmental surfaces, even after exposure to dry conditions and nutrient starvation during extended periods of time (Jawad et al., 1996; Wendt et al., 1997; Lee et al., 2006b; de Breij et al., 2009; Espinal et al., 2012). The survival of *A. baumannii* has also been attributed to resistance of this microorganism to antimicrobial drugs and desiccation (Jawad et al., 1998; Tomaras et al., 2003). Since *A. baumannii* can produce biofilm, the resistance phenotype could be attributed to the ability of *A. baumannii* clinical strains to form biofilms on abiotic surfaces, particularly in strains isolated from catheter-related urinary tract or bloodstream infections or even from a case of shunt-related meningitis (Tomaras et al., 2003; Rodríguez-Baño et al., 2008; Gaddy and Actis, 2009). In a recent study, Espinal et al. (2012) performed survival assays with biofilm- and non-biofilm-forming strains on glass coverslips in a desiccated environment. The survival times for the biofilm-forming strains were longer than for the non-biofilm-forming strains (36 versus 15 days, respectively, $P < 0.001$), demonstrating that *A. baumannii* strains can attach to glass coverslips and also form biofilm, allowing their survival under dry conditions for much longer lengths of time than non-biofilm-forming strains and may contribute to its persistence in the hospital environment, increasing the probability of causing nosocomial infections and outbreaks. A few previous reports have described the ability of clinical isolates of *A. baumannii* to attach to and form biofilms on glass surfaces comprising an amorphous material similar to exopolysaccharide (Vidal et al., 1996; Tomaras et al., 2003; Espinal et al., 2012). Scanning and transmission electron microscopy (**Figure 1**) studies have shown appendages and a polysaccharide layer covering the cells only in biofilm-forming strains (Espinal et al., 2012). This

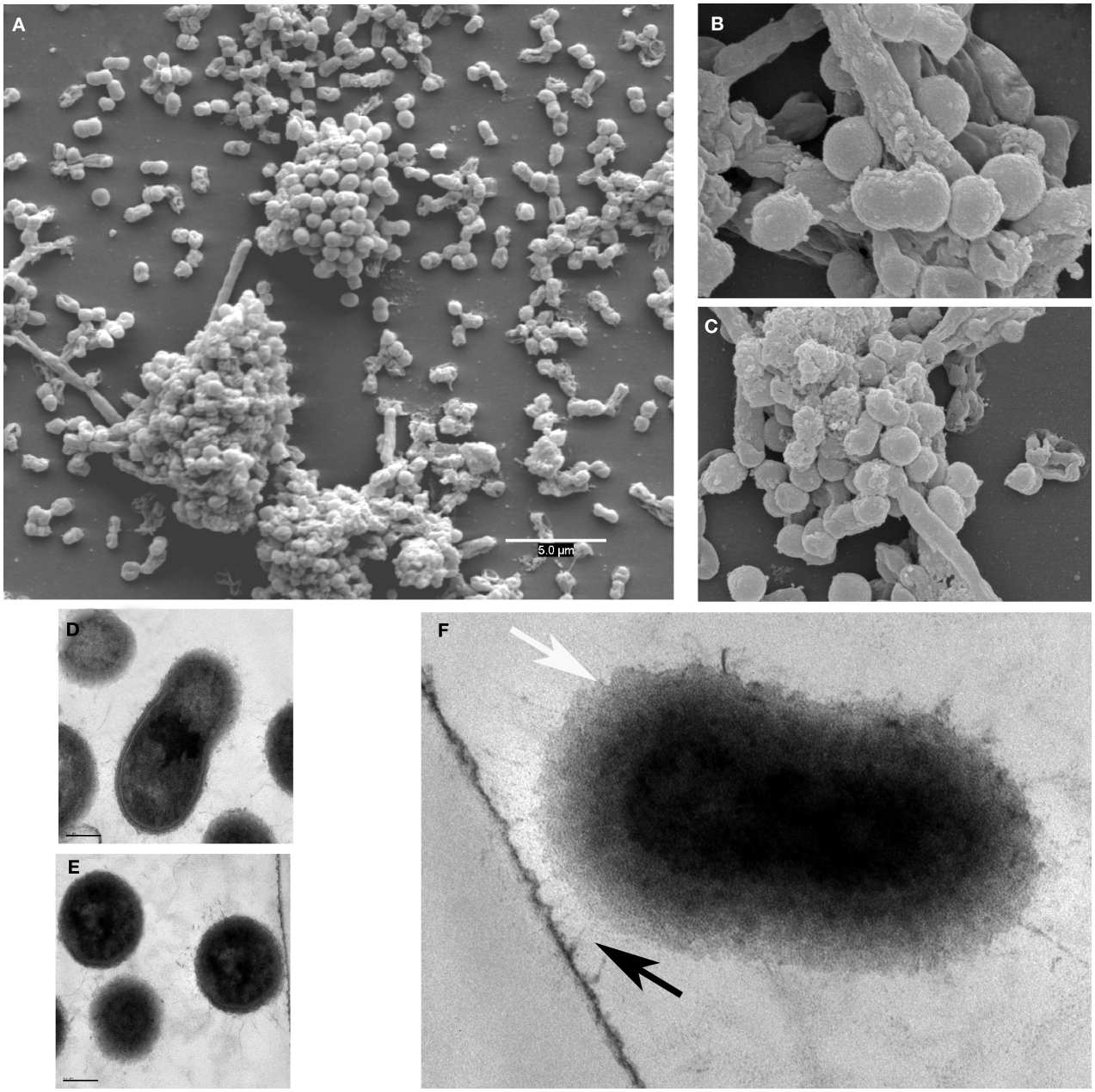

FIGURE 1 | Scanning electron microscopy (SEM) (A–C). Transmission electron microscopy (TEM) **(D–F)** of an *A. baumannii* biofilm-forming strain in liquid medium. Black arrow specifies appendage structures (pili or fimbriae), and white arrow the thick exopolysaccharide layer. All electron microscopy images are derived from the work by Espinal et al. (unpublished data)

highly hydrated layer may prevent lethal desiccation and, thus, protect against variations in humidity as well as contribute to mechanical stability, longer survival, and antimicrobial resistance (Sutherland, 2001; Donlan, 2002).

SURFACE POLYSACCHARIDES

Surface polysaccharide, such as capsule, is considered an important virulence trait in Gram-negative bacteria, but its role in the pathogenesis of *A. baumannii* is non-existent. Russo et al. (2010) obtained mutants from the *A. baumannii* strain AB307-0294 and identified two genes: *ptk*, that was predicted to be required for capsule polymerization and encodes a putative protein tyrosine kinase (PTK), and *epsA*, required for assembly and encoding a putative polysaccharide export outer membrane protein (EpsA). These genes are required for a capsule-positive phenotype and describe the participation of capsule in the pathogenesis of *A. baumannii*. This study demonstrated that the K1 capsule from the *A. baumannii* strain AB307-0294 was necessary for optimal

growth in human ascitic fluid and survival in human serum as well as in a rat tissue infection model. The active protection of the capsule allows bacterial resistance to the bactericidal activity of the complement.

On the other hand, the potential of PtK and EpsA as drug targets was observed when the loss of PTK or EpsA resulted in a complete and durable killing of AB307-0294 *in vivo*.

Other important extracellular polysaccharide is the poly-β-(1-6)-*N*-acetyl glucosamine (PNAG) described as a major component of biofilms in *Staphylococcus epidermidis* and *S. aureus* (Kropec et al., 2005). In *A. baumannii* clinical isolates the *pgaABCD* locus, encoding proteins for the synthesis of PNAG, has been proved critical for biofilm development (Choi et al., 2009). Besides the role in surface and cell-to-cell adherence, PNAG has been described as a virulence factor that also protects bacteria against innate host defenses. Although PNAG is not essential for biofilm formation under static conditions, it is required for maintaining the integrity of *A. baumannii* biofilms in dynamic and stressful environments. Indeed, PNAG has shown a role in the pathogenesis of *A. baumannii* and is a candidate vaccine against this pathogen (Choi et al., 2009; Cerqueira and Peleg, 2011).

LPS structures containing long O-specific sugar chains (smooth or S-type LPS) have been associated with virulence, and previous studies have shown that nosocomial isolates of *A. baumannii* produce rough R- and smooth S-type LPS. The differences in the ability of LPS from different isolates of *A. baumannii* to elicit mitogenic activity could be caused by variations in the fatty acid content of lipid A and in the O-antigens. LPS and lipid A from *Acinetobacter* have exhibited lethal toxicity in mice, pyrogenicity in rabbits, as well as complement inactivation *in vitro* (García et al., 1999). Pantophlet et al. (1998) suggested an important role for the LPS from nosocomial strains of *A. baumannii* as a virulence factor *in vivo* and that synthesis of endotoxin could be an important factor responsible for the severity of disease observed during sepsis by this microorganism.

Although little is known of the endotoxic potential of *A. baumannii* LPS with respect to human cells and its ability to stimulate inflammatory signaling via human toll-like receptors (TLRs), Erridge et al. (2007) investigated the biological activity of these endotoxins in human monocytic THP-1 cells and in TLR-deficient HEK-293 cells transfected with human TLR2 and TLR4 constructs. The results of this study showed that endotoxins derived from clinical isolates of *A. baumannii* and one *Acinetobacter* genomic species 9 are potent stimulators of inflammatory signaling in human monocyte cells and the responses to these bacteria are dependent on TLR2 and TLR4.

These results, however, are in apparent conflict with a recently published study by de Breij et al. (2010) that investigated the interplay among biofilm formation, adherence, and induction of an inflammatory response in human airway epithelial cells. De Breij and co-workers concluded that clinically relevant *A. baumannii* strains showing good adherence to human epithelial cells elicit a poor inflammatory response, allowing *Acinetobacter* strains to evade the host immune system and, therefore, explaining the exceptional survival and persistence capabilities of this microorganism.

These findings were in agreement with those reported in *Haemophilus influenzae* (Bresser et al., 1997).

OUTER MEMBRANE PROTEINS

Outer membrane proteins (OMPs) of Gram-negative bacteria have been related to antibiotic resistance, adaptation, and pathogenesis in the host cells. Some OMPs of the OmpA family have been characterized in *Acinetobacter* strains, and represent one of the major OMP in the genus (Vallenet et al., 2008).

Previous studies have determined that bacterial molecules secreted from *A. baumannii* are responsible for host cell death (Lee et al., 2001). Among these molecules, OmpA from *A. baumannii* (AbOmpA) makes up a potential virulence factor with multiple important effects in pathogenesis and signal processing (Perez et al., 2011). AbOmpA is the most abundant surface protein involved in the adherence to and invasion of epithelial cells and induces apoptosis in the early stages of *A. baumannii* infection (Gaddy et al., 2009).

Acinetobacter baumannii can induce cell death (Hep-2 cells) by means of cell surface death receptors and mitochondrial disintegration. Purified AbOmpA was identified in the mitochondria, where it induced the release of proapoptotic molecules such as cytochrome *c* and apoptosis-inducing factor (AIF), which mediates caspase-dependent and AIF-dependent apoptosis in epithelial cells and degrades chromosomal DNA (Choi et al., 2005). Apoptosis of epithelial cells can lead to the internalization of bacteria through the disrupted mucosal lining and, therefore, the outcome of infections caused by this microorganism depends on apoptosis induction in the epithelial cells (Choi et al., 2005).

AbOmpA is also implicated in resistance to complement and biofilm formation (Gaddy and Actis, 2009; Kim et al., 2009). Some *A. baumannii* strains are resistant to the killing activity of human serum by means of genetic components that increase *Acinetobacter* capabilities to cause bacteremia. The suggested serum-resistant mechanism involves the inhibition of host complement C3 protein cleavage and binding to the bacterial surface, which inhibit host phagocytic cell recognition and result in a serum-resistance phenotype (Kim et al., 2009). In addition, the binding of complement regulators to OMPs also plays a role in complement activation. Kim et al. (2009) demonstrated that an alternative complement pathway was responsible for the killing of *A. baumannii* in normal human serum. *In vitro* assays have indicated that factor H, the main regulator of this pathway, bound to the surface of this microorganism treated with normal human sera, may contribute to the persistence and dissemination of *Acinetobacter* in the host. Comparisons between *A. baumannii* ATCC19606^{T} and isogenic AbOmpA^{-} mutants have shown that mutant strains lose adherence and invasion capability in the host cells, demonstrating that AbOmpA participates in the evasion of the complement attack by interacting with factor H (Choi et al., 2008). In this sense, AbOmpA is considered the main complement regulator-acquiring surface protein (Choi et al., 2008) whereas secreted AbOmpA induces apoptosis in epithelial cells (Choi et al., 2005). In view of these results, AbOmpA stands out as an important virulence factor in *A. baumannii* and could be used as a target in the development of antibiotics and vaccines against this microorganism (Kim et al., 2009).

OUTER MEMBRANE VESICLES

In addition to OmpA, some Gram-negative bacterial species secrete outer membrane vesicles (OMVs) during bacterial growth. OMVs are spherical nanovesicles with a diameter between 20 and 200 nm which are composed of lipopolysaccharides, OMPs, lipids, and DNA or RNA (Kwon et al., 2009; Jin et al., 2011). Secreted OMVs have been shown to participate in QS, the transport of virulence factors, the inhibition of phagosome-maturation in macrophages, biofilm formation, and gene transfer (McConnell et al., 2011b; Rumbo et al., 2011). The surface factors of OMVs mediate adherence to host cells as well as the internalization of vesicular components suggesting that OMVs function as a transport vehicle of effector molecules into host cells (Kwon et al., 2009; Jin et al., 2011). Kwon et al. demonstrated that *A. baumannii* secretes OMVs during *in vitro* growth in association with some virulence-associated proteins and immune modulators suggesting that OMVs play a role in the pathogenicity of *A. baumannii*. AbOmpA, mediating the adherence to and invasion of *A. baumannii* to epithelial cells (Choi et al., 2008) was detected as a major protein component in the OMV fraction. Packaged in OMVs, AbOmpA induces cytotoxicity and host cell death. The results obtained in this study revealed that AbOmpA derived from OMVs was found in the cytoplasm of receiving cells, suggesting that OMVs can deliver virulence factors directly to host cells in the absence of bacteria.

Proteomic analysis of the OMVs from *A. baumanii* ATCC 19696^{T} and the clinical isolate DU202 identified more than 110 proteins derived from the outer membrane, periplasmic space, inner membrane, cytosol, and other additional undetermined sites. OMVs contained putative serine and Zn-dependent proteases, phospholipases, bacterioferritin, catalase, and a ferrichrome–iron receptor, as well as several proteins displaying secretion signals and pathogen-associated molecular patterns, such as LPS and lipoproteins (Kwon et al., 2009; Jin et al., 2011).

Since OMVs contain multiple antigenic proteins from the bacterial outer membrane, they could be used as vaccine antigens. Previous studies have shown that vaccination with OMVs resulted in the recovery of antibodies against multiple bacterial antigens displaying bactericidal activity and the ability to provide protection in animal models of infection (Schild et al., 2008). McConnell et al. (2011b) evaluated the immune response elicited after immunization with *A. baumannii* OMVs and found that the serum recovered from vaccinated mice reacted against multiple proteins present in the OMVs as well as against many proteins from the bacterial outer membrane. The robust and protective response elicited by this serum suggests that OMVs might constitute a viable immunization approach to control the infection and mortality caused by *A. baumannii*.

Vesicles have also been shown to be involved in the transfer of genetic material among similar bacterial species (Klieve et al., 2005). Rumbo et al. (2011) have demonstrated that OMVs vehiculate plasmids carrying carbapenem resistance genes such as the $bla_{\text{OXA-24}}$ gene, which can be readily transferred between different strains of *A. baumannii*. In this respect, OMVs might be considered as important virulence factors not only because they participate in host–pathogen interactions but also by allowing the spread of antibiotic resistance genes (such as those related to carbapenem resistance) to surrounding bacteria. The genetic material contained inside OMVs is protected from nucleases, thereby favoring the exchange of genetic material. These results might point toward a new mechanism for dissemination of resistance genes in addition to conjugation, transformation, and transduction processes.

HYDROLYTIC ENZYMES

Additional proteins proposed as virulence factors in *A. baumannii* also include Phospholipase D and Phospholipase C (Antunes et al., 2011a).

Phospholipase D, which is important for human serum resistance, epithelial cell invasion, and pathogenesis in a murine model of pneumonia, also plays a role in the systemic dissemination of bacterial pathogens within infected animals and is considered the main virulence determinant in *Corynebacterium pseudotuberculosis* (McKean et al., 2007). Jacobs et al. (2010) characterized a mutant *A. baumannii* strain harboring a transposon insertion within a putative phospholipase D (PLD) encoding-gene. In this study not only was phospholipase activity affected, but reduced *A. baumannii* epithelial cell invasion (*in vitro*) and serum proliferation were also reported. As a consequence, a decrease in bacteremia and colonization of visceral host organs was observed in a murine infection model. As suggested with AbOmpA, phospholipase D is an *A. baumannii* virulence factor that is required for wild-type levels of pathogenicity and is also a potential target for the therapeutic treatment of *Acinetobacter* infections (Jacobs et al., 2010).

Phospholipase C has also been shown to enhance the toxicity of epithelial cells. Camarena et al. (2010) demonstrated that an insertional mutant carrying a kanamycin cassette in the coding region of *plc1* displayed a reduction in the cytotoxic effect caused by *A. baumannii* on epithelial cells, thereby indicating that phospholipase C is an important factor in cellular damage.

ETHANOL-INDUCED PATHOGENESIS

In *A. baumannii*, ethanol is assimilated as a carbon source and enters the glyoxylate cycle which, in many pathogens, has been related to virulence. Smith et al. (2004) observed that ethanol promoted bacterial growth when *A. baumannii* was co-incubated with yeast and also demonstrated that low concentrations of ethanol stimulated *Acinetobacter* growth and contributed to endure salt stress. To understand the virulence of *A. baumannii* in the presence of ethanol, Camarena et al. (2010) characterized the transcriptional profile of this microorganism in the presence and absence of ethanol. They found 70 genes whose expression was affected by the presence of ethanol in the growth medium and suggested that virulence of *A. baumannii* in the presence of ethanol was due to increased metabolic capacity and the expression of some factors related to stress responses.

The genes that were induced by growth in the presence of ethanol during the exponential phase encoded proteins related to central metabolism or ethanol/acetate assimilation, such as ethanol dehydrogenase (AIS_2098) and aldehyde dehydrogenase (AIS_2102), genes encoding for *pta* (AIS_0481; phosphate acetyltransferase) and *ack*A (AIS_0482; acetate kinase), which are related to Acetyl-CoA synthesis.

Ethanol also induced genes involved in stress response and pathogenesis during exponential phase. Eleven genes encoded

hypothetical proteins; five unique to *A. baumannii* ATCC 17978 and, among the remaining six, AIS_2195 was exclusive of the *Acinetobacter* genus and AIS_2509 was only present in *A. baumannii* ATCC 17978 and the non-pathogenic *A. baylyi* ADP1. AIS_2509 was shown to contribute to ethanol stress survival together with AIS_2510 (HSP70-like), which is mildly induced by ethanol.

Ethanol can also enhance the virulence of *A. baumannii* by inducing heat-shock proteins such as Hsp90, GroEL, and Lon. Furthermore, ethanol promotes the expression of *plc1* (AIS_0043) which encodes the *A. baumannii* phospholipase C.

Additional genes detected in stationary phase cultures grown in the presence of ethanol include AIS_2381, which is required for acinetobactin synthesis, and AIS_2566 and AIS_2578, encoding a protein involved in siderophore synthesis and a siderophore receptor, respectively, which are required for the iron-uptake systems. Camarena et al. (2010) also demonstrated that ethanol induces a stress response enhancing bacterial fitness to survive in the host.

PENICILLIN-BINDING PROTEINS

Penicillin binding proteins (PBPs) are a family of enzymes that share a common evolutionary origin. These enzymes catalyze the synthesis of peptidoglycan, the primary component of the bacterial cell wall, and are also associated with cell morphogenesis and cell division complexes. The inhibition of PBPs causes instability in the cell wall, resulting in growth inhibition, or cell lysis (Cayo et al., 2011). PBPs have been classified as either high- or low-molecular mass PBPs. High-molecular mass PBPs enable peptidoglycan polymerization and insertion into the preexisting cell wall, and low-molecular mass PBPs contribute to cell separation and peptidoglycan remodeling (Sauvage et al., 2008). Although little is known about low-molecular mass PBPs, the putative low-molecular-mass penicillin-binding protein 7/8 (PBP-7/8) has been postulated to play a role in cell wall remodeling. PBP-7/8 is a hydrolase/endopeptidase. PBP-8 is an OmpT-mediated degradation product of PBP-7, and both PBPs stabilize and enhance soluble lytic transglycosylase 70 (*in vitro*). Although PBP-7/8 could be non-essential for normal elongation, it has been related to modulation of cell morphology and daughter cell separation. Russo et al. (2009) also demonstrated that a PBP-7/8-deficient mutant derivative of the wild *A. baumannii* AB307-0394 strain contributed to the pathogenesis of *A. baumannii* and participated in the growth and survival of *A. baumannii* in human ascites (*in vitro* and *in vivo*) in rat soft-tissue infection and pneumonia models. In addition, PBP-7/8 contributed either directly or indirectly to the serum resistance of AB307-0394.

IRON UPTAKE

Iron constitutes an important resource that it is not readily available in the human host. It is found complexed with iron-binding molecules such as heme, lactoferrin, and transferrin. A common alternative to more conventional human host defenses against bacterial infections involves the reduction of free extracellular iron concentration by means of iron-binding proteins. However, bacteria are able to survive and multiply under iron-limiting conditions found both in natural and host environments by exploiting a number of strategies for high-affinity iron acquisition, including production of ferric iron chelators (siderophores) that are released outside cells, uptake of exogenous chelators, such as heme and heterologous siderophores, and acquisition of ferrous iron (Dorsey et al., 2003a; Vallenet et al., 2008; Antunes et al., 2011a).

SIDEROPHORES

Siderophores are low-molecular-mass high-affinity iron chelating compounds classified according to their chemical structures (Dorsey et al., 2003a), as in catechols, high-affinity iron chelating molecules containing catecholate groups which are part of the iron-binding site.

The *A. baumannii* 8399 isolate, recovered during a nosocomial outbreak, was shown to contain a high-affinity iron-uptake system which included a catechol siderophore capable of scavenging iron from the high-affinity iron-binding proteins present in the human host (Dorsey et al., 2003b). The latter property was associated with the finding of *dhb* genes in the genome of *A. baumannii* 8399. Genetic complementation assays have proven that *dhb*A, *dhb*B, and *dhb*E genes encode active proteins that restore enterobactin biosynthesis and iron uptake when introduced in *Escherichia coli* mutants. These results demonstrated that *A. baumannii* 8399 harbors all the genetic determinants required for the biosynthesis of the catechol siderophore detected in iron-limited culture supernatants of this microorganism.

Acinetobacter baumannii 8399 also contains genes encoding proteins that are highly related to iron-transport proteins: OM73, an iron-regulated OMP detected in iron-starved *A. baumannii* 8399 cells; the P45 protein, the expression of which is regulated by both iron and Fur and could have a possible role in the secretion of the catechol siderophore produced by *A. baumannii* 8399; and the P114 protein, which may also participate in siderophore secretion in conjunction with the P45 protein (Dorsey et al., 2003b).

The mixed catechol-hydroxamate compound known as acinetobactin, (structurally similar to anguibactin and vibriobactin produced by *Vibrio cholerae*), was the first siderophore to be described in *A. baumannii*. Acinetobactin is produced by A. *baumannii* ATCC 19606^T and some clinical isolates (Yamamoto et al., 1994) but is absent in the non-clinical isolate SDF (Antunes et al., 2011b).

A comparison between the complete genomes derived from AYE, SDF, and ACICU clinical strains has demonstrated that AYE and ACICU express independent siderophore-mediated iron acquisition systems together with acinetobactin and hemin iron-capturing systems. Iron acquisition in the SDF strain, however, depends on the expression of a hemin system rather than on the expression of biosynthesis and transport functions for a particular siderophore. Several associated ABC transporters probably involved in the translocation of heme/hemoglobin from the periplasm to the cytosol have also been detected in this strain (Zimbler et al., 2009; Antunes et al., 2011a).

Furthermore, some *A. baumannii*-producing acinetobactin strains utilize 30% iron-saturated transferrin and 15% iron-saturated lactoferrin as the sole sources of iron for growth by scavenging iron bound to these proteins. None of these strains use heme or hemoglobin as an iron source. Insertional analysis has demonstrated that inactivation of *bau*A and *bas*D, encoding for acinetobactin transport and biosynthesis functions, respectively, affects the ability of isogenic derivatives to grow under

iron-limiting conditions (Mihara et al., 2004; Zimbler et al., 2009). These findings suggest that *A. baumannii* is a versatile pathogen with the ability to acquire iron and survive under iron-limiting conditions and highlights the role of acinetobactin-mediated iron acquisition mechanisms in the pathogenesis of *A. baumannii* infections (Mihara et al., 2004; Zimbler et al., 2009).

The ferric-siderophore complex is transported inside bacterial cells with the aid of specific outer-membrane receptors, periplasmic proteins, and inner-membrane-associated proteins, such as the iron-regulated outer membrane proteins system (IROMPs; Dorsey et al., 2003b; Mihara et al., 2004; Vallenet et al., 2008). In Gram-negative bacteria, these receptors are localized on the outer membrane, where internalization of siderophores or heme is associated with dissipation of the proton gradient on the inner membrane and is mediated by the TonB protein complex in the periplasmic space. The TonBExbBD energy-transducing system is required for hemin utilization in many bacteria. Once internalized, the ferric-siderophore complexes are reduced to release iron by an enzyme with ferric reductase activity (Vallenet et al., 2008; Zimbler et al., 2009; Antunes et al., 2011b).

In addition to the previously mentioned acinetobactin siderophore, Antunes et al. (2011b) have described two siderophore synthesis/transport gene clusters, which include genes for putative hydroxylase and acetyltransferase enzymes, suggestive of the synthesis of hydroxamate-type siderophores. One of these clusters was only present in the ATCC 17978, while the other was present in the ACICU, ATCC 17978, AYE, AB0057, AB307-294, and ATCC 19606^T *A. baumannii* strains, but not in the non-human SDF isolate.

Another alternative mechanism for bacterial iron assimilation involves the direct uptake of iron by the Feo system, a homolog of the *E. coli feo*B. This system, found in ACICU, ATCC 17978, AYE, AB0057, AB307-294, ATCC 19606^T, and SDF strains, consists of the cytosolic FeoA protein, the inner membrane Fe(II) permease FeoB, and the putative transcriptional repressor FeoC (Antunes et al., 2011b). The expression of the proteins implied in iron acquisition systems, including siderophore biosynthetic enzymes, depends on the transcriptional regulation of the global iron-binding repressor protein Fur, for ferric uptake regulator (Mihara et al., 2004; Vallenet et al., 2008), which acts as a transcription repressor of genes involved in the siderophore/heme(globin) systems. A bioinformatic search for Fur-binding sites revealed that putative Fur boxes were present in the intergenic regions of the abovementioned gene clusters, demonstrating the participation of these clusters in iron capture.

QUORUM SENSING

Bacteria elaborate chemical signals excreted from the cells to perform intercellular communication and environmental adaptation. This ability of bacteria to monitor cell density before expressing a phenotype is known as "quorum sensing" (Whitehead et al., 2001). QS is a widespread regulatory mechanism among Gram-negative bacteria and often takes place with the aid of acyl-homoserine lactone (AHL)-like signal molecules produced by the LuxI family of AHL synthases (Sarkar and Chakraborty, 2008; González et al., 2009). Modulation of the physiological processes controlled by acyl HSLs and, non-acyl HSL-mediated systems occurs in a cell density- and growth phase-dependent manner. AHLs-mediated QS has been linked with the production of virulence factors, motility, nodulation, plasmid transfer, antibiotic production, bioemulsan production, bioluminescence, and biofilm formation (Whitehead et al., 2001; Vallenet et al., 2008). These autoinducers bind to transcriptional regulatory proteins and activate or regulate gene expression in the organism (Bhargava et al., 2010). In a previous study, González et al. (2001) detected QS signal molecules capable of activating AHL biosensors in *A. baylyi* ADP1 cultures and in culture supernatants of some clinical *Acinetobacter* strains. A common AHL system of Gram-negative bacteria is mediated by two proteins: LuxI proteins which interact with the LuxR protein. This complex binds to a specific promoter sequence known as *lux*-box, which regulates the expression of QS target genes. In *Acinetobacter*, the *lux*-box is found upstream from the putative ATG of *aba*I, representing a binding site for AbaR. AbaI protein belongs to the LuxI family of autoinducer synthases, producing *N*-(3-hydroxydodecanoyl)-L-HSL (3-hydroxy-C(12)-HSL) as the primary signal and performing the function of signal transduction and QS. The AbaR protein, which is an autoinducer receptor in *A. baumannii*, interacts with AHL and controls gene expression (Niu et al., 2008).

Acinetobacter quorum signals have been shown to vary in virulent and non-virulent strains, thus differentiation between them on the basis of QS is difficult. On the other hand, communication among bacteria with respect to cell density is related to maturation of biofilm. It was observed that mutations in *abaI* led to a reduction in biofilm production compared with the isogenic parental strain but the original phenotype could be restored when a exogenous *Acinetobacter* AHL was added to the mutant (Niu et al., 2008; Bhargava et al., 2010). Thus, QS signal molecules influence biofilm formation which represents an important virulence factor related to the survival and antibiotic resistance of *A. baumannii* (Gaddy and Actis, 2009; Bhargava et al., 2010). Kang and Park (2010a) evaluated the effect of QS signals on hexadecane biodegradation and biofilm formation in the *Acinetobacter* spp. strain DR1, and found phenotypic changes in the strain associated with three putative QS signals that strongly indicated their relation in biofilm formation. Particularly, C_{12}-AHL, which was a major QS signal and controlled the biofilm formation in *A. baumannii* M2 previously described by Niu et al. (2008). The poor biodegradability of hydrocarbons was one of the significant changes observed when QS signals where eliminated, and consequently resulted in alterations of bacterial hydrophobicity, cellular motility by fimbriae or pili, biofilm formation, and other environmental factors (Clemmer et al., 2011). Thus, in the DR1 strain, the QS system and putative AHLs directly regulated the biofilm formation and growth on hydrocarbons. In another study, Kang and Park (2010b) described a trade-off between antibiotic resistance and biological fitness (Kang and Park, 2010b). The biological fitness of the *Acinetobacter* strain DR1 was influenced by the *in situ* acquisition of antibiotic resistance, probably attributable to the reduced ability to produce QS signals, lack of motility, and reduced substrate utilization.

As a concluding remark, it has been hypothesized that interference with bacterial virulence and/or cell-to-cell signaling

pathways, such as interruption of QS signals, known as quorum quenching (QQ; Chan et al., 2011), represents an important target for pathogenesis inhibition and development of novel therapeutics for the treatment of bacterial infections (Whitehead et al., 2001; Bhargava et al., 2010; Rasko and Sperandio, 2010).

PUTATIVE ALIEN ISLANDS

New insights into the pathogenesis of *A. baumannii* has demonstrated that mutagenesis and genomic sequencing can uncover and evaluate many virulence factors for a better understanding of the pathogenicity of this bacterium. Smith et al. (2007) performed a genomic analysis between *A. baumannii* and *A. baylyi* and found 55.74% homology at the protein level. One of the most interesting differences between both was the presence of 28 putative alien islands (pAs) in *A. baumannii* (Smith et al., 2007). These islands were found to contain the majority of the drug resistance and virulence factors, many acquired from their environment, indicating their role in the pathogenicity of *A. baumannii*. Some of these islands contained genes associated with pathogenesis, encoding heavy metal resistance, iron uptake and metabolism, fimbrial genes, autoinducer processing, and cell envelope biogenesis. Other pAs contained hypothetical genes and mobile elements that remain uncharacterized. Thus, gene shuffling mutagenesis revealed that pAs might (Camarena et al., 2010) play an important role in the pathogenesis of *A. baumannii*.

ANTIMICROBIAL RESISTANCE

While other human pathogens stand out due to their virulence and pathogenicity, *A. baumannii* is not known to be particularly virulent or to produce diffusible toxins or cytolysins, and only a few virulence factors have been described to date (Vallenet et al., 2008). There is, however, one feature at which this microorganism excels, and that is antimicrobial resistance. Indeed, *A. baumannii* has an outstanding ability to accumulate different resistance mechanisms which, together with its innate resistance to desiccation, contribute to the survival and persistence of *A. baumannii* under selective environmental pressure, making this microorganism a phenomenal nosocomial pathogen.

Acinetobacter baumannii has become resistant to almost all commonly used antimicrobial agents, including aminoglycosides, quinolones and broad-spectrum β-lactams, and multidrug or pan-drug-resistant strains are becoming a frequent problem in the clinical setting (Livermore et al., 2008; Rossolini and Mantengoli, 2008; Morgan et al., 2009; **Table 1**).

Overall, antimicrobial resistance can be achieved by means of two main mechanisms: acquisition of novel genetic information through horizontal gene transfer and genetic modification of endogenous genes.

Acquisition of novel genetic determinants in *A. baumannii* takes place by the combined effect of mobile genetic elements (insertion sequences, IS and transposons), integrons, and transferable plasmids, while the genetic modification of endogenous genes implies either spontaneous mutations that modify drug targets or the insertion/deletion of mobile elements that alter the expression of endogenous resistance mechanisms or modify membrane permeability.

β-LACTAMS

The mechanisms involved in resistance to β-lactams in *A. baumannii* typically include: (i) *enzymatic mechanisms* or production of β-lactam hydrolyzing enzymes (β-lactamases) and (ii) *non-enzymatic mechanisms* that involve modification of membrane permeability by either the loss of or decrease in the expression of OMPs or an increased expression of efflux pumps as well as sequence variation of PBPs.

ENZYMATIC MECHANISMS

The main mechanism of resistance to β-lactam antibiotics in *A. baumannii* lies in the production of β-lactamases encoded either chromosomally or in plasmids. The common genome of *A. baumannii* possesses two intrinsic β-lactam hydrolyzing enzymes; a non-inducible cephalosporinase (AmpC; Bou and Martinez-Beltran, 2000b) and a class D oxacillinase (OXA-51/69 variants; Brown et al., 2005).

Chromosomal cephalosporinases (AmpC)

AmpC enzymes are class C β-lactamases responsible for resistance to all penicillins and extended-spectrum cephalosporins, except cefepime, as well as to β-lactam-β-lactamase inhibitor combinations (Drawz et al., 2010). Several allelic variants of the AmpC enzyme have been reported and a new designation for this family of cephalosporinases has been defined (*Acinetobacter*-derived cephalosporinases, or ADC). According to sequence similarities as well as preferred substrates, AmpCs found within *Acinetobacter* have been classified in up to 56 different ADC types, with more than 25 variants being found in *A. baumannii* (Hujer et al., 2005; Zhao and Hu, 2012). Being non-inducible, the basal expression levels of AmpC enzymes from *A. baumannii* do not significantly alter susceptibility to β-lactams, although the presence of an upstream IS element (known as IS*AbaI*) promotes increased expression of bla_{ampC} and resistance to expanded-spectrum cephalosporins but not to cefepime and carbapenems. Expression of bla_{ampC} is apparently driven by promoter sequences within the IS*AbaI* element (Héritier et al., 2006).

Oxacillinases

Ambler Class D enzymes, also known as oxacillinases (OXA; Ambler, 1980), are distinguished by their ability to hydrolyze cloxacillin and oxacillin, and some also oxyimino-β-lactams, but not carbapenems and might be inhibited by clavulanic acid. The oxacillinase enzymes present in *Acinetobacter* spp., however, constitute an atypical subgroup of OXA enzymes since they present carbapenem-hydrolyzing activities (they hydrolyze imipenem and meropenem but not extended-spectrum cephalosporins and aztreonam) and, therefore, are also termed as carbapenem-hydrolyzing class D β-lactamases (CHDLs; Poirel and Nordmann, 2006a) and have their own group (2df) in the updated functional classification of β-lactamases recently published by Bush and Jacoby (2010). In *A. baumannii*, five phylogenetic subgroups of class D β-lactamases have currently been identified: the naturally occurring OXA-51/69 and four clusters of acquired CHDLs (OXA-23, OXA-24/40, OXA-58, and OXA-143), each with a variety of enzymes that represent different sequence substitutions.

Similar to bla_{ampC}, basal expression of the naturally occurring OXA-51 oxacillinase in *A. baumannii* only allows for

Table 1 | Antimicrobial resistance mechanisms in *A. baumannii*.

Antimicrobial	Resistance mechanism	Class/ family	Protein	Described in association with	Selected reference
β-Lactams	Chromosomal cephalosporinase	Class C	AmpC	IS	Hujer et al. (2005)
	Carbapenem-hydrolyzing class D β-lactamases	Class D	OXA-51-like	IS	Turton et al. (2006)
			OXA-23-like	IS, Tn, AbaR	Corvec et al. (2007), Adams et al. (2008)
			OXA-24/40-like	XerC/XerD	Merino et al. (2010)
			OXA-58-like	IS, Tn	Poirel and Nordmann (2006b)
			OXA-143-like		Higgins et al. (2009)
	Metallo-β-lactamases	Class B	IMP	Integron	Cornaglia et al. (2011)
			VIM	Integron	Cornaglia et al. (2011)
			SIM-1	Integron	Cornaglia et al. (2011)
			NDM	IS, Tn	Espinal et al. (2011a), Pfeifer et al. (2011)
	Minor relevance β-lactamases	Class A	TEM	AbaR	Adams et al. (2008), Shakil and Khan (2010)
			SHV		Naas et al. (2007)
			SCO-1		Poirel et al. (2007)
			CARB	IS, Tn, integron	Potron et al. (2009), Ramírez et al. (2010b)
			PER	IS, Tn, integron	Poirel et al. (2005a), Bonnin et al. (2011b)
			VEB	IS, integron, AbaR	Fournier et al. (2006), Poirel et al. (2009)
			CTX-M	Tn	Potron et al. (2011)
			GES	Integron	Moubareck et al. (2009)
			KPC		Robledo et al. (2010)
		Class D	OXA-2, 10, 20, 37	Integron, AbaR	Navia et al. (2002), Fournier et al. (2006), Adams et al. (2008)
	Decreased permeability		CarO	IS	Ravasi et al. (2011)
			47 kDa OMP		Quale et al. (2003)
			44 kDa OMP		Quale et al. (2003)
			37 kDa OMP		Quale et al. (2003)
			33–36 kDa OMP		del Mar Tomas et al. (2005)
			22–33 kDa OMP		Bou et al. (2000a)
			HMP-AB		Gribun et al. (2003)
			43 kDa OMP		Fernández-Cuenca et al. (2011)
	Efflux pump	RND	AdeABC	IS	Magnet et al. (2001)
			AdeIJK		Damier-Piolle et al. (2008)
	Modified penicillin-binding proteins		PBP		Cayo et al. (2011)
Aminoglycosides	Aminoglycoside-modifying enzymes		Acetyltransferases	IS, Tn, Integron, AbaR	Cho et al. (2009)
			Nucleotidyltransferases		Cho et al. (2009)
			Phosphotransferases		Cho et al. (2009)
	Target binding site modification		16S rRNA methylases	IS, Tn	Doi et al. (2007)
	Efflux	RND	AdeABC	IS	Magnet et al. (2001)
		MATE	AbeM		Su et al. (2005)
Quinolones	Target site mutations		GyrA/ParC		Hamouda and Amyes (2004)
	Efflux pump	RND	AdeABC	IS	Magnet et al. (2001)
			AdeIJK		Damier-Piolle et al. (2008)
			AdeFGH		Coyne et al. (2010b)
		MATE	AbeM		Su et al. (2005)
		SMR	AbeS		Srinivasan et al. (2009a)
Chloramphenicol	Efflux pump	RND	AdeABC	IS	Magnet et al. (2001)
			AdeIJK		Damier-Piolle et al. (2008)
			AdeFGH		Coyne et al. (2010b)

(Continued)

Table 1 | Continued

Antimicrobial	Resistance mechanism	Class/ family	Protein	Described in association with	Selected reference
		MFS	CmlA	AbaR	Fournier et al. (2006), Vila et al. (2007)
			CraA		Roca et al. (2009)
		MATE	AbeM		Su et al., 2005
		SMR	AbeS		Srinivasan et al. (2009a)
Tetracyclines	Efflux pump	MFS	TetA	IS, Tn, AbaR	Ribera et al. (2003a), Fournier et al. (2006)
			TetB		Vila et al. (2007)
	Ribosomal protection		TetM		Ribera et al. (2003b)
Tigecycline	Efflux pump	RND	AdeABC	IS	Magnet et al. (2001)
			AdeIJK		Damier-Piolle et al. (2008)
Polymyxins	Lipid A modification		PmrCAB		Arroyo et al. (2011), Beceiro et al. (2011)
	Loss of lipopolysaccharide		LpxABC	IS	Moffatt et al. (2011)
	Porin loss				Fernández-Reyes et al. (2009)

IS, Insertion sequence; Tn, transposon; AbaR, resistance island; RND, resistance–nodulation–cell division family; MFS, major facilitator superfamily; MATE, multidrug and toxic compound extrusion family; SMR, small multidrug resistance family.

weak hydrolysis of β-lactamic substrates, mainly penicillins and carbapenems (they are not active against expanded-spectrum cephalosporins; Héritier et al., 2005a) unless IS*AbaI* or IS*Aba9* elements are located upstream from *bla*$_{\text{OXA-51}}$-like genes to increase their expression (Turton et al., 2006; Figueiredo et al., 2009). Over 68 different OXA-51 sequence variants are classified as Class D enzymes (Zhao and Hu, 2012).

Originally named ARI-1, the acquired OXA-23 enzyme shares 56% identity at the protein level with OXA-51/69 and was initially identified in a plasmid from an *A. baumannii* isolate in Scotland, constituting the first oxacillinase with carbapenemase activity to be reported (Paton et al., 1993). Since then, the *bla*$_{\text{OXA-23}}$ gene has been identified all over the world, both in the chromosome or in plasmids, and it is apparently exclusive of the *Acinetobacter* genus, with a *Proteus mirabilis* isolate from France being the sole exception (Bonnet et al., 2002). OXA-27, OXA-49, and OXA-73 are also included within this cluster (Afzal-Shah et al., 2001; see also http://www.lahey.org/studies/).

The second cluster of acquired class D enzymes is named after the OXA-24/40 enzyme (OXA-24 and OXA-40 were initially identified as different enzymes but re-sequencing has shown that they are indeed indistinguishable; see http://www.lahey.org/studies/), which was originally isolated from the chromosome of a carbapenem-resistant *A. baumannii* isolate from Spain (Bou et al., 2000c). This class also includes the OXA-25, OXA-26, and OXA-72 (Afzal-Shah et al., 2001; Wang et al., 2007) enzymes and shares roughly 63 and 60% amino acid identity with the OXA-51/69 and OXA-23 clusters, respectively. Although the OXA-26 enzyme has been identified in Belgium (Afzal-Shah et al., 2001), the OXA-72 in isolates from Asia (Wang et al., 2007; Lee et al., 2009a; Lu et al., 2009) and more recently also from Croatia (Goic-Barisic et al., 2011), and the *bla*$_{\text{OXA-40}}$ gene has also been found in the United States (Lolans et al., 2006; Qi et al., 2008), the *bla*$_{\text{OXA-40}}$-like genes seem to be highly prevalent in Spain and Portugal (Quinteira et al., 2007; Ruiz et al., 2007) and they can also be found either in the chromosome or be plasmid-borne.

The third cluster of acquired CHDLs is represented by OXA-58, identified in *A. baumannii* by Poirel et al. (2005b), sharing 59% amino acid identity with OXA-51/69 and less than 50% amino acid identity with OXA-23 and OXA-24/40. The *bla*$_{\text{OXA-58}}$ gene has only been found in *Acinetobacter* spp. so far, and it has been detected in *A. junii* in Romania and Australia (Marqué et al., 2005; Peleg et al., 2006), in *A. pittii* and *Acinetobacter phenon* 6/ct 13TU in Spain (Martí et al., 2008a,b), in *A. nosocomialis* in Taiwan (Lin et al., 2010), and Wang et al. (2007) also reported the first identification of a *bla*$_{\text{OXA-58}}$-like gene in *Acinetobacter* genomic species 14TU in China.

The *bla*$_{\text{OXA-58}}$ gene is usually plasmid-encoded, which most likely accounts for its wide distribution throughout the world, although it is especially prevalent in Italy and Greece (D'Arezzo et al., 2009; Papa et al., 2009; Donnarumma et al., 2010; Di Popolo et al., 2011; Gogou et al., 2011), where carbapenem-resistant *A. baumannii* strains producing OXA-58 have caused several outbreaks in intensive care and pediatric units (Poirel et al., 2006; Pournaras et al., 2006; Tsakris et al., 2008). OXA-96 (Koh et al., 2007b) and OXA-97 (Poirel et al., 2008b) constitute point-mutation derivatives of OXA-58 included within the same cluster and with similar hydrolytic properties.

The fourth cluster of acquired CHDLs present in *A. baumannii* was identified more recently upon the isolation of a novel OXA-143 enzyme recovered from an *A. baumannii* clinical isolate in Brazil (Higgins et al., 2009). At the protein level, this enzyme is 88, 63, and 52% identical to OXA-24/40, OXA-23, and OXA-58 respectively, but exhibits a similar substrate profile to that of other CHDLs from *A. baumannii*.

Overall, the level of carbapenem-hydrolysis by CHDLs is considerably low, with imipenem being the preferred substrate over meropenem, thereby raising a debate as to the exact contribution of these enzymes to carbapenem resistance (Queenan and Bush, 2007). Héritier et al. (2005b) addressed this issue by studying changes in susceptibility to carbapenems using either knock-out mutants or transformation experiments with both natural

and recombinant plasmids containing $bla_{\text{OXA-23}}$, $bla_{\text{OXA-40}}$, and $bla_{\text{OXA-58}}$ in different hosts and genetic backgrounds. These experiments demonstrated that acquired CHDLs significantly contribute to the resistant phenotype, with OXA-40 and especially OXA-23 showing a significantly greater impact on resistance than OXA-58. In addition, they also noticed that CHDLs expressed from natural plasmids originated greater levels of carbapenem resistance than those expressed in recombinant vectors, pointing out the importance of IS elements to enhance the expression of acquired class D oxacillinases in *A. baumannii* (Héritier et al., 2005b). The role of mobile genetic elements in the expression and transfer of resistance determinants will be discussed further on.

Metallo-β-lactamases

Since their introduction back in 1985, carbapenems have probably been (and still are) the most important antimicrobial agents for the treatment of infections caused by multidrug-resistant *A. baumannii*. During the last decades, however, resistance to carbapenems is increasing worldwide (Gopalakrishnan and Sureshkumar, 2010; Davies et al., 2011; Gogou et al., 2011; Lee et al., 2011a) and carbapenem-resistant *A. baumannii* strains are commonly resistant to all other classes of antibiotics as well, showing intermediate susceptibility to rifampicin and only being susceptible to tigecycline and colistin, although resistance to both antimicrobials has also recently been reported (Al-Sweih et al., 2011; Taneja et al., 2011).

There is another class of acquired β-lactamases in *A. baumannii* which, together with Ambler class D enzymes, contributes to the carbapenem-resistant phenotype, the Ambler class B of metallo-β-lactamases or MBLs. This group of enzymes differs from other β-lactamases in its broad substrate profile (they are capable of hydrolyzing all β-lactams except the monobactam aztreonam), potential for horizontal transfer and the fact that they are zinc-dependent metalloproteins inhibited by EDTA but not by carbapenem or β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam (Bush and Jacoby, 2010).

Although MBLs are not as much widespread as class D enzymes in *A. baumannii*, they display a significantly higher hydrolytic activity toward carbapenems (100- to 1,000-fold; Poirel and Nordmann, 2006a). Of the several groups of MBLs described to date, only IMP, VIM, SIM, and the novel NDM have been found in *A. baumannii* and, with the exception of NDM, they are typically identified within class 1 integrons containing an array of resistance gene cassettes as well, usually including some aminoglycoside-resistance determinants (Riccio et al., 2000; Houang et al., 2003; Zarrilli et al., 2004; Lee et al., 2005; Tsakris et al., 2006). The importance of integrons and their contribution to the MDR phenotype will be further expanded later in this review.

IMP metallo-β-lactamases (named for being active on imipenem) were first described in Japan from a *Pseudomonas aeruginosa* isolate in 1990 (Watanabe et al., 1991). After that initial isolation, IMP enzymes have been reported worldwide in several Gram-negative bacteria, including *A. baumannii* (Queenan and Bush, 2007). So far there are up to 33 different IMP variants (according to http://www.lahey.org/studies/) but only 8 (IMP-1, -2, -4, -5, -6, -8, -11, and -19) have been identified in *A. baumannii*, mostly in Asia but also in Europe and parts of South America (Riccio et al., 2000; Chu et al., 2001; Da Silva et al., 2002; Towner et al., 2002; Gales et al., 2003; Lee et al., 2003, 2008; Yamamoto et al., 2011). Enzymes belonging to the IMP type have broad substrate specificity and are especially active against cephalosporins and carbapenems.

The first VIM enzyme (VIM-1) was initially described in 1999 from a *P. aeruginosa* isolate in the Italian location of Verona (hence, Veronese imipenemase; Lauretti et al., 1999) but the first VIM enzyme (VIM-2) in *A. baumannii* was not described until a few years later (Yum et al., 2002). VIM MBLs display <40% amino acid identity with IMP enzymes and share a similar substrate profile, albeit with a higher affinity toward carbapenems (Docquier et al., 2003). During the past decade several VIM variants (VIM-1, -2, -3, -4, and -11) have been identified in *A. baumannii* isolates, mainly in European and Asian countries (Yum et al., 2002; Lee et al., 2003, 2008; Tsakris et al., 2006; Wroblewska et al., 2007; Figueiredo et al., 2008) but also in clinical isolates of the closely related *A. nosocomialis* and *A. pittii* (Espinal et al., 2011b).

SIM-1 was originally found in *A. baumannii* in a tertiary care hospital in Seoul, Korea (hence, Seoul imipenemase), and so far this isolate contains the only $bla_{\text{SIM-1}}$ gene reported in this microorganism (Lee et al., 2005). SIM-1 exhibits 64–69% amino acid identity with IMP enzymes and, similar to IMP and VIM, it is capable of hydrolyzing penicillins, narrow-, and expanded-spectrum cephalosporins as well as carbapenems.

More recently, a novel class B metallo-β-lactamase enzyme has been reported. This enzyme was first identified in *Klebsiella pneumoniae* and *E. coli* clinical isolates recovered in Sweden from a traveler returning from India and has, hence, been termed NDM-1 for New Delhi metallo-β-lactamase (Yong et al., 2009). Similar to other MBL enzymes, NDM-1 confers resistance to all β-lactams except aztreonam. After its initial isolation, several reports have identified bla_{NDM} genes in Enterobacteriaceae worldwide due to their plasmidic localization that allows for rapid transfer and dissemination (Nordmann et al., 2011, 2012; Poirel et al., 2011a). In 2010 the $bla_{\text{NDM-1}}$ gene was first identified in an intensive care unit in India from an *A. baumannii* isolate that also contained the $bla_{\text{OXA-23}}$ gene and *armA* (Karthikeyan et al., 2010), a gene encoding a 16S rRNA methylase conferring resistance to aminoglycosides (Yamane et al., 2005). The chromosomal or plasmidic location of this gene, however, was not clear. Kaase et al. (2011) identified the first NDM variant in an *A. baumannii* isolate from Egypt, NDM-2, which differed from NDM-1 by a single amino acid substitution but shared an identical spectrum of hydrolysis. Although not fully demonstrated, $bla_{\text{NDM-2}}$ was thought to be chromosomally located. Several reports have subsequently described NDM enzymes in *A. baumannii* isolates from China, Germany, and Israel (Chen et al., 2011; Espinal et al., 2011a; Pfeifer et al., 2011) but also in an *Acinetobacter lwoffii* isolate from China (Hu et al., 2011). Interestingly, while the $bla_{\text{NDM-1}}$ and $bla_{\text{NDM-2}}$ from Germany (Pfeifer et al., 2011) and Israel (Espinal et al., 2011a), respectively, were found on the chromosome of *A. baumannii*, the $bla_{\text{NDM-1}}$ from Chinese isolates were located on several plasmids ranging from 30 to 50 kb in size (Chen et al., 2011) and as was the $bla_{\text{NDM-1}}$ gene found in *A. lwoffii* of Chinese origin.

Minor relevance β-lactamases

Additional β-lactamase enzymes belonging to the Ambler class A have also been described in *A. baumannii* but they are generally regarded of minor relevance since the prevalence of these enzymes as well as their specific contribution to the resistant phenotype is usually hindered by the presence of more prevalent mechanisms such as the intrinsic AmpC cephalosporinase or the naturally occurring OXA-51, among others (Poirel and Nordmann, 2006a).

Narrow-spectrum serine β-lactamases such as TEM-1, SCO-1, CARB-2, -4, and -8 as well as OXA-20, OXA-21, and OXA-37 have all been detected in *A. baumannii* (Vila et al., 1997a; Navia et al., 2002; Mammeri et al., 2003; Zarrilli et al., 2004; Koh et al., 2007b; Poirel et al., 2007; Wang et al., 2007; Ramírez et al., 2010b), but some other extended-spectrum β-lactamases and serine-carbapenemases are also present.

PER-1 was the first ESBL enzyme identified in *A. baumannii* (Vahaboglu et al., 1997) but also PER-2 and -7 (Pasterán et al., 2006; Bonnin et al., 2011b), VEB-1 (Poirel et al., 2003), TEM variants TEM-92, -116, and -150 (Naiemi et al., 2005; Endimiani et al., 2007; Shakil and Khan, 2010), GES-11, -12, and -14 (Bogaerts et al., 2010), the atypical expended-spectrum carbenicillinase CARB-10 (Potron et al., 2009), capable of hydrolyzing fourth generation cephalosporins but not ceftazidime or cefotaxime, at least three different SHV-type ESBLs (SHV-2, -5, and -12; Naiemi et al., 2005; Naas et al., 2007) as well as three different types of the plasmid-mediated CTX-M enzymes (CTX-M-2, -15, -43; Nagano et al., 2004; Celenza et al., 2006; Shakil and Khan, 2010) have been reported so far in this microorganism.

Interestingly, while PER and VEB are usually regarded as second-class β-lactamases in Enterobacteriaceae (Naas et al., 2008), they are more commonly found in *A. baumannii* and seem to be emerging in certain geographic regions where they are responsible for nosocomial outbreaks (Vahaboglu et al., 1997; Naas et al., 2006a,b). On the other hand, CTX-M variants, especially CTX-M-15, are widely disseminated among Enterobacteriaceae worldwide (Canton and Coque, 2006) but, until recently, were rare in *A. baumannii*. Recent reports seem to indicate an increase in *A. baumannii* strains bearing CTX-M variants within the Asia-Pacific region, most likely associated with transposon-mediated mobilization events (Nagano et al., 2004; Shakil and Khan, 2010; Potron et al., 2011).

It is worth mentioning that 10 KPC enzymes, including a novel variant, KPC-10, have also recently been identified among isolates belonging to the *A. calcoaceticus–A. baumannii* complex in Puerto Rico, although identification to the species level was not conclusive (Robledo et al., 2010).

NON-ENZYMATIC MECHANISMS

Membrane permeability

Resistance to β-lactams by means of non-enzymatic mechanisms includes any alteration in the permeability of bacterial membranes that either prevents the entry of antimicrobial agents or promotes their efflux. Very little is known about the OMPs of *A. baumannii*, but several studies have highlighted the relevance of such proteins in resistance to β-lactams. In 2002, Viale and co-workers demonstrated that the loss of a 29-kDa protein, named CarO, was associated with carbapenem resistance in *A. baumannii* in the absence of any known carbapenemase (Limansky et al., 2002). The loss of CarO in several carbapenem-resistant *A. baumannii* isolates was due to the presence of distinct insertion elements that disrupted the *carO* gene (Mussi et al., 2005). Structural studies on this protein, however, showed that although CarO presented some pore-forming properties, it did not contain any specific binding site for carbapenems and was rather a non-specific channel (Siroy et al., 2005). Several other investigations have also identified a variety of OMP proteins mainly involved in carbapenem resistance upon OMP-loss or reduced expression, including a set of endemic carbapenem-resistant *A. baumannii* isolates from New York presenting reduced expression of 47-, 44-, and 37-kDa OMPs together with an increased expression of the class C cephalosporinase (Quale et al., 2003); an *A. baumannii* isolate in Spain that had lost a 33- to 36-kDa OMP associated with carbapenem resistance (del Mar Tomas et al., 2005); and, also similar to what has already been described in Enterobacteriaceae (Kitchel et al., 2010; Pitart et al., 2011), the combined effect of reduced expression of 22- and 33-kDa OMPs and an acquired carbapenemase enzyme (OXA-23; Bou et al., 2000a).

Proteomic studies have also highlighted some OMPs that might have a relevant role in the resistant phenotype to β-lactams, such as the heat-modifiable HMP-AB, which constitutes the major OMP of *A. baumannii* and resembles the OmpA protein from Enterobacteriaceae (Gribun et al., 2003), OmpW (Siroy et al., 2006; Vila et al., 2007), and a 43-kDa protein similar to OprD from *P. aeruginosa* (Dupont et al., 2005).

More recently, Fernández-Cuenca et al. investigated the virulence phenotype of a pan-drug-resistant *A. baumannii* clinical isolate and found an association between attenuated virulence and the decreased expression of genes encoding CarO and OprD-like porins. Attenuated virulence in this work was attributed to a higher biological cost when losing certain OMPs (Fernández-Cuenca et al., 2011).

The role of efflux proteins in the antibiotic susceptibility profile of *A. baumannii* will be discussed in more detail within the following pages, however, it should be mentioned that the contribution to β-lactam resistance of the three resistance–nodulation–cell division (RND) efflux pumps identified so far in *A. baumannii* (AdeABC, AdeIJK, and AdeFGH) has not been extensively studied. Disruption of either *adeABC* or *adeIJK* has been shown to cause 4- and 12-fold decreases in the MICs of cefotaxime and cefepime, respectively, with that of imipenem remaining unchanged. AdeFGH, on the other hand, does not seem to affect susceptibility to β-lactams (Magnet et al., 2001; Damier-Piolle et al., 2008; Coyne et al., 2010b). Interestingly, Wong et al. (2009) showed that disruption of *adeB* in three *A. baumannii* strains significantly altered susceptibility to meropenem but not to imipenem and, more recently, Roca et al. (2011) characterized an AdeABC-type efflux pump in an isolate of the closely related *A. nosocomialis* which was able to extrude monobactams, third-generation cephalosporins, cefoxitin, and meropenem but, again, not imipenem.

Overall, β-lactam extrusion by means of efflux systems leads to small increases in the MIC, and high-level resistance can only be achieved in combination with additional resistance mechanisms (Héritier et al., 2005a).

Penicillin-binding proteins

Another non-enzymatic mechanism of resistance to β-lactams involves changes in the affinity or expression levels of PBPs. Several reports have identified differences in the expression patterns of PBPs between carbapenem-susceptible and carbapenem-resistant *A. baumannii* strains. In some of these strains, carbapenem resistance has been associated with the overexpression of PBP types with low affinity for carbapenem in the absence of any other known resistance mechanism (Gehrlein et al., 1991; Urban et al., 1995) but also with the reduced expression of PBP types together with the production of several β-lactamases or the loss of a 22.5-kDa OMP (Fernández-Cuenca et al., 2003; Cayo et al., 2011). Overall, the contribution of PBP variants to carbapenem resistance is marginal unless associated with β-lactamases, overexpression of efflux pumps or decreased expression of OMPs, and the mechanisms leading to altered expression levels of these proteins have yet to be elucidated (Yun et al., 2011).

AMINOGLYCOSIDES

Resistance to aminoglycosides in *A. baumannii* can be achieved by means of efflux proteins extruding these compounds (reviewed below) or by the expression of aminoglycoside-modifying enzymes (AMEs), specifically acetyltransferases, nucleotidyltransferases, and phosphotransferases (Cho et al., 2009). Activity of AMEs results in the modification of hydroxyl or amino groups present within aminoglycosides that decrease their affinity for the target site (Smith and Baker, 2002). AMEs can be located in either the bacterial chromosome or in plasmids and are usually associated with class 1 integrons and resistance islands where two or more aminoglycoside-resistance genes often occur in combination (Cho et al., 2009). Nemec et al. showed that more than 95% out of 106 European multidrug-resistant *A. baumannii* isolates contained at least one aminoglycoside-resistance gene and 84% contained a combination of two to five different genes with more than 12 distinct combinations. Seventy-eight percent of the strains contained class 1 integrons as well, highlighting the major role of horizontal gene transfer in the dissemination of AMEs in *A. baumannii* (Nemec et al., 2004).

More recently, 16S rRNA methylation mediated by *armA* has also been described in *A. baumannii*, conferring high-level resistance to all clinically relevant aminoglycosides, such as gentamicin, tobramycin, and amikacin (Doi and Arakawa, 2007). *armA* has been found in China, Korea, and the United States (Lee et al., 2006a; Doi et al., 2007; Yu et al., 2007), and it is also commonly found in combination with the $bla_{\text{OXA-23}}$ gene (Kim et al., 2008; Karthikeyan et al., 2010).

QUINOLONES

The mechanisms of fluoroquinolone resistance in *A. baumannii* parallel those of other Gram-negative bacteria and are mainly achieved by point mutations originating amino acid substitutions within the quinolone-resistance determining regions (QRDR) of DNA gyrase and DNA topoisomerase IV (Fàbrega et al., 2009). Mutations in both *gyrA* and *parC* have been described in *A. baumannii* with the most common substitutions being Ser83 to Leu83 for *gyrA* and Ser80 to Leu80 in *parC* (Vila et al., 1995, 1997b; Seward and Towner, 1998; Spence and Towner, 2003; Wisplinghoff et al., 2003; Hamouda and Amyes, 2004; Higgins et al., 2004; Valentine et al., 2008). It is worth mentioning that mutations in *parC* are always accompanied by a concurrent mutation in *gyrA*, probably suggesting that DNA gyrase is the preferred target for fluoroquinolones in *A. baumannii* (Vila et al., 1995, 1997b; Hamouda and Amyes, 2004).

Several efflux pumps in *A. baumannii* are also involved in fluoroquinolone resistance and their specific contribution will be discussed later. Interestingly, plasmid-mediated fluoroquinolone resistance mechanisms such as *qnr*, *aac(6′)-Ib-cr*, or *qepA* (Fàbrega et al., 2009) have not yet been described in *A. baumannii* (Yin et al., 2008; Srinivasan et al., 2009b).

POLYMYXINS

To date, most multidrug *A. baumannii* strains still remain susceptible to polymyxins, prompting an increased use of these compounds despite previous concerns regarding toxicity issues (Falagas and Kasiakou, 2005). Polymyxin E (Colistin), acts by modifying the negative charges of the outer membranes of Gram-negative bacteria, ultimately leading to the disruption of the bacterial membrane. There is very little information regarding the mechanisms of colistin resistance in *A. baumannii*, but it appears that colistin resistance might be multifactorial. By comparing the proteomes of colistin-susceptible versus colistin-resistant *A. baumannii* isolates Fernández-Reyes et al. (2009) were able to identify the differential expression of 35 proteins, including OMPs, chaperons, protein biosynthesis factors, and metabolic enzymes. Other studies, however, have focused on modifications in the bacterial lipopolysaccharide. It has been shown that mutations causing an up-regulated expression of the *pmrA* and *pmrB* genes lead to colistin resistance in *A. baumannii* (Adams et al., 2009). *pmrA* and *pmrB* constitute a two-component regulatory system that governs the expression of *pmrC*, encoding a phosphoethanolamine transferase enzyme involved in lipid A modification. Up-regulation of *pmrA* and *pmrB* also causes the overexpression of *pmrC* and the addition of phosphoethanolamine to the lipid A, which impairs the self-promoted uptake of colistin across the outer membrane (Arroyo et al., 2011; Beceiro et al., 2011).

On the other hand, Moffatt et al. (2010) demonstrated that colistin resistance could also be achieved by the complete loss of lipopolysaccharide production due to mutations affecting several genes involved in lipid A biosynthesis (*lpxA*, *lpxC*, or *lpxD*). Mutations in these genes ranged from single point mutations to large deletions or even the presence of an insertion element, IS*Aba11*, truncating either *lpxA* or *lpxC* (Moffatt et al., 2011). Interestingly, Moffatt et al. observed that colistin-resistant mutants lacking LPS had a 32- to 256-fold increase in susceptibility to other classes of antibiotics. The authors attributed this observation to a direct consequence of LPS loss causing an increased permeability of the outer membrane.

Interestingly, some reports seem to indicate that colistin resistance in *A. baumannii* is associated with lower bacterial fitness *in vivo* as well as decreased virulence, suggesting that colistin usage for the treatment of *A. baumannii* infections might constitute a safe and effective strategy (López-Rojas et al., 2011b). Nevertheless, reduced bacterial fitness in colistin-resistant *A. baumannii* strains does not always occur as acquisition of compensatory mutations

might contribute to maintain the virulence of resistant isolates (López-Rojas et al., 2011c; Rolain et al., 2011).

EFFLUX

Antimicrobial efflux is yet another important mechanism of resistance in *A. baumannii*. In general, efflux constitutes a resistance mechanism that involves the extrusion of antimicrobial agents (as well as other compounds) from the inner side of bacterial membranes to the external environment by means of specific proteins typically named efflux pumps. Although only a few efflux systems have been described in this microorganism, their importance should not be taken lightly since most are capable of pumping out several antimicrobial agents and, therefore, contribute to the greatly feared multidrug-resistant phenotype of nosocomial *A. baumannii*.

Efflux pumps in bacteria are classified into up to five distinct families according to amino acid sequence similarities, energy source, number of components, number of transmembrane-spanning regions and types of substrates: (i) The adenosine triphosphate (ATP)-binding cassette (ABC) superfamily, (ii) the multidrug and toxic compound extrusion (MATE) family, (iii) the small multidrug resistance (SMR) family, (iv) the major facilitator superfamily (MFS), and (v) the RND family (Piddock, 2006).

The RND family usually plays a predominant role in MDR in Gram-negative bacteria and species of *Acinetobacter* are not an exception (Li and Nikaido, 2009). Members of this family consist of a tripartite system including a transporter protein embedded within the inner membrane, an OMP channel, and a membrane fusion protein linking the other two (Magnet et al., 2001). The first RND pump to be identified in *A. baumannii* was named AdeABC, with AdeB constituting the multidrug transporter protein, AdeC being the OMP and AdeA the periplasmic linking protein (Magnet et al., 2001). The *adeABC* genes are also preceded by two additional genes in inverted orientation, *adeSR*, which encode a two-component regulatory system apparently involved in controlling *adeABC* expression (Marchand et al., 2004). Expression of AdeABC confers decreased susceptibility to a wide variety of antimicrobials such as kanamycin, gentamicin, tobramycin, netilmicin, amikacin, erythromycin, tetracycline, chloramphenicol, trimethoprim, sparfloxacin, ofloxacin, perfloxacin, norfloxacin, ethidium bromide and, more recently, tigecycline (Ruzin et al., 2007) and meropenem (Koh et al., 2007a). Not all *A. baumannii* isolates seem to carry this system, though: Huys et al. (2005a) detected the *adeB* gene in 49 out of 51 (96%) highly related *A. baumannii* strains, Chu et al. (2006) found *adeB* in 39 out of 56 (70%) *A. baumannii* isolates from Hong Kong, Nemec et al. (2007) in roughly 83% of 116 isolates tested and Courvalin and co-workers identified the *adeB* gene in 24 out of 27 *A. baumannii* strains (88%; Damier-Piolle et al., 2008), altogether indicating that although AdeABC might not be intrinsic to *A. baumannii*, it is indeed highly widespread among clinical isolates. Interestingly, the *adeSRABC* genes have recently been identified in one isolate of the closely related *A. nosocomialis* together with two additional RND pumps not found in *A. baumannii* (Roca et al., 2011). Very little is known about the resistance mechanisms in this particular microorganism but *A. nosocomialis* has appeared as a recent emergent pathogen with a great potential to cause disease and acquire a multidrug-resistant phenotype (Espinal et al., 2011b). Hence, the presence of three major efflux systems in an emerging multidrug-resistant pathogen is alarming.

Some studies also seem to suggest a correlation between the presence of this efflux system and the resistant phenotype of *A. baumannii* clinical isolates, since AdeABC is commonly associated with class 1 integron genes in resistant strains but missing in highly susceptible isolates (Fournier et al., 2006; Lin et al., 2009).

The second RND efflux system (AdeIJK) described in *A. baumannii* was reported in the same clinical isolate that led to the identification of AdeABC (Damier-Piolle et al., 2008). Overexpression of this pump either in *E. coli* or *A. baumannii* is apparently toxic, suggesting the presence of a tight regulation mechanism to maintain its expression levels low. No adjacent regulatory genes, however, have been found in the vicinity of *adeIJK*.

Inactivation of AdeIJK by allelic replacement showed decreased resistance to substrates similar to those of the AdeABC pump, and a double mutant (Δ*adeABC*/Δ*adeIJK*) displayed even lower MICs of chloramphenicol, tetracyclines, erythromycin, clindamycin, fluoroquinolones, and tigecycline than each of the single mutants, thus indicating both a cumulative effect of the pumps as well as overlapping substrate profiles, which is, nevertheless, interesting from a clinical point of view. However, opposed to AdeABC, AdeIJK did not seem capable of extruding ethidium bromide or azithromycin, despite the latter being a preferred substrate for AdeABC (Damier-Piolle et al., 2008). Unfortunately, extrusion of aminoglycosides could not be tested in this system since the allelic replacement mutants incorporated either kanamycin or apramycin resistance cassettes.

AdeIJK has so far been detected in all the *A. baumannii* strains tested (Damier-Piolle et al., 2008) and is therefore considered to have a more predominant role in the intrinsic low-level resistant phenotype of *A. baumannii*. The AdeIJK efflux transporter seems highly specific to *A. baumannii* and has not yet been described in other species of the *Acinetobacter* genus. It is worth mentioning, however, that *adeIJK* and *adeXYZ* (an RND efflux pump described in *A. pittii* and *A. nosocomialis*; Chu et al., 2006; Roca et al., 2011) are extremely alike, sharing 93% identity at the nucleotide level and 99% similarity at the protein level. Similarity percentages obtained when comparing already described *adeJ* sequences from different *A. baumannii* strains also provide similar figures which might suggest that they actually constitute the same efflux system that has been described twice in different genomic species and has been given different names.

Coyne et al. (2010a) reported a third RND-type efflux pump (AdeFGH) within a derivative mutant (Δ*adeABC*/Δ*adeIJK*) from the same *A. baumannii* strain in which AdeABC and AdeIJK had been characterized. Expression of AdeFGH was responsible for high-level resistance to chloramphenicol, clindamycin, fluoroquinolones, and trimethoprim as well as decreased susceptibility to tetracycline–tigecycline and sulfonamides, but β-lactams, erythromycin, and rifampin remained unchanged (Coyne et al., 2010b). Once again, substrate specificity toward aminoglycosides could not be assessed due to the presence of the respective kanamycin and apramycin resistance cassettes.

A small ORF encoding a putative LysR-type transcriptional regulator, named *adeL*, was also identified in inverted orientation upstream from *adeFGH* and mutations within the *adeL* gene have been associated with the overexpression of this efflux system (Coyne et al., 2010b).

As for the presence of efflux pumps included within the remaining families of efflux transporters in *A. baumannii*, there is even less information than that available for the RND family.

Roca et al. (2009) identified the MFS efflux pump CraA, for chloramphenicol resistance *Acinetobacter*, in an *A. baumannii* clinical isolate from Spain. This protein was similar in sequence and secondary structure to the MdfA efflux pump from *E. coli* but differed in its substrate profile. CraA was shown to be highly specific for chloramphenicol and has, so far, been found in all the *A. baumannii* strains tested and might be responsible for the intrinsic resistance of *A. baumannii* to this antimicrobial agent.

More recently, Rajamohan et al. described a second MFS efflux pump, AmvA, mainly involved in the extrusion of dyes, disinfectants, and detergents and also erythromycin, although only causing a fourfold decrease in the MIC of this antibiotic. AmvA is also present in all *A. baumannii* strains studied so far (Rajamohan et al., 2010).

The third type of MFS efflux pumps present in *A. baumannii* is made of the acquired tetracycline efflux systems (Tet) that are part of plasmids, transposons, or resistance islands (see below). TetA and TetB constitute the most prevalent Tet efflux pumps in *A. baumannii* with TetA conferring resistance to tetracycline and TetB to both tetracycline and minocycline (Vila et al., 2007), although the *tet(M)* gene, involved in tetracycline ribosomal protection, has also been identified in one clinical isolate of *A. baumannii* (Ribera et al., 2003b). *tet(A)*, together with the gene encoding its transcriptional regulator, *tetR*, is located in a Tn*1721*-like transposon that, in turn, might be part of a larger resistance island (Ribera et al., 2003a; Fournier et al., 2006). On the other hand, *tet*(B) is located in small plasmids ranging from 5- to 9-kDa (Srinivasan et al., 2009b). Prevalence studies seemed to indicate that *tet*(B) is more commonly found in multidrug-resistant *A. baumannii* clinical isolates, with at least 50% of the strains containing this determinant (Guardabassi et al., 2000; Huys et al., 2005b; Martí et al., 2006; Mak et al., 2009; Srinivasan et al., 2009b).

The only efflux pump of the MATE family described so far in *A. baumannii*, AbeM, was identified in Su et al. (2005) and it was shown to extrude aminoglycosides, fluoroquinolones, chloramphenicol, trimethoprim, ethidium bromide and several dyes. Although AbeM has also been found in all *A. baumannii* strains studied to date, the role of this pump in antimicrobial resistance remains unclear since substrate-profiling studies carried out in *E. coli* did not fins any correlation between antibiotic resistance and overexpression of this pump.

AbeS is yet another efflux pump identified in *A. baumannii* that confers low-level resistance to several antimicrobial agents, including chloramphenicol, fluoroquinolones, erythromycin, and novobiocin, as well as resistance to dyes and detergents, but it is not present in all fully sequenced *A. baumannii* genomes. AbeS belongs to the SMR family and retains a certain degree of similarity with the EmrE system of *E. coli* (Srinivasan et al., 2009a).

GENOME PLASTICITY

As already mentioned, probably one of the most intriguing characteristics of *A. baumannii* is its capacity to acquire, retain, and disseminate multiple resistance mechanisms which, combined with its ability to survive desiccation as well as most disinfectants, accounts for the prolonged survival of *A. baumannii* strains in the clinical setting and makes this microorganism almost impossible to eradicate.

Acquisition and dissemination of antimicrobial resistance determinants in *A. baumannii* are achieved by combining resistance genes with an array of mobile elements that mediate the exchange of genetic material and rearrange bacterial genomes, giving rise to multiple genetic combinations and providing an endless source of genetic adaptability.

Such an array includes IS, transposons, integrons, plasmids and, ultimately, resistance islands.

INSERTION SEQUENCES

Insertion sequences indeed have a predominant role in the acquisition of resistance within *A. baumannii*. They are generally defined as the smallest mobile DNA elements (<2.5 kb), carrying only the genetic information required for their mobilization. In practical terms IS are constituted by a pair of short inverted-repeat sequences (IR) bracketing one or perhaps two ORFs encoding a transposase, the enzyme involved in the transposition or "jumping" of the IS element.

More than 30 different types of ISs have been reported in *Acinetobacter* spp. (Siguier et al., 2006) being IS*AbaI* and IS*Aba125* the most prevalent ISs in this microorganism (Adams et al., 2010). The genome of the *A. baumannii* AYE strain has been shown to contain up to 21 copies of IS*AbaI* (Vallenet et al., 2008) but this IS has also been identified in additional *A. baumannii* isolates from around the world (Goic-Barisic et al., 2009; Andriamanantena et al., 2010; Culebras et al., 2010; Higgins et al., 2010; Koo et al., 2010; Adams-Haduch et al., 2011; Karunasagar et al., 2011; Kusradze et al., 2011; Nigro et al., 2011b; Zhou et al., 2011).

As a consequence of IS mobilization these genetic elements can contribute to resistance in three different ways:

(i) Insertion of an IS immediately upstream from a given ORF might provide additional promoters to enhance transcriptional levels of genes that are otherwise poorly expressed. In this respect, IS*AbaI* is commonly found upstream from bla_{ampC} and $bla_{\text{OXA-51}}$-like genes in *A. baumannii*, being responsible for ceftazidime and carbapenem resistance, respectively, in isolates lacking additional resistance mechanisms (Héritier et al., 2006; Turton et al., 2006). In addition, IS*AbaI*, IS*Aba2*, IS*Aba3*, IS*Aba4*, IS*Aba10*, IS*Aba16*, IS*Aba125*, IS*Aba825*, and IS*18* have also been found upstream from $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-58}}$ (Poirel and Nordmann, 2006b; Bertini et al., 2007; Corvec et al., 2007; Giannouli et al., 2009; Lee et al., 2011b; Ravasi et al., 2011; Lopes et al., 2012) and IS*Aba9* has been described upstream from both $bla_{\text{OXA-51}}$ and the gene encoding CARB-10 (Figueiredo et al., 2009; Potron et al., 2009).

CHDLs however, are not the only β-lactamases whose expression is enhanced by the presence of upstream ISs, both

bla$_{PER-1}$ and *bla*$_{PER-2}$ contain a novel IS element, IS*Pa12*, that has been shown to up-regulate their expression (Poirel et al., 2005a; Pasterán et al., 2006). Similarly, expression of *bla*$_{PER-7}$ is also driven by a promoter sequence located within an upstream IS*CR1* element (Bonnin et al., 2011b).

(ii) IS elements can also contribute to resistance by inserting within certain ORFs and disrupting their coding sequences. Insertional inactivation leading to resistance has been associated with the disruption of transcriptional regulators, such as the insertion of IS*Aba1* within the *adeS* gene leading to overexpression of the AdeABC efflux pump, but also with the disruption of genes involved in membrane permeability, such as IS*Aba10*, IS*Aba10*, IS*Aba825*, and IS*Aba125* insertion within the *carO* gene leading to carbapenem resistance (Lu et al., 2009; Lee et al., 2011b; Ravasi et al., 2011) or the insertion of IS*Aba11* within the *lpxA* or *lpxC* genes causing loss of lipopolysaccharide production and colistin resistance (Moffatt et al., 2011).

(iii) Without any doubt, however, the major contribution of ISs to the development of pan-resistant *A. baumannii* strains lies in their ability to mobilize gene cassettes, either within the bacterial chromosome or between the chromosome and plasmids, allowing for a rapid dissemination of such cassettes not only among different *A. baumannii* strains but also among isolates belonging to different taxonomic genera (Roberts et al., 2008).

Insertion sequences-mediated mobilization might take place when two IS elements bracket a gene cassette, usually containing antimicrobial resistance genes, and the terminal IR from each IS cooperate to displace the intervening DNA sequence, thereby forming a compound transposon (Roberts et al., 2008). Bear in mind that ISs involved in compound transposons can still provide promoter sequences for the transcriptional expression of the genes they carry.

A single IS can also mediate the mobilization of flanking DNA by a rare mechanism known as "one-ended" transposition that combines one *authentic* end together with a *surrogate* end displaying a certain degree of similarity and located nearby (Motsch et al., 1985). In addition, ISs belonging to the IS*91* family (which includes IS*CR1* and IS*CR2*) are able to transpose by means of a rolling-circle replication mechanism (Toleman et al., 2006) and mobilization of DNA elements adjacent to ISs can also be achieved due to homologous recombination between two ISs, although this mechanism feeds on the copy number of ISs.

In *A. baumannii*, mobilization of the *bla*$_{OXA-23}$ gene is mediated either by two copies of IS*Aba1* in inverted or direct orientation and generating a compound transposon designated Tn*2006* or Tn*2009*, respectively (Corvec et al., 2007; Adams et al., 2008; Zhou et al., 2011); by a single copy of IS*Aba1* forming a transposon-like structure named Tn*2008* (Adams-Haduch et al., 2008); or by a similar structure comprising a single IS*Aba4* copy forming another transposon-like structure named Tn*2007* (Corvec et al., 2007).

Of note, the chromosome of the non-pathogenic and environmental *Acinetobacter radioresistens* was recently identified as the original source of the acquired *bla*$_{OXA-23}$ gene of *A. baumannii*, and other OXA-23 variants have also been found in other *A. radioresistens* isolates (Poirel et al., 2008a), suggesting that the diversity of structures surrounding the *bla*$_{OXA-23}$ gene might have arisen from independent transposition events from the chromosome of commensal *A. radioresistens* strains to transferable plasmids that have subsequently been mobilized into *A. baumannii*.

The plasmid-encoded *bla*$_{OXA-58}$ gene is also commonly bracketed by distinct combinations of IS elements that usually contain IS*Aba3* downstream from *bla*$_{OXA-58}$ and differ in the presence of ISs in the 5′ flanking end. Specifically, IS*Aba3* seems to be more prevalent, but IS*Aba825*, IS*Aba1*, IS*Aba2*, IS*18*, and IS*26* can also be present (Poirel and Nordmann, 2006b; Bertini et al., 2007; Giannouli et al., 2009). The role of such upstream ISs in providing additional promoters has been clearly demonstrated, but their role in the genetic mobilization of *bla*$_{OXA-58}$ is not yet clear. Apparently, each of these IS elements shows a distinct geographic distribution and since the *bla*$_{OXA-58}$ is usually plasmid-borne they might have been acquired through horizontal gene transfer and homologous recombination (Poirel and Nordmann, 2006b; Giannouli et al., 2009). Of note, the *bla*$_{OXA-58}$ gene flanked by two IS*Aba3* elements has also been identified in plasmids recovered from *A. pittii* isolates but, then again, this probably reflects the inter-genus transferability of these plasmids rather than transposon-mediated acquisition (Evans et al., 2010; Huang et al., 2010).

Additional resistance determinants thought to have been mobilized by means of IS-mediated transfer include the ESBLs *bla*$_{PER-1}$, bracketed by IS*Pa12* and IS*Pa13* and forming a compound transposon named Tn*1213* (Poirel et al., 2005a), *bla*$_{PER-7}$ and *bla*$_{VEB-1}$ mobilized by the upstream IS*CR1* and IS*CR2*, respectively, by means of rolling-circle transposition (Poirel et al., 2009; Bonnin et al., 2011b), and also CARB-10, whose transfer is apparently due to a one-ended transposition event from an upstream IS*Aba9* (Potron et al., 2009).

More recently, Potron et al. (2011) characterized a novel compound transposon in the chromosome of *A. baumannii* isolates from Haiti containing the *bla*$_{CTX-M-15}$ gene flanked by IS*Ecp1* and a truncated IS*26*, reflecting a transfer event from Enterobacteriaceae.

Among MBLs, the *bla*$_{NDM}$ sequences from Enterobacteriaceae have been shown to contain different ISs upstream from the *bla*$_{NDM-1}$ gene, although the role of these ISs in the expression of NDM is not clear and they are most likely involved in the genetic mobilization of the resistance gene (Yong et al., 2009; Poirel et al., 2010; Ho et al., 2011). Sequence analysis of some of these *bla*$_{NDM}$ genes from Enterobacteriaceae identified a fragment of variable length containing the right-end repeat from the insertion sequence IS*Aba125* in-between the *bla*$_{NDM-1}$ gene and the corresponding IS element as a remnant of IS*Aba125* insertion, and the IS element IS*Ec33* from *E. coli* strain 271 is bracketed by the sequence upstream from the IS*Aba125* right-end (Poirel et al., 2011b; Solé et al., 2011). IS*Aba125* belongs to the IS*30* family and, until recently, had only been found in *A. baumannii*, either chromosomally or on a plasmid upstream from the *bla*$_{OXA-58}$ gene (Mussi et al., 2005; Evans et al., 2010) but never linked to *bla*$_{NDM}$ genes. Interestingly, Solé et al. recently identified a complete IS*Aba125* copy upstream from a *bla*$_{NDM-1}$ gene in *E. coli* and both Pfeifer et al. (2011) and Espinal et al. (2011a) have also found *bla*$_{NDM-1}$ and *bla*$_{NDM-2}$ sequences in *A. baumannii*

bracketed between two copies of IS*Aba125*, thereby defining a novel transposon. In view of these results, it has been postulated that some of the bla_{NDM} sequences found in Enterobacteriaceae might have originated from *Acinetobacter* by means of IS*Aba125*-mediated mobilization (Solé et al., 2011). IS*Aba125* has also been found flanking the aminoglycoside-resistance gene *aphA6* and constituting the compound transposon Tn*aphA6* (Nigro et al., 2011a).

Insertion sequences are also involved in the mobilization of the *tet(A)* and *tetR* genes, encoding the tetracycline efflux pump and its transcriptional regulator, respectively, and commonly found within a Tn*1721*-like transposon that might also be present in multiple copies within the bacterial chromosome (Ribera et al., 2003a; Fournier et al., 2006).

It is worth mentioning that conserved inverted repeats homologous to binding sites acting as targets for the XerC and XerD recombinases, usually involved in site-specific recombination mechanisms, have been described flanking $bla_{OXA-24/40}$ genes in different plasmids recovered from *A. baumannii* isolates (D'Andrea et al., 2009; Merino et al., 2010; Acosta et al., 2011; Tian et al., 2011). In view of these results, XerC/XerD recombinases have been postulated as a novel mechanism driving mobilization of the $bla_{OXA-24/40}$ gene (Merino et al., 2010).

Conjugative plasmids are also known to play a central role in the intra- and inter-species transfer of resistance determinants. However, although some resistance genes such as bla_{OXA-58} and bla_{OXA-23} are commonly plasmid-borne in *Acinetobacter* spp., a recent study of plasmid replicons in *A. baumannii* suggested that replicon types within this microorganism do not correspond to those circulating among Enterobacteriaceae, which might provide a plausible explanation for the observation that prevalent plasmid-encoded resistance mechanisms identified among Gram-negative bacteria (ESBLs, KPC, etc.) are rarely present in *Acinetobacter* spp. while this genus seems to contain its own type (CHDLs) of plasmid-borne carbapenemases (Bertini et al., 2010).

INTEGRONS

Integrons constitute another important vehicle for the spread and accumulation of resistance genes in *A. baumannii*, and several studies have demonstrated a positive correlation in *A. baumannii* between the carriage of integrons and the degree of MDR (Huang et al., 2008; Lee et al., 2009b). Integrons are genetic elements that mediate the integration of gene cassettes by a site-specific recombinational mechanism and direct their coordinated expression. All integrons bear three key elements: a specific recombination site (*attI*), a gene encoding an integrase (*intI*) and a common promoter region (P1–P2) oriented toward the site of integration. The integrase recombinates discrete units of circular DNA known as gene cassettes (often carrying resistance genes) into the *attI* site allowing the coordinated expression of the genes in the cassette from the common promoter (Mazel, 2006).

Although integrons are not mobile by themselves (but gene cassettes can readily be exchanged to other integrons), they can be spread to different chromosomal locations, plasmids or even other microorganisms by means of IS-mediated transposition or homologous recombination (Mazel, 2006). There are several classes of integrons mainly classified according to the sequence of the encoded integrases, which show 40–58% identity. Class 1 integrons displaying a wide variety of gene cassettes are commonly found in *A. baumannii*, and typically encode aminoglycoside-resistance genes as well as genes conferring resistance to antiseptics and sulfonamides (Nemec et al., 2004; Zhao and Hu, 2011). Other resistance determinants associated with class 1 integrons in *A. baumannii* include bla_{IMP}, bla_{VIM}, and bla_{SIM} MBL types (Lee et al., 2003, 2005, 2008; Yamamoto et al., 2011) as well as some Ambler class A β-lactamases such as CARB, GES, PER, VEB, and narrow-spectrum OXA-types (Vila et al., 1997a; Navia et al., 2002; Poirel et al., 2003; Zarrilli et al., 2004; Naas et al., 2006a; Moubareck et al., 2009; Potron et al., 2009; Bonnin et al., 2011a).

Class 2 integrons, which are embedded within the Tn7 transposon, display a narrower diversity of gene cassettes and those described in *A. baumannii* have been shown to contain several resistance determinants such as *aadB*, *catB2*, *dfrA1*, *sat2*, and *aadA1*, conferring resistance to aminoglycosides, chloramphenicol, trimethoprim, streptothricin, and streptomycin, respectively (Ramírez et al., 2010a). More recently, Ramírez et al. reported an *A. baumannii* isolate carrying bla_{CARB-4} in a class 2 integron together with 6 additional resistance determinants comprising four families of antibiotics. This novel integron constitutes the longest class 2 integron described so far and also represents the only example of a β-lactamase gene encoded within such a class (Ramírez et al., 2010b).

RESISTANCE ISLANDS

In addition to the huge diversity of resistance mechanisms displayed by *A. baumannii* isolates, this microorganism is also able to accumulate multiple resistance determinants in what has been termed as "resistance islands." Resistance islands are specific regions of the genome that harbor large clusters of horizontally transferred genetic DNA including a great deal of antimicrobial resistance genes. Such regions provide a "safe haven" for mobile elements since insertion at this site is not likely to cause any damage to the host cell. They are often inserted at the same locus on the *A. baumannii* chromosome and are assembled by discrete genes or gene packages usually associated with mobile elements (IS and Tn), integrons, or both.

The first resistance island described in *A. baumannii* was found in the multidrug-resistant *A. baumannii* AYE strain and was hence termed AbaR1. AbaR1 comprised an 86 kb region integrated within the *comM* gene and contained up to 45 antibiotic and heavy metal resistance genes. Interestingly, AbaR1 was flanked at both ends by 5 bp perfect direct repeats (DR) and included two ORFs near the 3′ *comM* end annotated as putative transposition genes, thereby suggesting acquisition by means of a transposition event.

At the time of this review, more than 22 resistance islands have been described (Fournier et al., 2006; Adams et al., 2008, 2010; Iacono et al., 2008; Post and Hall, 2009; Krizova and Nemec, 2010; Post et al., 2010; Rose, 2010; Krizova et al., 2011; Nigro et al., 2011b; Zhou et al., 2011) and, with only three exceptions (Adams et al., 2008; Rose, 2010), all have been found inserted within the *comM* gene (**Table 2**).

Shaikh et al. (2009) studied a collection of 50 multidrug-resistant *A. baumannii* isolates and found that 82% contained a truncated *comM* gene. Deeper sequence analysis of 10 such isolates

Table 2 | Resistance Islands in *A. baumannii*.

AbaR-type	Size (kb)	Strain	EC	*comM* Insertion	Reference
AbaR1	86	AYE	I	+	Fournier et al. (2006)
AbaR2	17	ACICU	II	+	Iacono et al. (2008)
AbaR3	63	AB0057	I	+	Adams et al. (2008)
AbaR4	18	AB0057	I	–	Adams et al. (2008)
AbaR5	56	3208	I	+	Post and Hall (2009)
AbaR6	27	D2	I	+	Post et al. (2010)
AbaR7	20	A92	I	+	Post et al. (2010)
AbaR8	29	D13	I	+	
AbaR9	39	AB056	I	+	Adams et al. (2010)
AbaR10	30	AB058	I	+	Adams et al. (2010)
AbaR11	20	NIPH470	I	+	Krizova et al. (2011)
AbaR12	38	LUH 6013	I	+	Krizova et al. (2011)
AbaR13	45	LUH6015	I	+	Krizova et al. (2011)
AbaR14	21	LUH5881	I	+	Krizova et al. (2011)
AbaR15	55	LUH6125	I	+	Krizova et al. (2011)
AbaR16	39	LUH7140	I	+	Krizova et al. (2011)
AbaR17	58	LUH8592	I	+	Krizova et al. (2011)
AbaR18	52	NIPH2713	I	+	Krizova et al. (2011)
AbaR19	30	NIPH2554	I	+	Krizova et al. (2011)
AbaR21	64	RUH875	I	+	Nigro et al. (2011b)
AbaR22	39	MDR-ZJ06	II	+	Zhou et al. (2011)
n.a.	n.f.	A473	I	–	Rose (2010)
n.a.	n.f.	A473	I	–	Rose (2010)

EC, European clone lineage; n.a., not assigned; n.f., not found.

identified AbaR-like sequences in 8, thus confirming that the *comM* is likely a preferred integration site for this sort of genetic structures (Shaikh et al., 2009).

In addition, all AbaR sequences identified in *A. baumannii* isolates belonging to the European Clone I (EC I) lineage seem to have a common genetic structure. They share a 16.3-kb backbone transposon, designated Tn*6019*, containing two ORFs annotated as putative transposition genes, an arsenate resistance operon (*arsHBRC*), a putative sulfate permease gene (*sup*), and a universal stress protein gene (*uspA*) that, in turn, is disrupted by a second compound transposon formed by two copies of a cadmium and zinc resistance transposon (Tn*6018*) bracketing a multiple antibiotic resistance region (MARR). The MARR region contains most of the variability found among all AbaRs, with only a few exceptions displaying additional differences within the left-hand copy of Tn*6018* and the Tn*6019* backbone region (Post et al., 2010; **Figure 2**).

Although AbaR1 is so far the largest resistance island described in *A. baumannii* (Fournier et al., 2006), there is general agreement that AbaR5 to AbaR21 are all derivatives of AbaR3 that have originated as a result of either deletions mediated by one of the three IS*26* elements present within AbaR3, deletions caused by a single homologous recombination event or arisen due to integron shuffling (Krizova et al., 2011; Nigro et al., 2011b). AbaR1 displays the same general structure as AbaR3 but contains an additional 29 kb region consisting of a very complex class 1 integron that might have been incorporated into AbaR3.

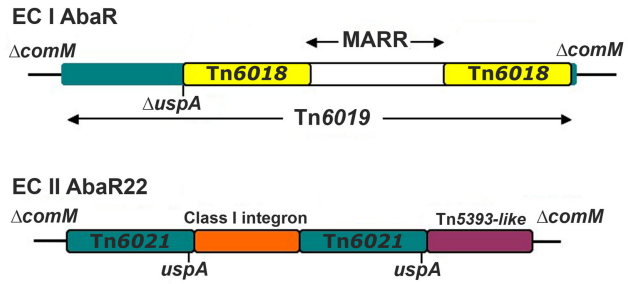

FIGURE 2 | Schematic overview of the conserved AbaR structure found in *A. baumannii* isolates belonging to the European clone I lineage and that of AbaR22, isolated from strain MDR-ZJ06 belonging to the European clone II lineage (Zhou et al., 2011).

Interestingly, some AbaR-related structures found in *A. baumannii* isolates belonging to the EC II lineage present some noticeable differences compared to AbaRs from EC I. Several EC II isolates have been shown to contain a Tn*6019*-related transposon (termed Tn*6021*) as well as some genes present in AbaR1 but they also possess an intact *uspA* gene and, therefore, lack the Tn*6018* compound transposon and the MARR region (Post et al., 2010; Zhou et al., 2011; Bonnin et al., 2012). These differences reinforce the delineation of *A. baumannii* isolates in different European clones since AbaRs structures seem to have appeared independently in each of these isolates (**Figure 2**).

Krizova et al. have recently suggested that the origin of AbaRs in strains of the EC I might lie in the antibiotic regimes administered in Europe during the 1970s and 1980s, since AbaRs seem to provide resistance to antimicrobials mostly used during that period, thereby facilitating the survival and spread of AbaRs-bearing strains. The subsequent modification of the treatment of choice might have then diminished the selective pressure on such determinants and accounted for the truncated AbaR3-derivatives in the current population of *A. baumannii* clinical isolates (Krizova et al., 2011).

The combination of IS, Tn, integrons, conjugative plasmids, and resistance islands accounts for the extreme genomic plasticity of *A. baumannii* and partly explains the successful emergence of this microorganism as a dreadful nosocomial human pathogen. Recent studies comparing the whole genome sequence of closely related *A. baumannii* isolates have demonstrated a unique genetic repertoire in terms of IS, plasmids, and AbaRs even in *A. baumannii* isolates belonging to the same sequence type (ST; Adams et al., 2010; Di Nocera et al., 2011; Snitkin et al., 2011).

CLINICAL IMPORTANCE AND TREATMENT OPTIONS

Acinetobacter baumannii is currently considered one of the most important nosocomial pathogens. In a prevalence study of infections in intensive care units conducted among 75 countries of the 5 continents, this microorganism was found to be the fifth most common pathogen, although with a high variability among the different countries (Vincent et al., 2009). Different surveillance studies have found this pathogen to be the fifth cause of pneumonia, after *P. aeruginosa*, in hospitalized patients, mainly in intensive

care units (Jones, 2010). In addition, these microorganisms are also frequently reported to cause other nosocomial infections such as bacteremia and urinary tract and surgical infections. In fact, *A. baumannii* was found to be third most frequent cause of nosocomial bloodstream infection in a large multicenter study with an estimation of 34% of all patients and 43% in patients in intensive care units (Wisplinghoff et al., 2004).

With respect to the treatment of *A. baumannii* infections it is important to take into account the resistant profile involved to thereby consider the different treatment options available. As mentioned above, *A. baumannii* is currently resistant to multiple antibacterial agents, including carbapenems and, occasionally, colistin. Therefore, in many cases, optimal treatment for nosocomial infections caused by this microorganism is sometimes not available. A decade ago sulbactam, which has intrinsic activity against *A. baumannii* and shows *in vivo* efficacy in a murine model of pneumoniae, was used to treat infections caused by carbapenem-resistant clinical isolates (Rodríguez-Hernández et al., 2001; Levin et al., 2003). However, nowadays the percentage of resistance to sulbactam has reached such a high level that its use as antimicrobial agent against infections caused by *A. baumannii* has been invalidated

Rifampicin has demonstrated consistent results related to *in vivo* efficacy in experimental models of infection and in some open studies of human infections, especially when combined with colistin. These studies suggest that the possible use of rifampicin for the treatment of multidrug-resistant *A. baumannii* infections should be analyzed in well-designed clinical trials (Pachón-Ibáñez et al., 2006; Pachón-Ibánez et al., 2010). Bassetti et al. (2008) studied the use of colistin in combination with rifampin in critically ill patients with pneumonia (19 patients) and bacteremia (10 patients) caused by *A. baumannii* resistant to all antibiotics except colistin in medical and surgical intensive care units. Clinical and microbiological response was observed in 22 of 29 cases (76%), suggesting that the combination of colistin and rifampicin appears to be an effective and safe therapy for severe infections due to multidrug-resistant *A. baumannii.*

There is some heterogenicity concerning the clinical data to determine the utility of tigecycline in the treatment of nosocomial infections caused by *A. baumannii.* The possible development of resistance during treatment with tigecycline suggests that it should only be used in combined regimens with other antimicrobials. Two studies have reported the combination of tigecycline with other antimicrobial agents. Principe et al. (2009) found that tigecycline showed synergism with levofloxacin, amikacin, imipenem, and colistin, whereas antagonism was observed for the tigecycline/piperacillin–tazobactam combination. Considering all antimicrobials in combination with tigecycline, checkerboard analysis showed 5.9% synergy, 85.7% indifference, and 8.3% antagonism. Tigecycline showed synergism with levofloxacin (four strains; 16.6%), amikacin (two strains; 8.3%), imipenem (two strains; 8.3%), and colistin (two strains; 8.3%). Synergism was detected only among tigecycline non-susceptible strains. Time-kill assays confirmed the synergistic interaction between tigecycline and levofloxacin, amikacin, imipenem, and colistin in five out of seven selected isolates. Ozbek and Senturk (2010) isolated six meropenem-resistant *A. baumannii* strains in which synergy of tigecycline/colistin and tigecycline/levofloxacin was observed using checkerboard analysis in just one strain each.

Other combinations have recently been described in the scientific literature. Liang et al. isolated 14 extensive drug-resistant *A. baumannii* strains from patients admitted to the intensive care units of a Chinese hospital. Most of the strains were resistant to all antimicrobials except colistin and minocycline, and the combinatory results by killing curves showed a synergistic effect between colistin/meropenem, colistin/rifampicin meropenem/minocycline and colistin/minocycline. It is worth mentioning that the combination of meropenem/minocycline could be a possible alternative when colistin is not available as in China (Liang et al., 2011). In a recent study the combination of teicoplanin/colistin was investigated against multidrug-resistant *A. baumannii* isolates (resistant to third-generation cephalosporins, quinolones, and aminoglycosides), with checkerboard analysis showing synergy between the two antibiotics. Bactericidal activity was shown in the first 4 h for both colistin alone and in combination with teicoplanin, however regrowth was observed when colistin was used alone (Wareham et al., 2011). Although the mechanism of action of colistin is not completely known, it is thought to act in the bacterial membrane by disrupting it (Lam et al., 1986). Therefore, the use of colistin in combination with antibiotics such as teicoplanin or vancomycin which are effective against Gram-positive microorganisms (Gordon et al., 2010), likely helps glycopeptides to reach the target.

Of particular concern are infections caused by pan-drug-resistant *A. baumannii* isolates, which are steadily increasing in various regions worldwide. There is an urgent need for new therapeutic strategies since isolates resistant to all antibacterial agents, including colistin, have been reported (Park et al., 2009) and this scenario will likely progress in association with the increased use of this antibiotic.

FUTURE POTENTIAL THERAPEUTIC ALTERNATIVES

The emergence of bacteria resistant to most of the antibiotics available has lead to the appearance of different terms concerning resistance. Only a short time ago, experts in the topic homogenized all these terms, in the case of *A. baumannii*, MDR may be referred when the strain is non-susceptible to $\geq$1 antimicrobial agent in $\geq$3 antimicrobial categories; an increase in resistance now refers to extensive drug resistance (XDR) when non-susceptible to $\geq$1 antimicrobial agents in all but $\leq$2 categories (i.e., bacterial isolates remain susceptible to only one or two categories), and pan-drug resistance (PDR) is considered when the microorganism is non-susceptible to all the antimicrobial agents in all antimicrobial categories (Magiorakos et al., 2012).

In regard to future potential therapeutic alternatives, the perspectives are not good, at least within the next decade (Payne et al., 2007). Nonetheless there are two main options for the design of new drugs to treat infections caused by PDR-resistant *A. baumannii.* The first is related to knowledge of the biochemical bases of resistance and has been used to design rational strategy to counteract resistance. This strategy can follow two approaches: (i) Modification of the basic structure of the antibacterial agent, circumventing antibacterial resistant mechanisms, and (ii) Development of compounds inhibiting the mechanisms of resistance

for an antibacterial agent, whereby the concomitant administration of the antibacterial agent plus the inhibitor, as a co-drug, potentiates this activity (Li and Heide, 2005). Some studies have used this strategy to develop drugs active against MDR *A. baumannii*. A derivative of ciprofloxacin (UB-8902) showed good activity against *A. baumannii* strains carrying a mutation in the *gyrA* gene, with a MIC_{50} of 4 mg/L (range <0.06–8 mg/L) in comparison with ciprofloxacin displaying a MIC_{50} of 64 mg/L (Vila et al., 2006). Further *in vivo* studies using this new quinolone have also been performed in a murine pneumonia model, showing that UB-8902 presented bactericidal activity against *A. baumannii* strains resistant to ciprofloxacin. Moreover, this quinolone was effective at reducing mortality with a dose lower than the toxic dose in a model of peritoneal sepsis (López-Rojas et al., 2011a). BAL30072 is a new monocyclic β-lactam belonging to the sulbactam class of antibiotics active against MDR *A. baumannii*. BAL30072 possesses a dihydropyrimidinone siderophore in its side chain and has shown better activity than meropenem in five MDR *A. baumannii* strains, with increased activity when a combination of the two was used (Russo et al., 2011).

The second option is to design a new compound involving antimicrobial peptides (AMPs) as the principal candidates. The use of AMPs as antibiotics has several advantages and disadvantages, the main disadvantage being the instability of the compound against proteases present in the blood and serum. Several methods may be used to overcome this issue although these may sometimes not be easy to apply due to a possible loss of the effectiveness of peptides upon changes in their structure. D-Amino acids, stable to proteases (Chen et al., 2006; Friedman, 2010) may be used as well as both methylation and fluorination of specific amino acids (Meng and Kumar, 2007; Fernández-Reyes et al., 2010). Another option, albeit sometimes less effective in terms of activity due to the loss of one positive charge which is important for the interaction with membranes, is cyclation (Molhoek et al., 2011). On the other hand, the advantages of AMPs are that they are a less specific target and, compared to antibiotics, acquisition of resistance is difficult (Marr et al., 2006).

Braunstein et al. (2004) used a gentamicin-resistant *A. baumannii* strain to test an AMP containing 33% of D-amino acids that showed better activity (5.6–11.2 mg/L) and greater stability to hemolysis (0–100% at 180 mg/L) than the L-amino acid form. The same effect was seen by Jiang et al. in which a totally synthetic D-amino acid AMP was tested against 550 clinical isolates involving 74% of MDR strains. Although no resistance profile was reported in the article, the AMP showed good values for both MIC_{50} and MIC_{90} (Jiang et al., 2011).

In another study using strains resistant to ciprofloxacin, the MIC_{50} and MIC_{90} values of pexiganan, an analog of magainin, were 2 and 8 mg/L, respectively (Ge et al., 1999). In another analog, but in this case a short version of the peptide of human lactoferrin, 11 residues from the N-terminal were studied. The *A. baumannii* strains used were resistant or intermediate to 16 and 19 out of 20 antimicrobial agents, including imipenem and meropenem. This peptide was tested *in vivo* and a reduction of 3–4 log CFU/ml was observed in an experimental infection in mice. However at peptide concentration ranging from 49.5 to 98.9 mg/L, this effect was not seen in the control. In addition the effect of this peptide was very rapid, achieving the minimal number of CFU in 5–15 min (Dijkshoorn et al., 2004). Other peptides present in the human body such as β-defensin 3 have been tested against *A. baumannii*. This AMPs belongs to the cysteine-rich peptides, and a bactericidal effect was achieved *in vitro* after 1.5 h using 4 mg/L of the peptide against MDR *A. baumannii* (Maisetta et al., 2006).

The origin of the peptides tested so far is different, some being totally synthetic, some isolated from humans or others from animals. In the case of animals, amphibians are a large source of peptides as in the case of the study published by Mangoni et al. (2008) in which most of the peptides were isolated from frogs and showed good MIC values against MDR *A. baumannii*. The same strategy was used in the report published by Conlon et al. (2009) however the MIC values were higher compared to the other peptides. Other peptides isolated from toad skin secretions were also tested against XDR strains obtaining values from 112.8 to 2.8 mg/L (Conlon et al., 2010). The A3-APO peptide is shown to be more effective and less toxic against *A. baumannii* than colistin, however this has only been observed *in vivo* and the effect could not be shown *in vitro*, suggesting that this peptide may prevent inflammation at the site of the infection (Ostorhazi et al., 2010, 2011).

Different AMPs have been tested against *A. baumannii*, however the peptide constituted by the mixture of cecropin A and melittin (Andreu et al., 1992) has been extensively studied. The first hybrid tested against MDR *A. baumannii* was cecropin A(1-8)melittin(1-18), or CA(1-8)M(1-18). This hybrid was tested under different experimental conditions, with all showing a lower MIC compared to polymixin B (Saugar et al., 2002). The next hybrid tested was CA(1-7)M(2-9) and, compared to the original peptides cecropin A and melittin as well as to other antibiotics, the MIC of this hybrid peptide was 0.25–8 mg/L, being the lowest value compared to other hybrid peptides. In addition, this hybrid peptide also showed synergy with co-amoxiclav, ceftazidime, piperacillin, and imipenem (Giacometti et al., 2003). Synergy of other AMPs and currently used antibiotics has also been studied by Giacometti et al. (2000), observing synergy of magainin II with co-amoxiclav, aztreonam, chloramphenicol, ceftazidime, piperacillin, and meropenem. CA(1-7)M(2-9) was also tested against PDR *A. baumannii* strains, Similar to other hybrids, CA(1-8)M(1-18), Oct-CA(1-7)M(2-9), and CA(1-7)M(5-9), the MIC values ranged from 2 to 8 mg/L compared to 4 to 64 mg/L for colistin. In addition, the hybrids showed bactericidal activity at 4 × MIC (Rodríguez-Hernández et al., 2006). Several commercial peptides have also been tested against colistin-resistant *A. baumannii* strains. Most did not show any activity, although indolicidin and particularly mastoparan showed MIC values of 2 and 1 mg/L, respectively, compared to the colistin MIC of 256 mg/L. Moreover, a bactericidal effect was found for mastoparan along all the killing curve for 8 × MIC (Vila-Farrés et al., 2012).

Although AMPs and AMPs in combination with antibiotics are potential future therapeutic alternatives against PDR *A. baumannii* clinical isolates, another therapeutic alternative might involve the use of vaccines, specifically those administered through OMVs (McConnell et al., 2011a,b).

CONCLUSION

Over the past few decades *A. baumannii* has emerged as one of the most successful nosocomial pathogens throughout the world. This is partly due to its intrinsic aptitude to persist in the hospital setting and acquire multiple resistance mechanisms, but also to its capacity to cause acute infections especially in severely ill patients. While we know a great deal about the mechanisms responsible for antimicrobial resistance in this microorganism we see ourselves unable to stop it. Pan-drug strains resistant even to colistin have already been reported and we are running out of options to deal with this novel menace. On the other hand, there is little knowledge regarding the mechanisms driving the pathogenesis of *A. baumannii* and there is much to learn on this issue. Novel therapeutic strategies are urgently needed and while research on novel antimicrobial agents does not seem very promising, the quest for novel drugs interfering with *A. baumannii* pathogenicity and not just bacterial growth opens up as a novel and challenging alternative.

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Extraintestinal pathogenic *Escherichia coli*: a combination of virulence with antibiotic resistance

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Escherichia coli represents an incredible versatile and diverse enterobacterial species and can be subdivided into the following; (i) intestinal non-pathogenic, commensal isolates. (ii) Intestinal pathogenic isolates and (iii) extraintestinal pathogenic *E. coli* or ExPEC isolates. The presence to several putative virulence genes has been positively linked with the pathogenicity of ExPEC. *E. coli* remains one of the most frequent causes of nosocomial and community-acquired bacterial infections including urinary tract infections, enteric infections, and systemic infections in humans. ExPEC has emerged in 2000s as an important player in the resistance to antibiotics including the cephalosporins and fluoroquinolones. Most importantly among ExPEC is the increasing recognition of isolates producing "newer β-lactamases" that consists of plasmid-mediated AmpC β-lactamases (e.g., CMY), extended-spectrum β-lactamases (e.g., CTX-M), and carbapenemases (e.g., NDM). This review will highlight aspects of virulence associated with ExPEC, provide a brief overview of plasmid-mediated resistance to β-lactams including the characteristics of the successful international sequence types such as ST38, ST131, ST405, and ST648 among ExPEC.

Keywords: *Escherichia coli*, virulence, antimicrobial resistance

INTRODUCTION

Escherichia coli is an important cause of urinary tract infections (UTIs), enteric infections, and systemic infections in humans (Mandell et al., 2005). The systemic infections include bacteremia, nosocomial pneumonia, cholecystitis, cholangitis, peritonitis, cellulitis, osteomyelitis, and infectious arthritis. *E. coli* is also leading cause of neonatal meningitis.

Escherichia coli comprises of non-pathogenic commensal isolates that forms part of the normal flora of humans and various animals (Kaper et al., 2004). However, several variants have been described that cause infection of the gastrointestinal system (intestinal pathogenic *E. coli*) while others cause infections outside the gastrointestinal system (extraintestinal pathogenic *E. coli* or ExPEC). ExPEC incorporates the following variants; avian pathogenic *E. coli*, uropathogenic *E. coli* (UPEC), and those isolates responsible for septicemia and neonatal meningitis (Kaper et al., 2004).

Uropathogenic *E. coli* are the primary causes of community-acquired UTIs with an estimated 20% of women over the age of 18 years suffering from at least one UTI in their lifetime (Foxman, 2010). UPEC is responsible for 70–95% of community-onset UTIs and approximately 50% of nosocomial UTIs, hence accounting for substantial morbidity, mortality, and medical expenses. Recurrent or relapsing UTIs are especially problematic in many individuals. The primary reservoir of UPEC is believed to be the human intestinal tract and employ diverse repertoire of virulence factors to colonize and infect the urinary tract in an ascending fashion (Foxman, 2010). However, community-onset clonal outbreaks of UTIs, possibly due to the consumption of food contaminated with UPEC have also been described (Wiles et al., 2008). Additionally, there is some evidence that UPEC isolates can also be transmitted via sexual activities (Wiles et al., 2008).

The aim of this article is to highlight aspects of virulence associated with ExPEC, to provide a brief overview of the most important plasmid-mediated resistance to β-lactams including the characteristics of international sequence types among ExPEC.

GENOMICS AND VIRULENCE FACTORS

Virulence factors involve mechanisms that enable pathogenic bacteria to cause infections. Genomics offers an interesting tool for defining virulence factors; it can be used to identify genes encoding for specific factors that contributes to virulence in pathogens. However, the presence of a single factor rarely makes an organism virulent; a combination of factors will determine if a bacterium can cause infection (Dobrindt, 2005). Moreover, the determining factor is not simply the presence or absence of virulence-associated genes, but also their levels of expression, which can vary between pathogenic and non-pathogenic isolates.

The *E. coli* genome is composed of a conserved core of genes that provides the backbone of genetic information required for essential cellular processes and a flexible gene pool that harbors genetic information which provides properties that enables the bacterium to adapt to special environmental conditions (Dobrindt, 2005). The size of the flexible gene pool is determined by the acquisition and loss of genomic DNA. The parallel gain and loss of mobile genetic elements in the flexible portion of

the gene pool such as bacteriophages, plasmids, genomic islands, transposons, insertion elements enabled the evolution of separate clones of *E. coli* (Dobrindt, 2005). A portion of the flexible gene pool consists of accessory genetic elements (including plasmids, transposons, insertion elements, prophages, and genomic islands), that can be either integrated into the chromosome or replicate independently as extrachromosomal elements. These elements can be laterally transferred to other species thus contributing to inter-, intra-species variability in genomic contents (Dobrindt, 2005).

The presence to several putative virulence genes has been positively linked with the pathogenicity of ExPEC. Phylogenetic analyses have shown that intestinal *E. coli* and ExPEC falls into four main phylogenetic groups namely A, B1, B2, and D (Herzer et al., 1990). ExPEC belongs mainly to group B2 and, to a lesser extent, to group D while intestinal commensal isolates tend to belong to groups A and B1. ExPEC isolates exhibit considerable genome diversity and possess a broad range of virulence-associated factors including toxins, adhesions, lipopolysaccharides, polysaccharide capsules, proteases, and invasins that are frequently encoded by pathogenic islands and other mobile DNA islands (**Table 1**). It seems that these putative virulence factors contribute to fitness (e.g., iron-uptake systems, bacteriocins, proteases, adhesins) of ExPEC and increase the adaptability, competitiveness, and ability to colonize the human body rather than being typical virulence factors directly involved in infection (Mokady et al., 2005).

Whether a commensal *E. coli* will develop into ExPEC requires the presence of functional genes directly contributing to pathogenesis and certain putative factors enabling the successful colonization of the host that enhances fitness and adaptation of the bacterium to its surroundings. This highlights the thin line between "virulence" and "fitness" and "colonization" factors in the definition of ExPEC virulence factors.

Table 1 | Virulence-associated factors present in ExPEC.

	Virulence factor
ADHESINS	
F10 *papA*	P fimbriae subunit variant
papC	*papACEFG*, genes of P fimbriae operon
papEFG	*papACEFG*, genes of P fimbriae operon
sfa/foc	S or F1C fimbriae
focG	F1C fimbriae adhesin
iha	Adhesion siderophore
fimH	Type 1 fimbriae
tsh	temperature sensitive hemagglutinin
hra	Heat-resistant agglutinin
afa/draBC	Dr-binding adhesins
TOXIN	
hlyD	α-Hemolysin
sat	Secreted autotransporter toxin
pic	Serine protease
vat	Vacuolating toxin
astA	Enteroaggregative *E. coli* toxin
cnf1	Cytotoxic necrotizing factor
SIDEROPHORES	
iroN	Salmochelin (siderophore) receptor
fyuA	Yersiniabactin (siderophore) receptor
ireA	Siderophore receptor
iutA	Aerobactin (siderophore) receptor
CAPSULE	
kpsM II	*kpsM* II group 2 capsule
K1	K1 group 2 capsule variants
K2	K2 group 2 capsule variants
K5	K5 group 2 capsule variants
kpsMT III	Group 3 capsule
MISCELLANEOUS	
usp	Uropathogenic-specific protein
traT	Serum resistance-associated
ompT	Outer membrane protease T
iss	Increased serum survival
H7 *fliC*	Flagellin variant
malX	Pathogenicity island marker

ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI*

The management of infections caused by ExPEC has been complicated by the emergence of antimicrobial resistance, especially since the late 1990s (Pitout and Laupland, 2008). The cephalosporins, fluoroquinolones, and trimethoprim–sulfamethoxazole are often used to treat community and hospital infections caused by *E. coli* and resistance to these agents is responsible for delays in appropriate therapy with subsequently increasing morbidity and mortality (Lautenbach et al., 2001; Tumbarello et al., 2007). Until the late 1990s ExPEC were relatively susceptible to first line antibiotics, however several surveillance studies during the 2000s across Europe, North and South America have shown that between 20 and 45% of ExPEC are resistant to first line antibiotics including the cephalosporins, fluoroquinolones, and trimethoprim–sulfamethoxazole (Foxman, 2010).

RESISTANCE TO β-LACTAMS

β-Lactam antibiotics, especially the third generation cephalosporins, are a major drug class used to treat serious community-onset or hospital-acquired infections caused by *E. coli* (Livermore and Woodford, 2006). Among *E. coli*, β-lactamase production remains the most important mediator of β-lactam resistance. β-lactamases are bacterial enzymes that inactivate β-lactam antibiotics by hydrolysis, which results in ineffective compounds (Livermore and Woodford, 2006).

Most importantly is the increasing numbers of ExPEC isolates producing "newer β-lactamases" that includes the plasmid-mediated AmpC β-lactamases (e.g., CMY types), extended-spectrum β-lactamases (ESBL; e.g., CTX-M types), and carbapenemases. The carbapenemases consists of three important classes; the class A (e.g., KPC types), the class B or metallo-β-lactamases (MBLs), and class D or OXA types (Jacoby and Munoz-Price, 2005). CMY-2, CTX-Ms, and NDM types of β-lactamase are mostly responsible for the emerging resistance to the β-lactam antibiotics in *E. coli*. The characteristics of these newer β-lactamases including the KPC and OXA types are summarized in **Table 2**. Various carbapenemases including VIM, IPM, KPC, and OXA-48 β-lactamases have been described in members of the

Table 2 | Newer β-lactamases in *Escherichia coli*.

Enzymes	Classification	Examples	Spectrum of resistance	Inhibition
Extended-spectrum β-lactamases (ESBLs)	Class A	CTX-M, TEM, SHV	Penicillins Cephalosporins Monobactams	Clavulanic acid Tazobactam Sulbactam
Plasmid-mediated AmpC β-lactamases	Class C	CMY, FOX, ACT, MOX, ACC, DHA	Penicillins Cephalosporins Monobactams Cephamycins	Cloxacillin Boronic acid
Metallo-β-lactamases (MBLs)	Class B	IMP, VIM, NDM	Penicillins Cephalosporins Cephamycins Carbapenems	Metal chelators, e.g., EDTA and dipicolinic acid
KPC carbapenemases	Class A	KPC	Penicillins Cephalosporins Cephamycins Carbapenems	Clavulanic acid (weak) Tazobactam Boronic acid
OXA-β-lactamases	Class D	OXA-48, -181	Penicillins Temocillin β-lactamase combinations Carbapenems	Clavulanic acid (weak) NaCl

Enterobacteriaceae (including *E. coli*). However, these enzymes are less common among *E. coli* and will not be addressed in this review. It is therefore important to remember that in certain areas of the world, KPC and OXA-48-producing *E. coli* can be important causes of carbapenem resistance.

EXTENDED-SPECTRUM β-LACTAMASES

The most well known of the "newer" β-lactamases was first described in 1983 and have been named the ESBLs. These enzymes have the ability to hydrolyse the penicillins, cephalosporins, and monobactams, but not the cephamycins and carbapenems, and are inhibited by "classical" β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam (Paterson and Bonomo, 2005). Although ESBLs have been identified in a range of Enterobacteriaceae, they are most often present in *E. coli* and *K. pneumoniae*. The majority of ESBLs identified in clinical isolates during the 1980s–1990s were the SHV or TEM types, which evolved from parent enzymes such as TEM-1, -2, and SHV-1 (Paterson and Bonomo, 2005). A different type of ESBL, named CTX-M β-lactamases, originated from environmental *Kluyvera* spp., and gained prominence in the 2000s with reports of clinical isolates of *E. coli* producing these enzymes from Europe, Africa, Asia, South and North America (Canton and Coque, 2006). Since the mid 2000s CTX-M β-lactamases had been identified in different members of the Enterobacteriaceae, but especially in *E. coli*, and have become the most widespread and common type of ESBL (Pitout and Laupland, 2008).

CTX-M-producing *E. coli* are important causes of community-onset UTIs, bacteremia and intra-abdominal infections (Canton and Coque, 2006). Risk factors associated with infections caused by CTX-M-producing *E. coli* include the following: repeat UTIs, underlying renal pathology, previous antibiotics including cephalosporins and fluoroquinolones, previous hospitalization, nursing home residents, older males and females, Diabetes Mellitus, underlying liver pathology, and international travel to high risk areas such as the Indian subcontinent (Rodriguez-Bano and Pascual, 2008). There are geographic variations in the prevalence of ExPEC that produce CTX-Ms. Surveys from several countries worldwide have illustrated an alarming trend of associated resistance to other classes of antimicrobial agents among CTX-M-producing *E. coli* that included trimethoprim–sulfamethoxazole, tetracycline, gentamicin, tobramycin, and ciprofloxacin (Pitout and Laupland, 2008).

Currently, the most widespread and prevalent type of CTX-M enzyme among human clinical isolates of *E. coli* is CTX-M-15. ExPEC producing this enzyme often belong to the international uropathogenic sequence type named ST131 and to a lesser extend ST38, ST405, and ST648 (Peirano and Pitout, 2010). It seems that the intercontinental dissemination of these sequence types have in part contributed to the worldwide emergence of CTX-M-15 producing *E. coli*. ST131 with CTX-M-β-lactamases when compared to other ESBL-producing *E. coli* was more likely to be resistant to antibiotics, to produce the aminoglycoside modifying enzyme *aac(6′)-Ib-cr*, and cause community-acquired infections including urosepsis (Pitout et al., 2009). ST131 is not a single entity and can be subdivided into different clusters by other typing methods such as pulsed-field electrophoresis (Peirano et al., 2011a). It is therefore possible that variants of ST131 may differ in virulence profiles and ability to spread. The global distribution of ST131 cam also reflects repeated selection of local variants that have acquired certain resistance plasmids. A recent study from Canada showed that investigated the molecular epidemiology of ESBLs-producing *E. coli* causing bacteremia over an 11-year period (2000–2010) showed that ST131 was the most common

and antimicrobial resistant sequence type and the influx of a single pulsotype of ST131 was responsible for a significant increase of ESBL-producing *E. coli* especially since 2007 (Peirano et al., 2011a).

Johnson et al. (2009) gave some insight into the origin of ST131 in North America. They studied 199 trimethoprim–sulfamethoxazole-resistant and fluoroquinolone-resistant *E. coli* isolated from urines in Canada during 2002–2004 and identified ST131 in 23% of isolates and nearly all were fluoroquinolone-resistant (i.e., 99%) but, notably, remained susceptible to the cephalosporins (i.e., only 2% of ST131 in that study were resistant to the cephalosporins; Johnson et al., 2009). Another study by Johnson et al. (2010) investigated the presence and virulence properties of ST131 among 127 ExPEC *E. coli* from the 2007 SENTRY and meropenem yearly susceptibility test information collection (MYSTIC) surveillance programs across the United States. Overall 54 (i.e., 17%) belonged to ST131, but interestingly this sequence type included 52% of isolates that showed resistance to ≥3 antimicrobial classes. ST131 has a significant higher virulence score that other ExPEC and certain virulence factors such as uropathogenic-specific protein (*usp)*; outer membrane protein (*ompT)*; secreted autotransporter toxin (*sat*), aerobactin receptor (*iutA*), and pathogenicity island marker (*malX*) were associated with this sequence type. Their results showed that ST131 had distinctive virulence and resistance profiles and concluded that the combination of antimicrobial resistance and virulence may be responsible for the epidemiological success of this sequence type.

Studies have recently identified different sequence types such as ST38, ST405, and ST648 among CTX-M-producing *E. coli.* ST38 with CTX-M-9, -14, and -15 has previously been described in clinical isolates from Japan (Suzuki et al., 2009), the Netherlands (van der Bij et al., 2011), Korea (Kim et al., 2011), and Tanzania (Mshana et al., 2011). This sequence type is also associated with OXA-48 (Poirel et al., 2011) and NDM-1 (Yamamoto et al., 2011). ST405 with various types of CTX-Ms has a worldwide distribution (Coque et al., 2008; Jones et al., 2008; Mihaila et al., 2010; Smet et al., 2010) while ST648 with CTX-M-15, -32 are present in poultry from Spain (Cortes et al., 2010) and humans in China (Zong and Yu, 2010) and the Netherlands (van der Bij et al., 2011). ST405 and ST648 are also associated with NDM β-lactamases (Hornsey et al., 2011; Mushtaq et al., 2011). These reports suggest that they might also play a role in the worldwide distribution and success of CTX-M-producing *E. coli.*

It is evident today that CTX-M-producing *E. coli* is a major player in the world of antimicrobial resistance. A report from the Infectious Diseases Society of America listed ESBL-producing *E. coli* as a priority drug-resistant microbe to which new therapies are urgently needed (Talbot et al., 2006).

PLASMID-MEDIATED AmpC β-LACTAMASES

Escherichia coli possess a chromosomal gene that encodes for an AmpC β-lactamase. Usually, low amounts of this β-lactamases are produced because the AmpC gene is regulated by a weak promoter and a strong attenuator (Olsson et al., 1982). Occasionally, cephamycin-resistant strains produce plasmid-mediated β-lactamases derived from bacteria with chromosomally encoded, AmpC-cephalosporinases such as *Enterobacter*, *Citrobacter feundii*, *Morganella morganii*, *Aeromonas* spp., and *Hafnia alvei* (Philippon et al., 2002). These β-lactamases at high levels, hydrolyse penicillins, most cephalosporins, cephamycins, and monobactams, but not the fourth generation cephalosporins (e.g., cefepime) and carbapenems. AmpC enzymes are not inhibited by "classical" β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam although boronic acids and cloxacillin have shown to be good inhibitors (Doi and Paterson, 2007).

Just like ESBL-producing bacteria, organisms with plasmid-mediated AmpC enzymes have mostly been responsible for nosocomial outbreaks on a worldwide basis during the late 1980s and 1990s, although the risk factors associated with infection are not as well defined as those associated with ESBL-producing bacteria (Philippon et al., 2002). In a study reported from Korea, patients infected by plasmid-mediated AmpC-producing organisms had similar clinical features and outcomes to those patients infected with TEM- or SHV-related ESBL producers (Pai et al., 2004).

The SENTRY Antimicrobial Surveillance Program in the USA found plasmid-mediated AmpC β-lactamases in 2% of 1429 *E. coli* isolates from 30 centers; with CMY-2, FOX-5, and DHA-1 being identified (Deshpande et al., 2006). A UK study found acquired cephamycinases in 49% of cefoxitin-resistant *E. coli* (Woodford et al., 2007).

It seems that CMY-2, derived ancestrally from *Citrobacter* spp., is the most common plasmid-mediated cephamycinase reported in *E. coli* from different areas of the world. Recent hospital surveys from Asia, North America, and Europe have shown that the CMY types are present in *E. coli* from Asia, North America, and Europe (Alvarez et al., 2004; Mulvey et al., 2005; Moland et al., 2006; Woodford et al., 2007; Li et al., 2008). A population-based study conducted in Calgary, concluded that in this large Canadian region, CMY-2-producing *E. coli* is an emerging pathogen in the community that commonly causes UTIs in older women (Pitout et al., 2007). This was followed by two reports from Washington and Nebraska which showed Enterobacteriaceae producing CMY, ACC, and DHA types of AmpC β-lactamases are present in clinical isolates from outpatient clinics in the USA (Hanson et al., 2008; Qin et al., 2008).

METALLO-β-LACTAMASES (NDM-1)

Recently, a new type of MBL, named NDM, was described in *K. pneumoniae* and *E. coli* recovered from a Swedish patient who was hospitalized in New Delhi, India (Yong et al., 2009). MBLs have the ability to hydrolyse a wide variety of β-lactams, including the penicillins, cephalosporins, and carbapenems, but not the monobactams (i.e., aztreonam), and are inhibited by metal chelators such as EDTA (**Table 2**). The majority of NDM-1-producing bacteria are broadly resistant to various drug classes and also carry a diversity of other resistance mechanisms (e.g., to aminoglycosides and fluoroquinolones), which leaves limited treatment options (Nordmann et al., 2011a).

Kumarasamy et al. (2010), provide compelling evidence that NDM-producing Enterobacteriaceae (mostly *K. pneumoniae* and *E. coli*) are widespread in India and Pakistan. They also found that many UK patients infected with NDM-producing bacteria had recently traveled to India to undergo several types of medical procedures. The patients presented with a variety of hospital-

and community-associated infections with UTIs being the most common clinical syndrome. Recent reports from the subcontinent (including India, Pakistan, and Bangladesh) show that the distribution of NDM β-lactamases among Enterobacteriaceae are widespread through these countries (Castanheira et al., 2011a,b; Lascols et al., 2011): e.g., a hospital in Varanasi in Northern India identified NDM-1 prevalence rate of 6% among *E. coli* (n = 528) from outpatients and hospitalized patients between February 2010 and July 2010 (Seema et al., 2011), 7% among *E. coli* from a major hospital in Mumbai, India (Deshpande et al., 2010), whereas 15% (30/200) of in- and outpatients in Rawalpindi, Pakistan carried *E. coli* with NDM-1 in their gut flora (Perry et al., 2011).

Since mid-August 2010, NDM-1-positive bacteria have been reported worldwide, except in Central and South America (Nordmann et al., 2011b). Most are Enterobacteriaceae from patients hospitalized in 2009 and 2010 with an epidemiological link to the Indian subcontinent. Recent findings suggest that the Balkan states and the Middle East might act as secondary reservoirs for the spread of NDM-1, which may or may not initially have reached these countries from the Indian subcontinent. Enterobacteriaceae with NDM-1 have been recovered from many clinical settings, reflecting the disease spectra of these opportunistic bacteria, including hospital and community-onset UTIs, septicemia, pulmonary infections, peritonitis, device-associated infections, and soft tissue infections (Castanheira et al., 2011a; Nordmann et al., 2011a,b). NDM-1-positive bacteria have been recovered from the gut flora of travelers returning from the Indian subcontinent and undergoing microbiological investigation for unrelated diarrheal symptoms (Leverstein-Van Hall et al., 2010). There is also widespread environmental contamination by NDM-1-positive bacteria in New Delhi (Walsh et al., 2011).

There is no evidence that *E. coli* that produce NDM are more virulent than other isolates, however recent studies described presence of NDM β-lactamases in the very successful *E. coli* sequence type ST131 with an identical virulence genotype than ST131 that produce CTX-M β-lactamases (Peirano et al., 2011b). Of interest, ST131 with VIM and KPC carbapenemases have also recently been described (Mantengoli et al., 2011; Morris et al., 2011). Due to the very resistant nature of these NDM-producing *E. coli*, the treatment of infections due to these bacteria will remain a challenge to physicians. Antibiotics such as colistin, tigecycline, and fosfomycin show the best activity against NDM-producing bacteria (Nordmann et al., 2011b). However, Stone et al. (2011) recently described a breakthrough bacteremia caused by *E. coli* that produce NDM while the patient was treated with tigecycline; the isolate developed resistance to tigecycline.

SUMMARY

Escherichia coli is an important cause of UTIs, enteric infections, and systemic infections in humans. The presence to several putative virulence genes has been positively linked with the pathogenicity of ExPEC including toxins, adhesions, lipopolysaccharides, polysaccharide capsules, proteases, and invasins that are frequently encoded by pathogenic islands and other mobile DNA islands.

Up to the late 1990s ExPEC was relatively susceptible to first line antibiotics, however several surveillance studies during the 2000s across the world have shown increasing resistance to first line antibiotics including the cephalosporins, fluoroquinolones, and trimethoprim–sulfamethoxazole. The "newer β-lactamases" that consists of plasmid-mediated AmpC β-lactamases (e.g., CMY types), ESBL (e.g., CTX-M types), and carbapenemases (e.g., NDM) are important causes of resistance to β-lactam antibiotics among ExPEC.

Recent reports suggest that certain sequence types such as ST38, ST131, ST405, and ST648 might play an important role in the worldwide distribution and success of CTX-M-producing *E. coli*. Future investigations should be undertaken to study the microbiological and ecological factors that make CTX-M and NDM-producing *E. coli* such successful pathogens including well-designed epidemiological, clinical, and molecular studies to understand the dynamics of transmission, risk factors, and reservoirs for ST131.

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CTX-M enzymes: origin and diffusion

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CTX-M β-lactamases are considered a paradigm in the evolution of a resistance mechanism. Incorporation of different chromosomal bla_{CTX-M} related genes from different species of *Kluyvera* has derived in different CTX-M clusters. *In silico* analyses have shown that this event has occurred at least nine times; in CTX-M-1 cluster (3), CTX-M-2 and CTX-M-9 clusters (2 each), and CTX-M-8 and CTX-M-25 clusters (1 each). This has been mainly produced by the participation of genetic mobilization units such as insertion sequences (IS*Ecp1* or IS*CR1*) and the later incorporation in hierarchical structures associated with multifaceted genetic structures including complex class 1 integrons and transposons. The capture of these bla_{CTX-M} genes from the environment by highly mobilizable structures could have been a random event. Moreover, after incorporation within these structures, β-lactam selective force such as that exerted by cefotaxime and ceftazidime has fueled mutational events underscoring diversification of different clusters. Nevertheless, more variants of CTX-M enzymes, including those not inhibited by β-lactamase inhibitors such as clavulanic acid (IR-CTX-M variants), only obtained under in *in vitro* experiments, are still waiting to emerge in the clinical setting. Penetration and the later global spread of CTX-M producing organisms have been produced with the participation of the so-called "epidemic resistance plasmids" often carried in multi-drug resistant and virulent high-risk clones. All these facts but also the incorporation and co-selection of emerging resistance determinants within CTX-M producing bacteria, such as those encoding carbapenemases, depict the currently complex pandemic scenario of multi-drug resistant isolates.

Keywords: bla_{CTX-M} genes, *Kluyvera* spp., gene mobilization, IS*Ecp1*, IS*CR1*, plasmid, bacterial clones, antibiotic selective force

INTRODUCTION

Antibiotic resistance is a topic in continuous review and the several studies published every year with new data can modify or redefine our perspective about this problem. β-lactam resistance and particularly overviews about β-lactamases, enzymes able to hydrolyze β-lactam antibiotics, have undoubtedly been the most frequently studied topics in this field. β-lactams, the antibiotics most widely used all over the world, have also given rise to a continuous increase of resistant isolates due to their permanent selective force driving diversification of the resistance mechanisms.

β-Lactamases have been studied from many different aspects, including classification, hydrolytic profiles and kinetic properties, plasticity, frequency, and dissemination. Moreover they have been used as an example of the accelerated evolution following the Darwinian paradigm, where the strong antibiotic selective pressure favors the survival of the most adapted. According to a recent review from Davies and Davies (2010), a dramatic increase in the number of β-lactamases has been described since the 1980s of the last century but this increase is due almost exclusively to class A and D β-lactamases (Bush and Jacoby, 2010). Among the class A β-lactamases, the extended-spectrum β-lactamases (ESBLs) which are able to hydrolyze expanded-spectrum cephalosporins (such as cefotaxime, ceftriaxone, ceftazidime, or cefepime) and monobactams (aztreonam) represent a public health concern (Coque et al., 2008a; Pitout and Laupland, 2008). ESBLs of class A mainly include TEM, SHV, CTX-M, VEB, and GES enzymes. Among them, the highest number of variants described in the last years corresponds to the CTX-M family (123 variants until 2011, last accession December 5, 2011)[1]. This explosive dissemination of CTX-Ms around the world has been referred as the "CTX-M pandemic" due to their increasing description worldwide (Cantón and Coque, 2006). Although different updates about CTX-M β-lactamases have been published (Bonnet, 2004; Livermore et al., 2007; Rossolini et al., 2008; Hawkey and Jones, 2009; Naseer and Sundsfjord, 2011), new data about dispersion and clonality of CTX-M producing isolates, molecular epidemiology, protein plasticity, evolution and origin of the bla_{CTX-M} genes, influence of antibiotic use, and patients risk factors, justify this new review. **Figure 1** illustrates different factors fueling the emergence, maintenance, and spread of the CTX-M ESBLs. We will focus our attention on the epidemiology, origin and evolution of bla_{CTX-M} genes, and influence of surrounding genetic structures participating in the maintenance and spread of these genes and hence organisms producing CTX-M enzymes.

[1] www.lahey.org/Studies/other.asp

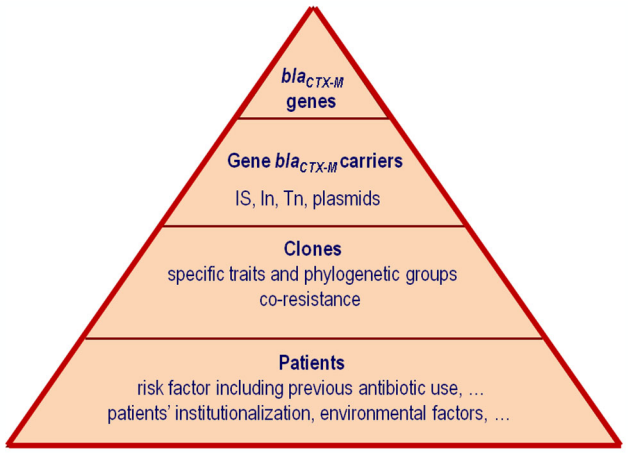

FIGURE 1 | Factors fueling the emergence, maintenance, and spread of the CTX-M extended-spectrum β-lactamases (ESBLs).

CLASSIFICATION OF β-LACTAMASES AND CTX-M ENZYMES

β-Lactamases are by far the most important resistant mechanisms in Gram-negative bacilli. With the popularization of molecular techniques, an increasing number of these enzymes have been characterized differing in amino acid sequences and hydrolytic activity against β-lactam antibiotics (Bush, 2010). They are mainly classified using the Ambler scheme (Ambler, 1980), which grades to the so-called molecular classes (A through D) and also currently functional groups which use the characteristic of the enzymes, including their hydrolytic substrate profile, and response to inhibitors (Bush and Jacoby, 2010). Classically, genetic location of the corresponding β-lactamase genes (*bla*) in plasmids or chromosome was also used to differentiate these enzymes. Nevertheless, this genetic characteristic is no longer used, as chromosomal *bla* genes can be mobilized and integrated into plasmids or transposons, but a reverse situation of initially describes plasmid-mediated β-lactamases into the chromosome is also increasingly found (Toleman et al., 2006; Coelho et al., 2010). In addition protein regulation characteristics (i.e., constitutive or inducible expression) are also noted when referring to different β-lactamases groups (Livermore, 1995) but this trait depends on the surrounding genes. In Gram-negative organisms, inducible expression of β-lactamases is commonly found in chromosomal β-lactamases whereas plasmid-mediated enzymes are generally constitutively expressed. Enhancement expression of their hydrolytic activity is often regulated by promoters present in upstream genes.

Within the β-lactamases, the ESBLs have been worthy of the attention of the scientific community and clinicians over the last three decades since their first description in 1983 (Knothe et al., 1983). In general, ESBLs are located in plasmids and are characterized for their ability to hydrolyze oxy-imino-cephalosporins (third and fourth generation cephalosporins) and monobactams but not cephamycins such as cefoxitin and carbapenems including imipenem, ertapenem, meropenem, or doripenem (Philippon et al., 1989; Jacoby and Muñoz-Price, 2005; Bush and Fisher, 2011). In addition, they are generally susceptible to β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Mainly, they are included in the 2be Bush-Jacoby and Medeiros functional group belonging to the molecular class A β-lactamases from the Ambler's classification (Ambler, 1980; Bush et al., 1995; Bush and Jacoby, 2010). Enzymes showing an "ESBL phenotype" have been mainly described in TEM-, SHV-, CTX-M, GES, and VEB families into class A β-lactamases, and OXA-ESBLs into class D β-lactamases (Paterson and Bonomo, 2005).

The TEM-, SHV-, and OXA-type ESBL enzymes derive from point mutations from the classical plasmid-mediated enzymes TEM-1, TEM-2, SHV-1, and OXA-10 which are not of greater importance in hydrolyzing extended-spectrum cephalosporins (Paterson and Bonomo, 2005; Bush and Jacoby, 2010). The TEM- and SHV-ESBLs were predominant in the ESBL landscape over the 1980s and 1990s in the past century, mainly associated with outbreaks in hospitals involving *Klebsiella pneumoniae* and to a lesser extent in *Escherichia coli* and other Enterobacteriaceae whereas the CTX-M were less prevalent (Bradford, 2001; Paterson and Bonomo, 2005). Although first discovered in 1989 (Bauernfeind et al., 1990, 1992), the CTX-M enzymes did not reach prominence over the other ESBL enzymes until the first decade of the 2000s when accelerated evolution and extraordinary dispersion of these enzymes were observed (Cantón, 2008). They were confined not only to the hospital setting but also to the community, with *E. coli* being the most important pathogen producing these enzymes (Cantón and Coque, 2006; Coque et al., 2008a).

Unlike other ESBLs, CTX-M family constitutes a complex and non-homogeneous group of enzymes. The first view and alignment of the amino acid sequence of the different CTX-M variants allowed to classify these enzymes in five clusters but recent publications report at least two more additional clusters (Bonnet, 2004; Rossolini et al., 2008; see also below). Phylogenetic analyses suggest that CTX-Ms were not originated by mutations from previous plasmid meditated enzymes but through mobilization of chromosomal *bla* genes from *Kluyvera* spp. when they were incorporate into mobile genetic elements (Cantón, 2008). These original mobilized $bla_{\text{CTX-Ms}}$ genes affected cefotaxime to a higher degree than ceftazidime. Nevertheless and from an evolutionary point of view, CTX-Ms as other ESBLs later diverged by punctual mutations probably as consequence of antibiotic selective pressure once *Kluyvera* spp. $bla_{\text{CTX-M}}$ genes were mobilized and were incorporated into mobile genetic elements. This also gave the CTX-M enzymes the opportunity to enhance the hydrolytic activity against ceftazidime and a new variants were described (Bonnet, 2004; Poirel et al., 2008).

EPIDEMIOLOGY OF CTX-M β-LACTAMASE-PRODUCING BACTERIA

Studies over the last 10 years have revealed that unlike some exceptions, the CTX-M enzymes have nearly displaced other ESBLs enzymes in Enterobacteriaceae, including TEM and SHV ESBL variants (Cantón, 2008; Coque et al., 2008a; Angel Díaz et al., 2009; Hawkey and Jones, 2009; Bush, 2010; Rodriguez-Villalobos et al., 2011). This displacement might have occurred not only as a consequence of the extraordinary dissemination of the corresponding

bla$_{CTX-M}$ genes in highly mobilizable genetic platforms, including plasmids and transposons, but also because of these platforms within successful clones (Cantón and Coque, 2006; Rogers et al., 2011; Woodford et al., 2011; **Figure 2**). Another reason for this increase is the co-resistance phenomenon in CTX-M producing organisms, particularly to aminoglycosides and fluoroquinolones, which might facilitate co-selection processes (Morosini et al., 2006; Cantón and Ruiz-Garbajosa, 2011).

Apart from this general overview, within the CTX-M enzymes, the CTX-M-15, and CTX-M-14 are by far the most important ones, virtually invading all human and animal compartments as well as the environment all over the world (Cantón et al., 2008; Hawkey and Jones, 2009; Dolejska et al., 2011; Hiroi et al., 2012). Nevertheless, temporal emergence and penetration of these enzymes in different epidemiological scenarios might also explain the current epidemiology of CTX-M enzymes. Antibiotic consumption and dissimilar risk factors in different geographic areas and groups of patients and particularities of different compartments might have also contributed to the current CTX-M scenario (Carattoli, 2008; Rodríguez-Baño and Navarro, 2008; Rodríguez-Baño and Pascual, 2008; Oteo et al., 2010a; Naseer and Sundsfjord, 2011). In this sense three different periods of CTX-M can be differentiated. The first one includes emergence of different CTX-M β-lactamases in different and distant geographic areas and might have occurred until the mid-1990s of the last century. The second was characterized by the emergence of the most widespread CTX-M enzymes, including CTX-M-3, CTX-M-9, CTX-M-14, and CTX-M-15 enzymes and might have occurred from 1994 to 2000. Some of these enzymes, but not exclusively, are variants of previously described CTX-M β-lactamases. Finally, the third period through 2000 is characterized by the universal dispersion and globalization of these β-lactamases. The second and third periods will be presented together bellow.

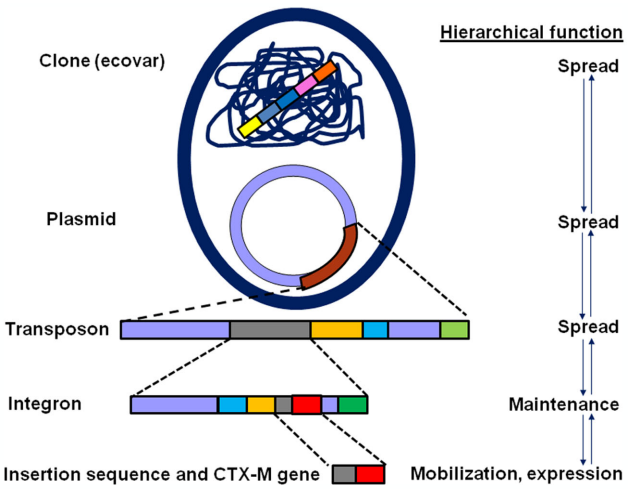

FIGURE 2 | Hierarchical complexity of *bla*$_{CTX-M}$ genes within genetic structures and bacterial clones participating in the mobilization, spread, and maintenance of these genes (see the text for further explanation).

THE EMERGENCE OF CTX-M β-LACTAMASES: DIFFERENT CTX-M ENZYMES IN DIFFERENT COUNTRIES

The first recognition of the emergence of CTX-M β-lactamases occurred nearly simultaneously in Europe and South America at the beginning of 1989 (Bauernfeind et al., 1990, 1992). The first publication recognizing an ESBL from the CTX-M group was that reporting a cefotaxime-resistant but ceftazidime susceptible *E. coli* isolate recovered from the ear of a 4-month-old child suffering from otitis media in Munich (Germany; Bauernfeind et al., 1990). The enzyme responsible for this particular ESBL phenotype not affecting ceftazidime was named as CTX-M-1 in reference to its preferential hydrolytic activity against cefotaxime (CTX as its acronym, -M from Munich). This enzyme inaugurated a group of enzymes that during the 1990s were also grouped as cefotaximases due to their particular phenotypic and hydrolytic profile (Bonnet, 2004). In South America, the first isolates with a "cefotaximase pattern" were detected in *Salmonella typhimurium* recovered in hospitalized patients suffering from meningitis, septicemia, or enteritis (Bauernfeind et al., 1992). The enzyme responsible for this phenotype had a different isoelectric point (pI 7.9) than the one (pI 8.9) described in Germany and was named as CTX-M-2. Later sequencing of *bla*$_{CTX-M-2}$ gene in 1996 confirmed a different amino acid sequence of these two enzymes but a shared homology of 84% (Bauernfeind et al., 1996).

These CTX-M-1 and CTX-M-2 β-lactamases were the first reported CTX-M like enzymes but not chronologically the enzymes belonging to this group. They were preceded by the isolation in 1986 of a cefotaxime-resistant *E. coli* isolate recovered from the fecal flora of a laboratory dog which was used for pharmacokinetic studies of β-lactam antibiotics in Japan (Matsumoto et al., 1988). The enzyme responsible for this phenotype was named as FEC-1 (Fecal *E. coli*), which was later recognized to be related with the CTX-M-3 enzyme initially found in Poland (Bonnet, 2004). Moreover, and also at the beginning of 1989 but in France, an *E. coli* with an identical phenotype was isolated from a Italian patient (Barthélémy et al., 1992; Bernard et al., 1992). The β-lactamase, named as MEN-1 (named after the patient in who it was isolated), was the first available sequence of a CTX-M β-lactamase (Barthélémy et al., 1992). In 1996 sequencing of both the original CTX-M enzymes, CTX-M-1 and CTX-M-2, revealed that MEN-1 was identical to CTX-M-1 (Bauernfeind et al., 1996).

Other CTX-M related β-lactamases that were contemporarily isolated during the 1990s were Toho-1 and Toho-2 enzymes (Toho refers to Toho University School of Medicine Omori Hospital in Tokyo). The former were characterized in a cefotaxime-resistant *E. coli* isolate recovered in 1993 from a 1-year-old female child who developed cystitis in Japan (Ishii et al., 1995) and the latter also in a cefotaxime-resistant *E. coli* isolate recovered in Japan in 1995 from the urine of a 69-year-old male who suffered from colon cancer (Ma et al., 1998). Sequence alignment revealed their linkage with CTX-M-2. Both enzymes were later renamed and designated as CTX-M-44 (Toho-1) and CTX-M-45 (Toho-2; last accession December 5, 2011)[2]

[2]http://www.lahey.org/Studies/.

All these earliest descriptions of CTX-M enzymes showed hot areas for their emergence (Central Europe, South America, and Japan) that could reflect not only specific selective driving forces such as antibiotic consumption but also geographic specificity for different enzymes that could be related with their presence in specific clones or abundance of organism from which they derived (see below). Unfortunately, clonality of ancestral CTX-M producing isolates was not performed as well as plasmid characterization. These studies would have been relevant for tracing the emergence and dissemination of the first CTX-M ESBLs. Moreover, epidemiological surveys including molecular characterization at the end of the 1980s from other geographic areas, such as South East Asia that is nowadays relevant for emerging β-lactamases, including carbapenemases (enzymes that are able to hydrolyze carbapenems), were absent. These would also have been important for the better understanding of the current dissemination of epidemiologically important CTX-Ms that were described through 1994, such as CTX-M-3, CTX-M-9, CTX-M-14, and CTX-15. **Table 1** includes epidemiological data of the earliest descriptions of different CTX-M ESBLs during the 1980s and 1990s. This data are relevant for understanding current epidemiological scenario.

PENETRATION AND GLOBALIZATION OF CTX-M ENZYMES ALL OVER THE WORLD

During the 1990s, different reports showed the appearance nearly at the same time of the same enzymes in very distant countries, denoting the potential independent emergence of these enzymes but also suggesting a rapid dissemination. During these years, diversification was also noted with the ever increasing description of new CTX-M enzymes from different groups. This has been well illustrated with CTX-M-3, a closely related enzyme with CTX-M-1 differing in four amino acid positions (V77A, D114N, S140A, and N288D). This enzyme was detected in *E. coli* and *K. pneumoniae* isolates recovered in 1996–1997 in Poland (Gniadkowski et al., 1998; Palucha et al., 1999), in 1998 in France (De Champs et al., 2000; Doucet-Populaire et al., 2000) and in 1999 in Taiwan (Yan et al., 2000). Unlike genetic determinants harboring $bla_{\text{CTX-M-3}}$ gene which were not sequenced at that time, CTX-M plasmid size and plasmid restriction patterns showed similarity demonstrating the potential diffusion of the same plasmids within isolates obtained from Poland and Taiwan (Yan et al., 2000). Despite this result, importation of this enzyme to Taiwan from Europe or vice versa was not possible to rule out as clonality was not addressed. Nevertheless, in both countries rapid dissemination of this enzyme was illustrated after its emergence (Baraniak et al., 2002; Yu et al., 2002; Ma et al., 2005).

Diversification of CTX-M-1 cluster was exemplified with increasing descriptions of new variants over the 1990s. For instance, CTX-M-10 was mainly described in the Mediterranean area (Spain and France; Oliver et al., 2001; Leavitt et al., 2009) and the CTX-M-15, initially identified in 1999 in enteric isolates recovered from a hospital in New Delhi, India (Karim et al., 2001), but nowadays found all over the world (Hawkey and Jones, 2009; Rogers et al., 2011). The CTX-M-10 differs from CTX-M-3 in two amino acids (A27V and R38Q) whereas CTX-M-15 in only one amino acid position (D240G). Nevertheless, diversification of CTX-M-3, CTX-M-10, and CTX-M-15 should have been produced from a common ancestor (Novais et al., 2010).

A similar example is the CTX-M-9 enzyme, which was initially described in Spain and Brazil in 1996 (Sabaté et al., 2000; Bonnet et al., 2001), followed by China in 1997 (Chanawong et al., 2002). A retrospective study performed in France revealed the presence of this enzyme in strains isolated during 1994 (Saladin et al., 2002). In Spain, this enzyme became one of the most prevalent ESBL but co-existing with CTX-M-14 enzyme (a variant of CTX-M-9 differing in one amino acid, A231V). This enzyme emerged in Korea in 1995 (Pai et al., 2001) and was rapidly disseminated over the world. In China, it was detected in 1997–1998 (Chanawong et al., 2002), in Taiwan in 1998–1999 (Yu et al., 2002), in France in 1999 (Dutour et al., 2002) and in Brazil in 1999 (Bonnet, 2004).

Surveys performed in different countries during that period revealed that once a CTX-M β-lactamase penetrates in a specific geographic area it becomes prevalent with displacement or superimposition over TEM and SHV ESBL variants. This was the case of CTX-M-2 in South America, CTX-M-3 in Poland and CTX-M-14 in Spain, Taiwan and China (Bonnet, 2004; Livermore and Hawkey, 2005). This landscape was also the case for CTX-M-15 in United Kingdom (Paterson and Bonomo, 2005; Cantón and Coque, 2006; Hawkey and Jones, 2009). As previously noted, it was first isolated in India in 1999 (Karim et al., 2001) but also emerged in the United Kingdom in 2001 and probably in Japan early in the 2000s [designated as UOE-1 in GeneBank (accession number AY013478; Mushtaq et al., 2003; Livermore and Hawkey, 2005)]. Moreover, in Poland a retrospective study of a collection of Enterobacteriaceae resistant to expanded-spectrum cephalosporins revealed the presence of the CTX-M-15 in *Serratia marcescens* and *E. coli* since 1998 (Baraniak et al., 2002). Interestingly plasmid analysis suggested that this enzyme could have evolved directly from CTX-M-3 rather than being imported from another country and independently in different hospitals.

In the United Kingdom, isolation of CTX-M was rare before 2001 and only sporadic cases of CTX-M-9 were described as well as an important outbreak due to CTX-M-26, a variant of CTX-M-25 with three amino acid substitutions (V80A, Q225R, and G242D), which was previously described in Canada (Munday et al., 2004). The CTX-M-15 rapidly disseminated in the United Kingdom since its first detection. Isolates were particularly associated with different *E. coli* clones, A to F, the first one representing the majority and later identified as belonging to the international O25:H4-ST131 clone that has been detected all over the world (Lau et al., 2008). Again a dramatic increase of CTX-Ms was demonstrated mainly due to the CTX-M-15 enzymes (Coque et al., 2008b).

In the United States, CTX-Ms were rare during the 1990s but increased from 25% in 2000 to nearly 90% in 2005 (Lewis et al., 2007). Interestingly, CTX-M-15 rapidly penetrated over a minority of CTX-Ms that include CTX-M-8, CTX-M-14, and CTX-M-16 (Lewis et al., 2007; Johnson et al., 2010; Peirano et al., 2010). Within this country and as in other parts of the world, the CTX-M-15 enzyme has been identified in hospitalized and extrahospitalary patients and also in companion animals in which CTX-M-15 was the most prevalent CTX-M enzyme (Castanheira et al., 2008; Urban et al., 2010; Shaheen et al., 2011).

Table 1 | Initial description of CTX-M enzymes belonging to different CTX-M groups.

Year	Country	CTX-M	CTX-M group	Comment	Reference
1986	Japan	FEC-1	CTX-M-1	*Escherichia coli* from the fecal flora of laboratory dogs used for pharmacokinetic studies and previously administered cephem antibiotics	Matsumoto et al. (1988)
1989	Germany	CTX-M-1	CTX-M-1	*Escherichia coli* isolate recovered from the ear of a 4-month child suffering from otitis media in Munich. The β-lactamases involved were firstly named as cefotaximases	Bauernfeind et al. (1990)
1989	Argentina	CTX-M-2	CTX-M-2	*Salmonella* Thypimurium isolates from hospitalized patients with meningitis, septicemia, or enteritis	Bauernfeind et al. (1992)
1989	France	MEN-1	CTX-M-1	*Escherichia coli* isolate in France in an Italian patient. Later sequencing reveals identity with CTX-M-1	Bernard et al. (1992), Barthélémy et al. (1992)
1993	Japan	Toho-1	CTX-M-2	*Escherichia coli* isolate recovered from a 1-year-old female child who developed cystitis. Toho-1 was later renamed as CTX-M-44	Ishii et al. (1995)
1996	Poland	CTX-M-3	CTX-M-1	*Citrobacter freundii* and *Escherichia coli* isolates recovered in Warsaw in patients with urinary tract infections	Gniadkowski et al. (1998)
1999	Taiwan	CTX-M-3	CTX-M-1	Different *Escherichia coli* isolates from several sources in hospitalized and non-hospitalized patients	Yan et al. (2000)
1994	France	CTX-M-9	CTX-M-9	A retrospective study showed presence of *Escherichia coli* with this enzyme in this country before its first recognition in Spain	Saladin et al. (2002)
1996	Spain	CTX-M-9	CTX-M-9	First description of CTX-M-14 in an *Escherichia coli* isolate from a patients with an urinary tract infection	Sabaté et al. (2000)
1996	Brazil	CTX-M-9	CTX-M-9	*Escherichia coli* isolate recovered from an hospitalized patient in Rio de Janeiro	Bonnet et al. (2001)
1997	China	CTX-M-9	CTX-M-9	*Escherichia coli* and *Klebsiella pneumoniae* recovered as part of an antimicrobial resistance monitoring project	Chanawong et al., 2002
1996	Korea	CTX-M-14	CTX-M-9	Detected in *Shigella sonnei* and in blood isolates of *Escherichia coli* and *Klebsiella pneumoniae*	Pai et al. (2001)
1997–1998	China	CTX-M-14	CTX-M-9	*Escherichia coli*, *Klebsiella pneumoniae* and *Enterobacter cloacae* recovered as part of an antimicrobial resistance monitoring project	Chanawong et al. (2002)
1998	Taiwan	CTX-M-14	CTX-M-9	*Escherichia coli* recovered during a multicenter antimicrobial resistance surveillance study	Ma et al. (2002)
1998	Poland	CTX-M-15	CTX-M-1	A retrospective study showed the presence of *Serratia marcescens* and *Escherichia coli* before the first description of this enzyme in India. Sequence and plasmid analysis revealed potential evolution from CTX-M-3	Baraniak et al. (2002)
1999	India	CTX-M-15	CTX-M-1	First description of this enzyme	Karim et al. (2001)
2001	United Kingdom	CTX-M-15	CTX-M-1	Spread in the community of *Escherichia coli* isolates with CTX-M-1. A major clone was later identified as belonging to the international 25:H4-ST131 clone	Mushtaq et al. (2003), Woodford et al. (2004)
1996–1997	Brazil	CTX-M-8	CTX-M-8	*Enterobacter cloacae*, *Citrobacter amalonaticus*, and *Enterobacter aerogenes* from different sources in hospitalized patients	Bonnet et al. (2000)
2000	Canada	CTX-M-25	CTX-M-25	*Escherichia coli* isolate recovered from an hospitalized patient	Munday et al. (2004)

The model of CTX-M-15 dissemination could have been different in countries in which other CTX-M β-lactamases were well established prior to the detection and penetration of this enzyme. This is the case of Spain and South America. In our institution in Spain, CTX-M-10 was first detected in 1990 in *K. pneumoniae* and in 1991 in *E. coli*. The first detection of CTX-M-9 was in *E. coli* in 1996 and in *K. pneumoniae* in 1999, CTX-M-14 in 2001 in *K. pneumoniae* and in *E. coli* and CTX-M-15 in 2001 in *E. coli* and in 2002 in *K. pneumoniae*. It is of note that in a national survey in 2000 in Spain involving 40 centers, CTX-M-15 was not

found. However, in 2006 in a similar study, CTX-M-15 was found in different regions and was one of the most prevalent enzymes after CTX-M-14 and SHV-12 (Díaz et al., 2010). More recently, in our institution CTX-M-15 represents 34% of total ESBL producing *E. coli* (Novais et al., unpublished data), 37% of CTX-M-14, and 20% of SHV-12, denoting the increasing prevalence of CTX-M-15 but still co-existing with other CTX-M enzymes. This data illustrates that when CTX-M-15 penetrates in a landscape where other ESBLs are present the increment of this enzyme and displacement of other ESBLs is a slow process. This model can also be the case of South America where CTX-M-2 has been the most prevalent CTX-M over years. Nevertheless, reports of CTX-M-15 producing isolates are increasing in this continent, including Colombia (Valenzuela de Silva et al., 2006; Ruiz et al., 2011), Brazil (Cergole-Novella et al., 2010), Uruguay (Bado et al., 2010), and Peru (Pallecchi et al., 2004, 2007).

At present, we cannot rule out that international travel and immigration might have contributed during the 1990s and the early 2000s to the rapid emergence and dissemination of CTX-M enzymes in different countries and geographic areas. This has recently been demonstrated with carbapenemases and particularly with the NDM-1 metalo-β-lactamase-producing organisms (Kumarasamy et al., 2010). Nevertheless, the presence of CTX-M enzymes in animals and most importantly in uncooked food products that were transported from one country to another suggests potential routes for dissemination (Matsumoto et al., 2007; Warren et al., 2008; Dhanji et al., 2010).

Finally, globalization of CTX-M enzymes is illustrated by their presence not only in food and companion animals but also in wild animals (Bonnedahl et al., 2010; Literak et al., 2010; Gonçalves et al., 2011; Silva et al., 2011). Additionally, they have been isolated in environmental compartments (Chen et al., 2010; Dhanji et al., 2011a).

CTX-M ENZYMES NOT ONLY IN ENTEROBACTERIACEAE

As previously stated CTX-M enzymes were initially described in *E. coli*, *K. pneumoniae*, and *Salmonella* spp. but rapidly emerged in other Enterobacteriaceae. This event occurs in bacterial isolates with chromosomal mechanisms such as inducible chromosomal AmpC in *Enterobacter* spp., *S. marcescens*, *Citrobacter* spp., and *Morganella morganii* able to destroy oxy-imino-cephalosporins (Cantón et al., 2002; Paterson and Bonomo, 2005). This find suggests the facility for transmission of $bla_{\text{CTX-M}}$ genes and also that the acquisition of these genes could have lower fitness cost for the bacteria than selection of derepressed AmpC β-lactamase variants which finally also contribute to cephalosporin resistance.

As CTX-M enzymes increased in prevalence in Enterobacteriaceae in the early 2000s, they also increased in non-fermentative Gram-negative bacilli and other non-Enterobacteriaceae species. The first CTX-M enzyme described in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* was observed in isolates recovered in 2004 from sputum samples of a cystic fibrosis patient in Amsterdam (al Naiemi et al., 2006). The CTX-M enzyme from these isolates was characterized as CTX-M-1. In the same 2004 year, CTX-M-43 (a variant of CTX-M-44 carrying the D240G change and belonging to the CTX-M-2 group), was also observed in *P. aeruginosa* and *Acinetobacter* spp. in isolates recovered from Bolivian hospitals (Celenza et al., 2006). CTX-M-2 was also found in a *P. aeruginosa* isolate recovered in a patient hospitalized in Brazil in 2005 (Picão et al., 2009). Despite these descriptions, prevalence of CTX-M enzymes in non-fermenter bacilli is still rare. This fact could be due to incompatibility of plasmids carrying these enzymes or might reflect phenotypic difficulties in recognizing non-fermenter isolates expressing CTX-Ms. On the other hand, *Aeromonas* spp. or *Vibrio* spp. isolates with CTX-M enzymes have been also recognized (Soler Bistué et al., 2006; Ye et al., 2010; Chowdhury et al., 2011; Gómez-Garcés et al., 2011).

ORIGIN AND MOBILIZATION OF THE $bla_{\text{CTX-M}}$ GENES

According to phylogenetic trees performed with all CTX-M β-lactamases described, the CTX-M lineage could be differentiated at in least five main clusters (**Figure 3**). Traditionally, each CTX-M cluster has been related to the chromosomal *bla* genes present in different *Kluyvera* species, which are part of the human normal intestinal microbiome, usually at low bacterial counts, and is considered as a saprophytic and an opportunistic pathogen (Farmer et al., 1981). Furthermore, they have been occasionally isolated in humans associated with different infections, mainly affecting the urinary tract and skin and soft tissues (Sarria et al., 2001) and is also present in the environment as free-living organisms in water, soil, sewage, and food products of animal origin (Farmer et al., 1981).

Chromosomal *bla* gene, *kluC*, present in *Kluyvera cryocrescens* has been considered the ancestor of the CTX-M-1 cluster (Decousser et al., 2001), *kluA* gene from *Kluyvera ascorbata* the origin of the CTX-M-2 cluster (Humeniuk et al., 2002) and three different chromosomal *bla* genes from *Kluyvera georgiana*, *kluG*, *kluY*, and $bla_{\text{CTX-M-78}}$ the origin of CTX-M-8 (Poirel et al., 2002), CTX-M-9 (Olson et al., 2005), and CTX-M-25 clusters (Rodríguez et al., 2010) respectively. Interestingly, the CTX-M-74 and CTX-M-75, characterized in a survey conducted in Brazil and with single one amino acid change with respect to CTX-M-2 (Minarini et al., 2009), showed a degree of nucleotide divergence that could be considered as a new cluster, reflecting a convergent evolution of these β-lactamases (Stepanova et al., 2008). All these data suggest that the environmental source of CTX-M β-lactamases was the genus *Kluyvera*. Although the scientific community has unequivocally accepted this point, several questions still arise to guarantee this line of evidence. First, the comparison of the topologies of phylogenetic trees between $bla_{\text{CTX-M}}$ and available 16S rDNA sequences of *Kluyvera* species reveals that the evolutionary relationships among CTX-M clusters is not completely correlate with the phylogenetic distance among the different species of *Kluyvera* (**Figure 3**). Whereas all *Kluyvera* species diversified at the same time from the same ancestral bacteria, this topology is not observed among $bla_{\text{CTX-M}}$ clusters, suggesting different evolutionary trajectories. Second, if each chromosomal gene from *Kluyvera* was the source of a determined CTX-M cluster, why have at least three chromosomal genes from *K. georgiana* (bla_{kluG}, bla_{kluY}, and $bla_{\text{CTX-M-78}}$) been proposed as precursor of three different CTX-M clusters? On the contrary, CTX-M-1 cluster has been related to different *Kluyvera* species (*K. ascorbata* and *K. cryocrescens*; Decousser et al., 2001; Rodríguez et al., 2004).

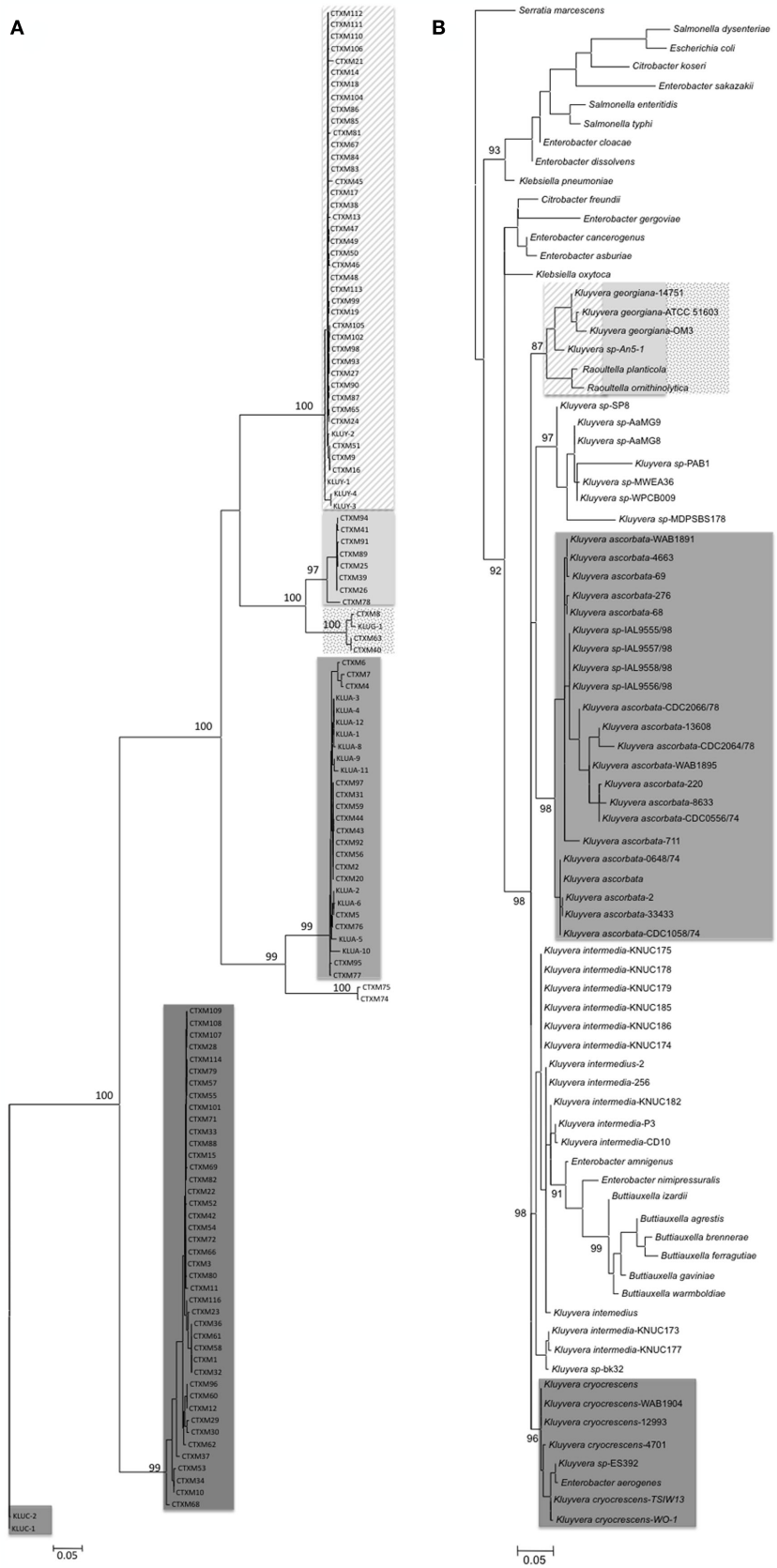

FIGURE 3 |

(Continued)

FIGURE 3 | Continued
Maximum likelihood trees of *bla*$_{CTX-M}$ genes and 16S rDNA of *Kluyvera* spp. in order to compare their respective phylogenetic topologies. (A) Phylogenetic tree of *bla*$_{CTX-M}$ genes ($n = 109$ and 17 chromosomal genes from *Kluyvera*) was obtained using PhyML_3.0 program (846 nt). The Tamura Nei nucleotide substitution model, used as evolutionary model was selected with the jModeltest program. The robustness of the relevant nodes was estimated with 1000 bootstrap pseudorandom replicates. We considered nodes valid when bootstrap value was >95%. **(B)** Phylogenetic tree of 16s rDNA of *Kluyvera* spp. ($n = 55$) and related species download of www.ncbi.nlm.nih.gov, using PhyML_3.0 (1310 nt). The Hasegawa–Kishino–Yano was the evolutionary model inferred by jModeltest program and invariant site rate of 77.6% (HKY + I + G). We considered nodes valid when bootstrap value was >85%. The phylogenetic trees were represented using MEGA 5.0 program.

These data could also suggest that *bla*$_{CTX-M}$ genes have been also circulating among different *Kluyvera* species. Third, upstream of *bla*$_{klu}$ gene identical genome organization was found in *Enterobacter aerogenes*, suggesting that *Kluyvera* could have gained *bla*$_{klu}$ gene after these species diverged, therefore most of the isolates must carry *bla*$_{klu}$ gene, including *Kluyvera intermedia*, where no *bla*$_{klu}$ gene has been identified. However, despite the presence of the *bla*$_{klu}$ gene in most *Kluyvera* isolates, 90% are susceptible to cefotaxime (Sarria et al., 2001). Obviously, *bla*$_{klu}$ gene could be present but weakly expressed. Unfortunately the presence of *bla*$_{klu}$ gene in *Kluyvera* susceptible isolates or their genetic environment has not been searched. These studies could help us to understand previous observations of *Kluyvera* spp. as the precursor of the CTX-M enzymes and responsible for the current pandemic.

It is well known that chromosomal *bla*$_{klu}$ genes are weakly expressed in their *original* context and require the presence of a strong promoter upstream to increase the MIC values and consequently be considered as phenotypically resistant. The insertion sequences (IS) have provided this promoter in clinical cefotaxime-resistant *Enterobacteriaceae* strains, the most prevalent being IS*Ecp*1 and IS*CR*1, while IS26 has been only detected in few isolates. Curiously, these IS have not been described in natural isolates of *Kluyvera*, although the capture of IS*Ecp*1 upstream of chromosomal *bla*$_{klu/CTX-M}$ genes occurs at high frequency in laboratory conditions. This was demonstrated in an elegant *in vitro* experiment performed by Lartigue et al. (2006). When IS*Ecp*1 was inserted upstream of *bla*$_{klu/CTX-M}$ gene, stress situations such as high temperature (40°C) or that exerted by antimicrobials such as ceftazidime or cefotaxime, the mobilization of IS*Ecp*1 was increased, carrying the downstream sequences. It has been speculated that global warming or higher temperature than humans in the intestinal compartment of certain animals and the increasing use of antimicrobials might have also contributed to the extraordinary increase and spread of the *bla*$_{klu/CTX-M}$ genes (Lartigue et al., 2006; Nordmann et al., 2008). Apart from these speculations, an interesting question is if mobilization of *bla*$_{klu}$ occurred, how often was it in the nature? Initially and accepting the genus *Kluyvera* as progenitor of the CTX-M enzymes, we can suspect mobilization of the same number of occasions has CTX-M clusters has been described. Barlow et al. (2008) using phylogenetic trees based on gene distance suggest that this occurred at least eight times (three times each for CTX-M-1 and CTX-M-2 clusters and only one each for CTX-M-9 and CTX-M-8; Barlow et al., 2008). However the description of different IS such as IS*Ecp1* and IS*CR1* related to CTX-M-9 (Valverde et al., 2009), allow us to suspect that more mobilization events may have occurred (see below).

EVOLUTION AND DIVERSIFICATION OF CTX-M β-LACTAMASES

Cumulative representation of the new variants described among the most prevalent β-lactamases shows that the highest slope during the last years correspond to the CTX-M and OXA-type β-lactamases (Bush and Fisher, 2011). This might indicate that they have recently suffered the most spectacular diversification process in the β-lactamases world. CTX-M phylogenetic trees suggest an explosive diversification from the original *bla* genes in a short time when they arrived in the clinical scenario (Novais et al., 2010). The first CTX-Ms identified were able to confer cefotaxime resistance although their capacities to efficiently hydrolyze ceftazidime was very low or null, but nowadays more than 60% of CTX-M variants are able to confer cefotaxime and ceftazidime-resistance simultaneously. Thus, ceftazidime could have been one of the main selective forces contributing to the diversification of CTX-Ms.

An evolution experiment was designed in our laboratory to obtain more efficient CTX-M variants against ceftazidime; the ceftazidime-resistant CTX-M variants carried the changes A77V, P167S, and D240G (Novais et al., 2008). These changes have been described in the nature in several cases including CTX-M-52 and CTX-M-87, which derived from CTX-M-15 and CTX-M-14 respectively (Yin et al., 2009). These results reinforce the idea that ceftazidime has driven the selection of new CTX-M variants. Moreover, we calculated the ratio of the number of non-synonymous substitutions with respect to synonymous substitutions ($\varpi = dN/dS$) among the *bla*$_{CTX-M}$ gene variants in order to define positive (accelerate evolution) or purified (slow evolutionary rate) selection processes during the diversification of CTX-Ms. In the deep branches of the CTX-Ms phylogenetic tree, processes of purified selection occurred while positive selection was the mechanism of molecular evolution in the more recent branches. In our experiments, six positions were identified to be under positive selection, including the amino acid positions 77, 167, and 240, which were affected under ceftazidime regimens. Nevertheless, our results also suggest the presence of more evolutionary forces involved in the selection of new and better adapted variants. The construction of mutants carrying combinations of these mutations revealed that the simultaneous presence of cefotaxime and ceftazidime in the environment has modeled the explosive evolution and diversification of the CTX-M enzyme (Novais et al., 2010).

Recombination events also accelerate the evolution. These events have also been described among *bla*$_{CTX-M}$ genes. As an example, the CTX-M-64 enzyme is the result of a recombination between members of the *bla*$_{CTX-M-9}$ and *bla*$_{CTX-M-1}$ derived genes, probably *bla*$_{CTX-M-14}$ and *bla*$_{CTX-M-15}$, as they are the most prevalent variants in the world (Nagano et al., 2009). The recombination

requires the simultaneous presence of both *bla*$_{CTX-Ms}$ in a same habitat. Recently, we described in an Ethiopian 1-year-old boy both CTX-M-14 and CTX-M-15 (Morosini et al., 2010). Moreover, in a national surveillance performed in China, the CTX-M-64 β-lactamase was recovered from three patients co-existing with other CTX-Ms (Sun et al., 2010). As the concomitant presence of different CTX-M β-lactamases in the same bacteria is no longer an unusual event, other recombinant enzymes might emerge in the near future.

bla$_{CTX-M}$ GENE PLASTICITY AND OTHER STRATEGIES TO INCREASE THE SPECTRUM OF ACTION OF CTX-M β-LACTAMASES

β-Lactamases are highly modeling proteins as few positions are critical to hydrolyze β-lactam compounds. Nowadays, at least 136 polymorphic positions have been found among all CTX-Ms described. Some of these changes play a main role increasing the β-lactam hydrolytic spectrum while others modulate or restore the synergic or antagonist effects caused by other modifications. Furthermore, some mutations are selected depending on the genetic background due to the interactions among the mutations present (epistasis) whereas other changes have an unknown effect on the enzyme and they could be only selected by random drift. For instance, directed mutagenesis studies have revealed new mutations that have not yet been described in nature, which could be selected under ceftazidime challenge such as M135I and A219V (Pérez-Llarena et al., 2011). These results suggest that the adaptive possibilities of CTX-M enzymes have not yet reached their evolutionary stasis.

In this section we explore if other mutations or adaptive possibilities could be expected in the CTX-M lineage. Two attractive issues are raised in relation to the capacities of CTX-Ms to confer resistance to non-oxyimino-β-lactam antibiotics, including β-lactam-β-lactamase inhibitor combinations (IR-CTX-M) and/or carbapenems (Girlich et al., 2008; Ripoll et al., 2011), as these variants have not yet been found in nature. In laboratory conditions, IR-CTX-M mutants carrying the S130G, K234R, and S237A mutations have been described (Gazouli et al., 1998; Aumeran et al., 2003; Ripoll et al., 2011). The selection of these mutants conferring resistance to β-lactam plus β-lactamase inhibitors showed an antagonistic pleiotropic effect with a simultaneous loss of the hydrolytic activity against cefotaxime and ceftazidime. Nevertheless, the L169S mutation that was recovered after the S130G was selected, slightly increase the hydrolytic efficiency against cefotaxime and ceftazidime, suggesting that mutations at position 169 could have a restoring effect, similar to the selection of A77V mutation after the emergence of P167S or D240G driving ceftazidime-resistance (Novais et al., 2008; Ripoll et al., 2011). Interestingly, a clinical isolate carrying a novel CTX-M variant (CTX-M-93), which differs from CTX-M-27 in a single L169Q mutation, shows a reduced activity against cefotaxime but increased activity against ceftazidime when compared with its progenitor (Djamdjian et al., 2011). This finding confirms that the mutations in 169-position confers increased hydrolytic activity against ceftazidime and this improvement is higher when the S130G mutation is also present. On the other hand, a recent study using a site-directed mutagenesis strategy found that the V103D and V260L mutations also endow an IR-CTX-M phenotype (Pérez-Llarena et al., 2011), again suggesting that new mutants could be selected in the future.

No mutations in *bla*$_{CTX-M}$ gene have been involved in resistance to carbapenems. However, clinical carbapenem-resistant strains carrying CTX-M β-lactamases have been isolated. Two types of mutants are related to this carbapenem-resistant phenotype: loss of porins and co-existence with other β-lactamases (Gülmez et al., 2008). The loss of OmpK35 and OmpK36 porins is the most frequent mechanism of carbapenem-resistant associated with CTX-M producing *K. pneumoniae* (Leavitt et al., 2009) and its functional restoration significantly decreased the MICs of all carbapenems (Doumith et al., 2009). *In vivo* resistance development to carbapenem has been previously described in two *K. pneumoniae* strains producing CTX-M-1 due to the lack of OmpK36 expression (Mena et al., 2006). However, the loss of this porin reduced *in vitro* fitness and attenuated virulence in a murine model (García-Sureda et al., 2011). Moreover, detection of carbapenem-resistant *E. coli* clinical strains carrying CTX-M due to reduced expression of OmpF and OmpC porins, homologous of OmpK35 and OmpF36, are increasingly recognized (Girlich et al., 2009; Bennett et al., 2010). These results illustrated that porin deficiency is more efficient than potential CTX-M mutation for the increase of carbapenem MICs.

On the other hand, the high worldwide distribution of CTX-M enzymes is giving rise to the co-existence of two or more β-lactamases in the same strain. This possibility is becoming a common bacterial strategy to enhance antibiotic resistance. CTX-Ms have been described associated with VIM-1 (Miró et al., 2010), KPC-2 (Chen et al., 2011), or OXA-48 carbapenemases (Cuzon et al., 2011; Pitart et al., 2011). In fact, the clone belonging to ST395 coproducing CTX-M-15 and OXA-48 has been demonstrated widespread throughout Europe (Potron et al., 2011). An extreme description was the co-existence of KPC-2, VIM-19, CMY-2, and CTX-M-15 in a *K. pneumoniae* strain isolated in Greece (Pournaras et al., 2010). Moreover, the recently described NDM-1 carbapenemase is normally present in isolates also expressing the CTX-M-15 ESBL and also OXA-type enzymes (Poirel et al., 2011a; Solé et al., 2011). This supports the concept of the genetic capitalism of the multi-drug resistant bacteria where the more resistant bacteria have the higher opportunity to become more resistant (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

GENETIC ENVIRONMENTS OF *bla*$_{CTX-M}$ GENES PARTICIPATING IN THE MOBILIZATION, SPREAD, AND MAINTENANCE OF THESE GENES

A linkage of different *bla*$_{CTX-M}$ genes with specific surrounding genetic elements, including IS, integrons, and transposons has been described. These genetic backbones are integrated in more complex structures such as different plasmid replicon types as well as specific clones defining a complex hierarchical organization with the possibility of interchanging different modules (Cantón and Coque, 2006; Carattoli, 2011; Woodford et al., 2011). **Figure 2** shows hierarchical complexity of *bla*$_{CTX-M}$ genes within different genetic structures and bacterial clones participating in the mobilization, spread, and maintenance of these genes. As previously described, *bla*$_{CTX-M}$ genes have been hypothetically mobilized

from *Kluyvera* spp. by IS and to a lesser extent by bacteriophages. The IS also participate in the over-expression of these genes and some of them (i.e., IS*CR1*) are adjacent to integron structures that are also integrated in transposition units. These supra-structures are often incorporated within conjugative plasmids that might be present in successful clones (or the so-called high-risk clones). Moreover, they can operate as evolutionary units and might individually act as units of selection (Baquero, 2004; Cantón and Ruiz-Garbajosa, 2011).

Some of the genetic elements and plasmids harboring $bla_{\text{CTX-M}}$ genes also harbor other resistance genes, including those encoding AmpC β-lactamases (plasmid bla_{AmpC}) and carbapenemases, plasmid-mediated quinolone resistance (PMQR) genes (i.e., *qnr* genes), or methylases affecting aminoglycosides. All these genes might also give advantage to $bla_{\text{CTX-M}}$ for maintenance due to co-selection processes.

INSERTION SEQUENCES AND PHAGE-RELATED SEQUENCES INVOLVED IN THE MOBILIZATION OF $bla_{\text{CTX-M}}$ GENES

There are a huge number of scientific articles describing *new* or *unique* genetic rearrangement associated with $bla_{\text{CTX-M}}$ genes but further analysis has revealed that they are only variations of a few different genetic structures (Lartigue et al., 2004; Eckert et al., 2006; Dhanji et al., 2011b; Toleman and Walsh, 2011). The experimental mobilization of $bla_{\text{CTX-Ms}}$ genes have been demonstrated by IS located upstream such as IS*Ecp*1 (Lartigue et al., 2006). Moreover, the effect of P_{out} promoter of these IS increasing the $bla_{\text{CTX-Ms}}$ expression suggests that the IS located upstream of these genes have a role in the selection and dissemination of CTX-Ms. Therefore, we have mainly considered in this part of the review the genetic upstream sequences of the $bla_{\text{CTX-M}}$ genes. The information of downstream sequences of $bla_{\text{CTX-M}}$ genes is less available and less knowledge has been accumulated.

Different IS have been identified upstream of the $bla_{\text{CTX-Ms}}$ genes, including IS*Ecp*1, IS*CR*1, IS*10*, and IS*26*. Although other IS elements have also been described upstream, they were the result of subsequent integration events. For instance, IS*1* and IS*10*, and even IS*CR1* and IS26, have been described interrupting IS*Ecp*1 (Eckert et al., 2006; Bae et al., 2008). IS*Ecp*1 is the most widely found IS upstream of different $bla_{\text{CTX-M}}$. Although it was first recorded adjacent to the $bla_{\text{CTX-M-15}}$ in 1999, it has been found associated with all clusters of these β-lactamases except CTX-M-8. Moreover, IS*CR1* has been related to several members of $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$ clusters and IS*10* with $bla_{\text{CTX-M-8}}$. The number of new genetic environments upstream of $bla_{\text{CTX-M}}$ must be related to the number of mobilization events but unfortunately in many $bla_{\text{CTX-M}}$ cases the upstream sequence is unknown (Barlow et al., 2008). Nowadays, we have been able to identify at least nine mobilization events (**Figure 4**): three in CTX-M-1 cluster ($bla_{\text{CTX-M-10}}$, $bla_{\text{CTX-M-53}}$, and other known $bla_{\text{CTX-M}}$ genes included in this cluster), two in CTX-M-2 cluster ($bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-5}}$), two in CTX-M-9 cluster ($bla_{\text{CTX-M-9}}$ and $bla_{\text{CTX-M-14}}$), and one each in CTX-M-8 and CTX-M-25 ($bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-25}}$, respectively).

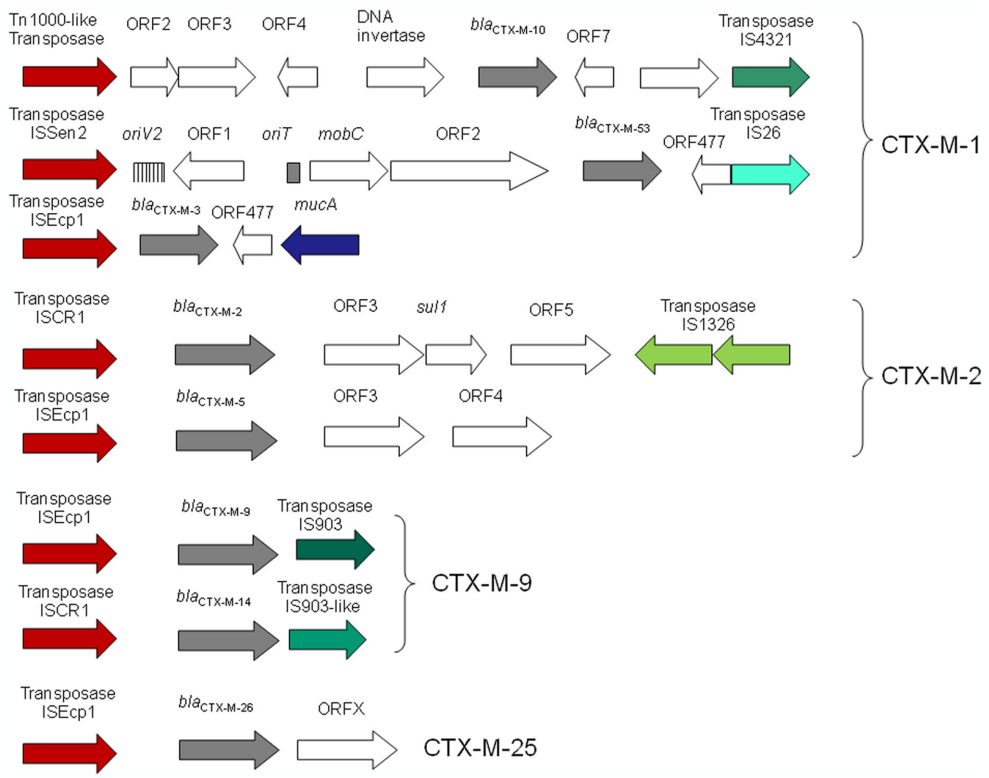

FIGURE 4 | Schematic representations of genetic environments surrounding $bla_{\text{CTX-M}}$ genes corresponding to suspected cases of mobilization events within different CTX-M groups (CTX-M-1, CTX-M-2, CTX-M-9, and CTX-M-25).

On the other hand, spacer sequences between IS*Ecp*1 and $bla_{\text{CTX-M}}$ genes have been well studied. The genetic distance between IS and $bla_{\text{CTX-M}}$ gene is related to cephalosporin MIC values (Ma et al., 2011). These distances ranged between 48 and 127 bp in $bla_{\text{CTX-M-1}}$ gene cluster, between 34 and 42 bp in $bla_{\text{CTX-M-9}}$ gene cluster (but more than 300 bp if IS*CR*1 is upstream of $bla_{\text{CTX-M}}$) and around 40–52 in $bla_{\text{CTX-M-25}}$ and $bla_{\text{CTX-M-8}}$ gene clusters respectively. When the homology among spacer sequences of $bla_{\text{CTX-M-1}}$ gene cluster was analyzed, all but $bla_{\text{CTX-M-10}}$ and $bla_{\text{CTX-M-53}}$ maintained a common region (known as V and W sequences). This suggests that these $bla_{\text{CTX-M}}$ genes belonging to CTX-M-1 cluster could derive from a single transposition event. A similar conclusion can be obtained when considering IS*Ecp*1 and $bla_{\text{CTX-M}}$ genes belonging to $bla_{\text{CTX-M-9}}$ (Y sequence), $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-25}}$ (**Figure 5**). In all these clusters, the ancestral source was *K. georgiana*. However the lack of homology among spacer sequences of these $bla_{\text{CTX-M}}$ gene clusters suggests a different source in each case. These results reveal that non-described *Kluyvera* species (or another organism source) could be the origin of clusters ascribed to *K. georgiana* (see **Figure 3** about the phylogenetic tree of 16S rDNA of *Kluyvera* spp. where more species of *Kluyvera* can be suggested according to bootstrap values).

Finally, $bla_{\text{CTX-M-10}}$ gene has been associated with phage-related sequences, which underlines bacteriophages involvement in the potential mobilization of this gene (Oliver et al., 2001). Although the occurrence of this event has not been frequently demonstrated, the importance of bacteriophages in the diffusion of *bla* genes was evaluated in sewage, showing that these sequences may contribute to the spread of some β-lactamases (Muniesa et al., 2004; Colomer-Lluch et al., 2011). Part of these sequences, also known as "phage-like elements," could have facilitated mobilization of resistance genes into the chromosome (Wozniak and Waldor, 2010; Guglielmini et al., 2011). With the exception of the previous description of the CTX-M-10 enzyme (Oliver et al., 2001), these structures have not been associated with other CTX-Ms despite the fact that these enzymes have also been observed integrated into the chromosome (Coelho et al., 2010; Song et al., 2011). Future *in silico* analysis might reveal a more frequent presence of these phage-like elements associated with *bla* genes.

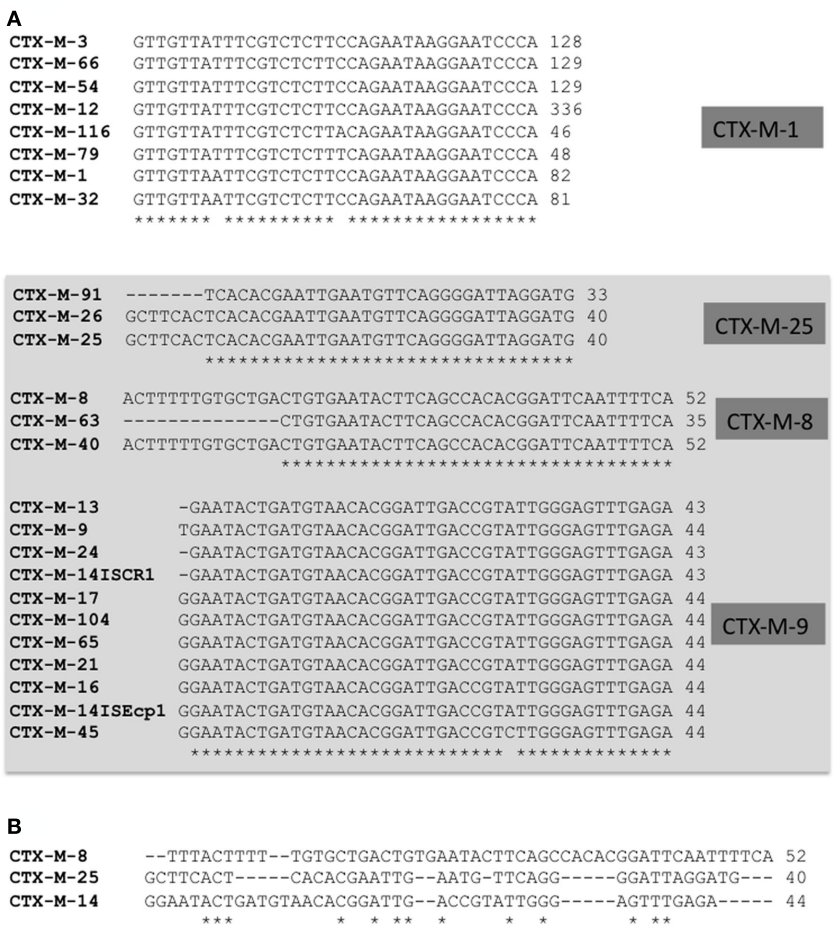

FIGURE 5 | Alignment of upstream sequences in different $bla_{\text{CTX-M}}$ gene clusters. Different $bla_{\text{CTX-M}}$ genes belonging to the same cluster share an identical DNA sequence in upstream region, suggesting the same origin **(A)**. Nevertheless, the loss of alignment among different upstream sequences from different $bla_{\text{CTX-M}}$ genes belonging to different clusters (included in the box squared) but theoretically derived from the same ancestral source in *Kluyvera georgiana* suggesting that these clusters had different sources **(B)**.

OTHER INTEGRATIVE AND MOBILIZATION UNITS

Recently, there has been an increasing interest for the so-called "integrative and conjugative elements" (ICEs) in the mobilization of resistant determinants. They are not homogenous structures and include the "conjugative transposons" or the so-called "integrated conjugative plasmids." These elements are a diverse group of mobile genetic elements found in both Gram-positive and Gram-negative bacteria. They primarily reside in the chromosome, but retain the ability to excise from and re-integrate into a new chromosome like bacteriophages and to transfer between bacterial cells by conjugation like plasmids. It has been recently stressed that they are closely linked to some of the more powerful resistance mechanisms such as the extended-spectrum-, metallo-, and AmpC type β-lactamases (Toleman and Walsh, 2011).

A characteristic of the ICE elements, as well as complex class 1 integrons and some IS is the inability to consistently recognize one of their own terminal sequences, while they recognize more genetically distant surrogate sequences. This has the effect of mobilizing the DNA sequence found adjacent to their initial location. This process is known as one-ended transposition mechanisms and is exemplified by some IS such as IS*CR1* and IS*Ecp1* as well as Tn*3* and Tn*21* derivatives, including Tn*5090*-like transposon (also called Tn*402*) that in turn harbor the class 1 integrons, IS*CR1* and certain $bla_{\text{CTX-M}}$ genes. A detailed review of the importance of these elements in the emergence and dispersion of resistance traits, including that exerted by $bla_{\text{CTX-M}}$ genes has been recently reviewed (Toleman and Walsh, 2011).

A novel structure named IMU that resides in a plasmid 7-kb IncQ-type plasmid has recently been associated with mobilization of *bla* genes (Poirel et al., 2009). The IMU structure resembles that of miniature inverted transposable elements (MITEs), which have been identified in eukaryotes such as plants (Chen et al., 2008) and also in bacteria (Buisine et al., 2002; Filée et al., 2007). In Enterobacteriaceae, they form a composite like element consisting of 288-bp elements located in opposite orientation able to mobilize defective class 1 integron structures carrying *bla* genes. Although they have not yet been associated with mobilization of $bla_{\text{CTX-M}}$ genes, they might participate in the future in the mobilization of these genes.

PLASMIDS AND SPREAD OF $bla_{\text{CTX-M}}$ GENES

The importance of plasmids in the spread of $bla_{\text{CTX-M}}$ genes is exemplified with certain CTX-M β-lactamases and has been stressed in several publications (Carattoli, 2009). The case of CTX-M-15 enzyme is of particular interest. It has been shown that the $bla_{\text{CTX-M-15}}$ gene is mainly associated with incompatibility group FII plasmids (Coque et al., 2008b). They are a paradigm of narrow host-rage plasmids characterized by a low-copy number in the bacteria. They are mainly found in Enterobacteriaceae and have recently been termed as "epidemic resistance plasmids" due to their propensity to acquire resistance genes and transfer among bacteria (Carattoli, 2011). The FII plasmids are not a homogeneous group and might have evolved by recombinatorial events among different plasmids. It has been shown that within these plasmids different replicon types are present that might facilitate fast evolution and plasmid diversification. Interestingly, initial studies on IncFII plasmids suggest that they were widely distributed within Enterobacteriaceae before antimicrobial use provided that they were well adapted to these organisms (Datta et al., 1980). This fact has undoubtedly facilitated persistence and spread of resistant determinants, including $bla_{\text{CTX-M}}$ genes after this acquisition. Moreover, incorporation of genetic determinants mediating non β-lactam resistance mechanisms would have also contributed to the maintenance of these plasmids within the bacteria and hence the $bla_{\text{CTX-M}}$ genes under co-selection process (Novais et al., 2007; Woodford et al., 2009).

On the other hand, broad host-range replicon plasmids such as IncN, IncI1, and IncL/M have also been involved in the dissemination and spread of CTX-M enzymes. Some of these plasmids, such as IncN, might have their reservoir in animals as they have been isolated in *Salmonella* spp. and *E. coli* from food producing animals (Carattoli, 2011). They often carry *qnr* and *aac(6′)-Ib-cr* genes determining low level fluoroquinolone resistance (Shen et al., 2008; Bado et al., 2010). More recently, these plasmids have been able to acquire genes coding the emerging carbapenemase such as $bla_{\text{NDM-1}}$ (Poirel et al., 2011b) and have been identified as epidemic plasmids in countries with a high prevalence of carbapenem resistance (Psichogiou et al., 2008). Other plasmids, such as IncL/M group are also able to carry $bla_{\text{CTX-M}}$ genes (i.e., $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-15}}$) as well as those producing methylase encoding genes involved in aminoglycoside resistance (i.e., *armA*; Sabtcheva et al., 2008; Naas et al., 2011).

Other plasmid types that have been preferentially associated with $bla_{\text{CTX-M}}$ genes are IncK plasmids with $bla_{\text{CTX-M-14}}$ in southern Europe (Valverde et al., 2009; Cottell et al., 2011) and IncHI2 plasmids with $bla_{\text{CTX-M-2}}$ and $bla_{\text{CTX-M-9}}$ in other geographic areas (García-Fernández et al., 2007). Nevertheless, these plasmids have also been responsible for the dispersion of other resistance genes such as IncK and bla_{CMY} genes (Baudry et al., 2009) or IncHI2 and bla_{VIM} and *qnr* genes (García-Fernández and Carattoli, 2010; Oteo et al., 2010b). These results denote ability of these plasmids for recruitment resistance genes, hence facilitating the increasing prevalence and persistence of the resistance traits of the bacteria. Furthermore, it is expected that persistence over time also gives opportunity to the bacteria to generate gene variation and an increasing the number of $bla_{\text{CTX-M}}$ variants in the coming years as has occurred with OXA-type β-lactamases. Some of these variants have been obtained *in vitro* under antibiotic selective pressure but have not yet been found in clinical isolates (Novais et al., 2008, 2010; Ripoll et al., 2011).

CLONAL DISPERSION AND CO-SELECTION PROCESSES: INVOLVEMENT OF MULTI-DRUG RESISTANT AND VIRULENT HIGH-RISK CLONES

One of the most interesting issues in the dispersion of CTX-M enzymes is the participation of specific clones, particularly those from *E. coli* and *K. pneumoniae*. Recent approaches based on MLST typing have demonstrated that despite a high diversity among CTX-M producers, a few clones or sequence types (ST) grouped in clonal complexes (CC) have been repeatedly found linked to CTX-M enzymes. This suggests that they are involved in the dissemination of these enzymes and that the adaptive success of some CTX-M enzymes could also depend on specific ST or CC where they are frequently present. These clones have been

named as high-risk clones (Woodford et al., 2011). The case of worldwide CTX-M-15 producing *E. coli* isolates exemplifies this issue as most of these isolates belong to the internationally spread ST131 clone (Coque et al., 2008b; Woodford et al., 2011). ST131 is the predicted founder of a clonal cluster that comprises 13 single locus variants (SLVs) and three double-locus variants (DLVs). It is related to serotype O25:H4 as well as to phylogenetic group B2, which grouped virulent extraintestinal isolates (Coque et al., 2008b; Peirano and Pitout, 2010). In addition, isolates belonging to this clone or CC frequently carry IncFII plasmids suggesting a common co-evolutionary process.

Different population structure studies of *E. coli* demonstrated that ST131 clone is also not only associated with CTX-M enzymes belonging to group 1 (CTX-M-1, CTX-M-3, and CTX-M-10) but also with others from distinct groups, such as CTX-M-9 or CTX-M-14 (Mora et al., 2010; Cao et al., 2011). This spread is present among human and animal isolates. In addition, very recent studies also showed that ST131 is over represented within non-ESBL producing isolates (Blanco et al., 2011) and that this clone can recruit emerging β-lactamases such as NDM-1 (Peirano et al., 2011). Multiresistance might have facilitated globalization of this clone. Moreover, at individual level the presence of specific virulence traits facilitating host adherence and receptor epithelial binding also contribute to persistence (Peirano and Pitout, 2010).

Other lineages of *E. coli* that have also contributed to the spread of CTX-M enzymes are ST38, ST393, and ST405 clones and their corresponding single- and DLVs (Coque et al., 2008a; Blanco et al., 2011). These lineages belong to serotype D, which represents also virulent extraintestinal strains (Picard et al., 1999). ST38 and ST393 seem to have been more preferential for the CTX-M-9 and CTX-M-14 enzymes whereas ST405 have been more preferentially with CTX-15 enzyme. Again these clones might also be overrepresented within non-ESBL producing isolates increasing the opportunity to recruit the corresponding $bla_{\text{CTX-M}}$ genes. These clones also express other emerging β-lactamases such as OXA-48 and NDM-1 as illustrated with ST38 (Poirel et al., 2011a; Yamamoto et al., 2011).

Many isolates belonging to these *E. coli* clones are fluoroquinolone-resistant uropathogenic isolates that have their natural environment in the intestinal microbiota. Fluoroquinolones might favor co-selection phenomena that could enrich the intestinal microbiota with these isolates. It has even been speculated that fluoroquinolone resistance either due to plasmid-mediated genes, including *qnr* variants and *acc(6′)-Ib-cr*, or to topoisomerase mutations preceded the acquisition of $bla_{\text{CTX-M}}$ genes (Jones et al., 2008; Naseer and Sundsfjord, 2011). In addition to this resistant trait, high-risk clones expressing CTX-M enzymes are also more favorable for acquiring other resistance mechanisms, such as those affecting aminoglycosides, trimethoprim–sulfamethoxazole and fosfomycin. Aminoglycoside resistance is related both with aminoglycoside modifying enzymes and methylases (Mushtaq et al., 2011). Fosfomycin resistance involving different resistance mechanisms has been more recently describe in isolates from the community in countries with increasing use of this antibiotic (Oteo et al., 2009). This again illustrates the genetic capitalism of the resistant bacterias (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

Together with these points, these CTX-M associated *E. coli* clones are highly virulent, easily transmitted among family members in colonized or infected patients (Mihaila et al., 2010) and have been found in wild-life, companion and food animals (Mora et al., 2010; Platell et al., 2011).

In *K. pneumoniae* more attention has been paid to carbapenemases, mainly KPCs, and the involvement of ST258 clone in their dissemination (Woodford et al., 2011). Nevertheless, different publications have studied the implication of different clones in the dissemination of CTX-M enzymes associated with *K. pneumoniae*. This is for instance the case of CC11, which has been identified widely spread in Asia associated with different CTX-M enzymes, including CTX-M-14 and CTX-M-15 (Ko et al., 2010; Lee et al., 2011). In Hungary this CC and also ST15 and ST147 were found to be responsible for the country-wide dissemination of ciprofloxacin-resistant CTX-M-15 producing *K. pneumoniae* (Damjanova et al., 2008). Also in the Copenhagen area of Denmark a *K. pneumoniae* ST16 clone harboring the $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-28}}$ genes was recognized as producing hospital outbreaks. (Nielsen et al., 2011). Some of these clones were able to acquire carbapenemases and other resistant traits due to their particular preferential for specific plasmids, including IncFII, IncN and IncL/M (Andrade et al., 2011). This observation exemplifies once more the genetic capitalism concept for which resistant organisms tend to be more resistant (Baquero et al., 2003; Cantón et al., 2003; Baquero, 2004).

CONCLUDING REMARKS

Although in recent years more interest has given to carbapenemases than ESBLs, the CTX-M family still deserve the close attention of researchers and constitute a paradigm of the emergence and spread of a resistant mechanism. Previous descriptions of hierarchical structures that include the $bla_{\text{CTX-M}}$ genes delineated the importance of these structures for maintenance and spread of these genes. Comparison of $bla_{\text{CTX-M}}$ sequences with sequence databases hypothetically defined that $bla_{\text{CTX-M}}$ genes have their ancestors in different *Kluyvera* spp. Nevertheless, *in silico* analyses also suggest that $bla_{\text{CTX-M}}$ genes might also have circulated among different *Kluyvera* species and that the incorporation to these organisms might well have occurred after these species diverged. In Enterobacteriaceae, the great adaptive success of $bla_{\text{CTX-M}}$ genes has been associated with few surrounding genetic structures, few plasmids and few bacterial clones. Successful associations of these units and co-existence of $bla_{\text{CTX-M}}$ genes with other resistance determinants might have contributed to the extraordinary spread of the CTX-M enzymes deserving an uncontrolled pandemic scenario. Future persistence of $bla_{\text{CTX-M}}$ genes within bacterial communities will be assured by co-selection processes as other resistance genes, including emerging ones such as carbapenemases, are now often present in CTX-M producing organisms. Persistence will also give new opportunities for evolution.

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Plasmid-mediated quinolone resistance; interactions between human, animal, and environmental ecologies

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Resistance to quinolones and fluoroquinolones is being increasingly reported among human but also veterinary isolates during the last two to three decades, very likely as a consequence of the large clinical usage of those antibiotics. Even if the principle mechanisms of resistance to quinolones are chromosome-encoded, due to modifications of molecular targets (DNA gyrase and topoisomerase IV), decreased outer-membrane permeability (porin defect), and overexpression of naturally occurring efflux, the emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998. Although these PMQR determinants confer low-level resistance to quinolones and/or fluoroquinolones, they are a favorable background for selection of additional chromosome-encoded quinolone resistance mechanisms. Different transferable mechanisms have been identified, corresponding to the production of Qnr proteins, of the aminoglycoside acetyltransferase AAC(6′)-Ib-cr, or of the QepA-type or OqxAB-type efflux pumps. Qnr proteins protect target enzymes (DNA gyrase and type IV topoisomerase) from quinolone inhibition. The AAC(6′)-Ib-cr determinant acetylates several fluoroquinolones, such as norfloxacin and ciprofloxacin. Finally, the QepA and OqxAB efflux pumps extrude fluoroquinolones from the bacterial cell. A series of studies have identified the environment to be a reservoir of PMQR genes, with farm animals and aquatic habitats being significantly involved. In addition, the origin of the *qnr* genes has been identified, corresponding to the waterborne species *Shewanella* sp. Altogether, the recent observations suggest that the aquatic environment might constitute the original source of PMQR genes, that would secondly spread among animal or human isolates.

Keywords: Quinolone resistance, Qnr, plasmid, efflux pump

INTRODUCTION

Quinolones are fully synthetic and bactericidal antibacterial agents used widely in both human and veterinary medicine. The clinically available quinolones have been classified into several generations based of their spectrum of activity (Ball, 2000). The first generation quinolone (Q1G), nalidixic acid, has been discovered in 1962 (Lesher et al., 1962). Other Q1G, such as pipemidic acid and oxolinic acid, had been developed, the latter being used for in veterinary medicine. The quinolones of the second generation are made of addition of a fluorine atom at position C-6 to the quinolone nucleus, yielding to the fluoroquinolones (FQ; Paton and Reeves, 1988). The early FQ (e.g., norfloxacin, ofloxacin, pefloxacin, ciprofloxacin, or enrofloxacin) achieved higher serum levels and showed potent activity against Gram-negative bacteria, several Gram-positive bacteria (such as *Staphylococcus aureus*), and intracellular bacteria. In addition, ciprofloxacin is active against *Pseudomonas aeruginosa*. Newer FQ (third generation quinolones) were subsequently developed and presented increased activity toward Gram-positive bacteria, in particularly to *Streptococcus pneumoniae* (e.g., sparfloxacin, levofloxacin, or moxifloxacin), and potent activity against anaerobic bacteria (e.g., trovafloxacin, gatifloxacin, or gemifloxacin; Van Bambeke et al., 2005).

Even if the main factors leading to resistance to quinolones and FQ related to chromosomal mutations in the drug target genes, the discovery during the last decade of a series of plasmid-encoded resistance mechanisms has contributed to speculate about the origin and enhancing factors of that transferable resistance. In particular, the interplay between an environmental and animal source on one side, and the human clinical pathogens on the other side (in which the emergence of resistance to quinolones is a matter of fact) remains to be further explored and understood.

That review aims to present some of the current available data from which speculations can be established.

MECHANISM OF QUINOLONE ACTION

The targets of quinolone molecules are the type II topoisomerases: DNA gyrase (topoisomerase II) and DNA topoisomerase IV (Drlica and Zhao, 1997). As opposed to type I topoisomerases that transiently cleave one strand of the DNA double helix, type II topoisomerases break transiently both strands of a duplex and pass another double-helical segment through the break by ATP

hydrolysis (Drlica and Zhao, 1997; Hawkey, 2003). The DNA gyrase introduces negative supercoils into DNA whereas topoisomerase IV exhibits a potent decatenation activity. Those enzymes are essential for bacterial growth by controlling the topological status of the chromosomal DNA to facilitate replication, transcription, recombination, and DNA repair (Drlica and Zhao, 1997; Hawkey, 2003). The DNA gyrase and the DNA topoisomerase IV are the main targets of quinolones in Gram-negatives and Gram-positives, respectively. Quinolones inhibit the activity of type II topoisomerases by trapping these enzymes on DNA as drug-enzyme-DNA complexes. Ternary complex formation is responsible for inhibition of bacterial growth (bacteriostatic action) by a rapid inhibition of DNA synthesis and a slower inhibition of RNA synthesis (Drlica and Zhao, 1997; Hawkey, 2003). Eventhough these drug–enzyme–DNA complexes block cell growth, they are not directly responsible for the lethal effect of quinolones. Indeed, bactericidal activity is due to the releasing of double-stranded DNA breaks from those complexes, but the detailed mechanism of action of quinolones still needs to be fully understood.

CHROMOSOME-ENCODED RESISTANCE

Resistance to quinolones in Enterobacteriaceae most commonly results from the accumulation of mutations primarily in DNA gyrase (GyrA) then in topoisomerase IV (ParC; Hooper, 2000; Ruiz, 2003; Hopkins et al., 2005; Jacoby, 2005). Alterations in GyrA of *E. coli* predominantly occur within the N-terminus of the protein in the so-called quinolone resistance determining region (QRDR) located between amino acids Ala67 and Gln106. Mutations appear most frequently at codons Ser83 and Asp87, which are located near the active sites of enzyme (Tyr122). In addition, quinolone resistance can be associated with a decreased membrane permeability and/or an overexpression of efflux pump systems (Hooper, 2000; Hopkins et al., 2005).

PLASMID-MEDIATED RESISTANCE

Although considered as impossible due to the plasmid curing effect of quinolones (Courvalin, 1990), plasmid-mediated quinolone resistance (PMQR) was first reported in 1998 from a *Klebsiella pneumoniae* isolate in the USA (Munshi et al., 1987). Indeed, a plasmid-mediated resistance to nalidixic acid in *Shigella dysenteriae* has been reported previously in 1987 (Munshi et al., 1987), but the reality of this phenomenon was later rejected (Courvalin, 1990). To date, several PMQR mechanisms have been identified: Qnr proteins, the aminoglycoside acetyltransferase AAC(6′)-Ib-cr, and the efflux pumps QepA and OqxAB.

Qnr PROTEINS

Qnr structure and nomenclature

The first identified PMQR determinant corresponded to the Qnr protein, lately termed QnrA1 (Martinez-Martinez et al., 1998). The corresponding gene was identified on a broad-host range conjugative plasmid recovered from a ciprofloxacin-resistant *K. pneumoniae* isolate (Martinez-Martinez et al., 1998). QnrA1 is a 218-amino-acid protein that belongs to the pentapeptide repeat family, of which more than 500 members are known, distributed in prokaryotics and eukaryotics (Vetting et al., 2006). Those proteins are made of tandemly repeated amino acid sequences with a consensus sequence [S, T, A, or V] [D or N] [L or F] [S, T, or R] [G] (Vetting et al., 2006). Six other QnrA variants (QnrA2 to QnrA7) have been identified, and differ from QnrA1 by a few amino acid substitutions (Jacoby et al., 2008).

Four distantly related Qnr-like determinants belonging to the pentapeptide repeat family have also been identified in Enterobacteriaceae: QnrB, QnrC, QnrD, and QnrS (Hata et al., 2005; Jacoby et al., 2006; Cavaco et al., 2009; Wang et al., 2009). To date, there are 42 QnrB variants, 1 QnrC, 1 QnrD, and 5 QnrS (http://www.lahey.org/qnrStudies/). QnrB1, QnrC1, and QnrS1 share 40, 60, 47, and 59% amino acid identity with QnrA1, respectively.

In addition, QnrVC-like proteins have been identified in *Vibrio cholerae*, sharing 57% amino acid identity with QnrA1 (Fonseca et al., 2008). Even if the *qnrVC* genes have been identified as acquired resistance genes, they are not plasmid-located, thus not considered as PMQR genes.

Mechanism of action

QnrA1 shares 20 and 19% amino acid identity with McbG and MfpA, respectively, two other members of the pentapeptide repeat family both involved in resistance to gyrase inhibitors (Cattoir and Nordmann, 2009). Qnr proteins may supplement resistance to quinolones due to altered quinolone target enzymes, efflux pump activation, or deficiencies in outer-membrane porins (Martinez-Martinez et al., 2003; Jeong et al., 2008). In addition, Qnr proteins facilitate selection of quinolone resistance mutants by raising the level at which they can be selected with a frequency more than 100-fold higher (Martinez-Martinez et al., 1998). The presence of Qnr determinants facilitates the selection of low-level of resistance to quinolones due to chromosome-encoded mechanisms. From a clinical point of view, Qnr determinants may increase the mutant prevention concentration (MPC) of ciprofloxacin by more than 10-fold, facilitating recovery of mutants with higher level of resistance to quinolones (Rodriguez-Martinez et al., 2007). Therefore, Qnr-positive isolates may be a favorable background for an *in vivo*-selection of additional chromosome-borne mechanism(s) of resistance to quinolones after treatment by fluoroquinolones (Poirel et al., 2006).

Epidemiology of Qnr determinants

Qnr in human clinical isolates. All types of Qnr determinants have been identified worldwide in many different enterobacterial species but mostly in *K. pneumoniae*, *Enterobacter* spp., *E. coli*, and *Salmonella enterica* from community and nosocomial isolates (Rodríguez-Martínez et al., 2011). Their overall prevalence may range from 0.2 to up to 94% depending on selection criteria of studied strains (resistance to ceftazidime, nalidixic acid, FQs,. . .; Strahilevitz et al., 2009; Rodríguez-Martínez et al., 2011). The prevalence of *qnrB* genes seems to be overall higher than that of the other *qnr* genes. However, the *qnrS* genes are very frequently identified in *Salmonella* sp., suggesting that they could represent a significant resistance trait along the food chain. For instance, a recent international survey (13 European countries) identified a *qnrS* gene in 10% of the *Salmonella* sp. collection (Veldman et al., 2011).

Very few studies have been performed to evaluate the prevalence of the *qnrC* and *qnrD* genes since those genes have been recently

identified. However, the *qnrC* has been identified from a *Proteus mirabilis* isolate from China, and its prevalence seems to be very low, at least in China (Wang et al., 2009). The *qnrD* gene has been identified in 22 out of 1215 *Salmonella* isolates obtained from different European countries, being either of human or animal isolates (Veldman et al., 2011).

Qnr in animal isolates. QnrS1 was first identified from a transferable plasmid carried by a clinical isolate of *Shigella flexneri* 2b as a source of a foodborne outbreak in Aichi prefecture, Japan (Hata et al., 2005). As described for the *qnrA1* gene, the *qnrS1* gene has been identified from several enterobacterial isolates (particularly in *Salmonella* spp.) in many countries. It has been identified in porcine *E. coli* (Szmolka et al., 2011) in Hungary, in equine *E. coli* in Czech Republic (Dolejska et al., 2011), and in poultry *E. coli* in China (Yue et al., 2011). The *qnrS2* gene was identified in a single non-Typhi *Salmonella* clinical isolate from the USA (Gay et al., 2006). Finally, the *qnrS3* variant has been identified in a single veterinary clinical *E. coli* isolate from China (GenBank accession no. EU077611).

In an interesting study including 1215 *Salmonella* and 333 *E. coli* isolates, six variants of *qnrB* were identified from 138 *qnrB*-positive isolates, most of them being obtained from turkeys (Veldman et al., 2011). The *qnrD* gene was identified in 22 *Salmonella* of eight different serovars, being mostly identified in Spain but also in Italy.

Whereas there is so far no report of *qnrA*-like genes in non-enterobacterial species, *qnrB*- and *qnrS*-like genes have been identified for instance in *Pseudomonas fluorescens* and *Aeromonas* spp. isolates, respectively (Ahmed et al., 2007; Cattoir et al., 2008b; Sanchez-Cespedes et al., 2008). Interestingly, *qnrS* and mostly *qnrB* genes were identified from zoo animals, mostly including reptiles (Ahmed et al., 2007).

Qnr determinants in aquatic environments. Overall, the *qnrS*-type genes seem to be the most commonly identified acquired *qnr* genes in the environment. They have been mainly identified from waterborne species, and in particular *Aeromonas* spp. The *qnrS2* gene was identified from a mobilizable IncQ-related plasmid (pGNB2) isolated from an activated sludge bacterial community of a wastewater treatment plant in Germany (Bonemann et al., 2006), in two strains of *Aeromonas* spp. (*Aeromonas punctata* and *A. media*) isolates from the Seine river in France (Cattoir et al., 2007b), and lately in a single clinical *Aeromonas veronii* isolate from Spain (Sanchez-Cespedes et al., 2008). In Italy, a *Citrobacter freundii* strain producing the ESBL TEM-116 was recovered from a sewage effluent (Forcella et al., 2010). This ESBL gene was encoded on a plasmid that co-harbored the *qnrB9* gene. This constitutes one of the few example showing the occurrence of a *qnr* gene from an enterobacterial isolate recovered from the environment.

The environmental species that have been found to carry *qnr* genes were mainly *Aeromonas* spp. or *Vibrio* spp. In China, an *A. punctata* strain recovered from a wastewater sample in the Shandong province carried the *qnrVC4* gene on a plasmid (Xia et al., 2010). That strain was resistant to nalidixic acid but susceptible to fluoroquinolones.

Mobile genetic vehicles

All the *qnr* genes have been identified on plasmids that vary in size ranging from ca. 7 to 320 kb (Cattoir and Nordmann, 2009; Strahilevitz et al., 2009) Those plasmids, and especially the *qnrA*- and *qnrB*-positive ones, often harbor other antibiotic resistance genes conferring resistance to β-lactams, aminoglycosides, chloramphenicol, tetracycline, sulfonamides, trimethoprim, and rifampin.

The *qnrA*-like genes are usually dentified as part of complex *sul1*-type class 1 integrons, that exhibit duplicated 3′-conserved sequences (3′-CS) containing the *qacEΔ1* and *sul1* genes. Immediately upstream of *qnrA* genes, the *orf513* gene which constitutes the transposase gene of insertion sequence IS*CR1* is systematically identified (Toleman et al., 2006). The *qnrB*-like genes have been associated with either the *orf1005* gene encoding a putative transposase for *qnrB1* (Jacoby et al., 2006), the IS*CR1* element for *qnrB2* (Garnier et al., 2006; Jacoby et al., 2006; Minarini et al., 2008), *qnrB4* (Cattoir et al., 2007b; Hu et al., 2008), *qnrB10* (Quiroga et al., 2007), and *qnrB12* (Kehrenberg et al., 2008), or an IS*Ecp1* element for *qnrB19* (Cattoir et al., 2008a). Although *qnrS*-like genes are not embedded in *sul1*-type integrons, two different genetic environments have been described, with the *qnrS1* genes being identified in association with Tn*3*-like transposon structures or the insertion sequence IS*Ecl2* (Poirel et al., 2007), and the *qnrS2* gene being part of a transposon-like structure, named mobile insertion cassette (MIC), and inserted in an ORF coding for a zinc metalloprotease (MpR) in *Aeromonas* spp. (Cattoir et al., 2008b; Sanchez-Cespedes et al., 2008).

The qnr genes originate from environmental species

By screening for a collection of 48 Gram-negative clinical and environmental bacterial species (Enterobacteriaceae, Aeromonadaceae, Pseudomonadaceae, Xanthomonadaceae, Moraxellaceae, and Shewanellaceae), the origin of the *qnrA* gene was identified as being the chromosome of *Shewanella algae* (Poirel et al., 2005b). Indeed, three QnrA-like determinants (termed QnrA3, QnrA4, and QnrA5) have been identified in *S. algae*, and differ by a few amino acid substitutions from QnrA1. *S. algae* is widely distributed in aquatic environments and rarely involved in human infections. As opposed to what it has been described for the plasmid-mediated *qnrA1* gene, the chromosomal *qnrA*-like genes were not associated with the IS*CR1* element in the chromosome of *S. algae* (Poirel et al., 2005b). Finally, The G + C content (52%) of the *qnrA*-like of *S. algae* matched exactly that of the genome of *S. algae* (Poirel et al., 2005b).

It has been shown that *Vibrio splendidus* is a source of QnrS-like determinants since chromosomal-encoded Qnr-like proteins shared about 84 and 88% amino acid identity with the plasmid-mediated determinants QnrS1 and QnrS2, respectively (Cattoir et al., 2007a). In addition, the G + C contents of *qnrS*-like genes from *V. splendidus* (ca. 45%) are close to those of *qnrS1* and *qnrS2* (ca. 44%). Although the exact progenitor species of the plasmid-encoded QnrS determinant remains unknown, the bacterial species should be closely related to *V. splendidus* and likely waterborne.

Recently, the progenitor of the *qnrB*-like genes was identified to be *Citrobacter* spp. which are enterobacterial species known to

be widely present in the aquatic environment, being either human commensal bacteria or opportunistic pathogens depending on the species (Jacoby et al., 2011).

Noteworthy, it has been shown that some bacterial species belonging to the Vibrionaceae family (such as *Vibrio vulnificus*, *Vibrio parahaemolyticus*, or *Photobacterium profundum*) also possess intrinsically chromosome-encoded Qnr-like determinants (sharing 40–67% identity with the plasmid-mediated Qnr determinants) and conferring resistance to quinolones (Poirel et al., 2005a). That means that those waterborne species may also constitute potential sources of emerging PMQR genes.

Several Qnr-like pentapeptide repeat proteins have been identified in the chromosome of Gram-positive bacteria (*Enterococcus faecalis*, *Enterococcus faecium*, *Listeria monocytogenes*, *Clostridium perfringens*, *Clostridium difficile*, *Bacillus cereus*, and *Bacillus subtilis*; Arsene and Leclercq, 2007; Rodriguez-Martinez et al., 2008). Amino acid sequences of these Qnr-like proteins are identical from 16 to 22% with the PMQR determinants QnrA1, QnrB1, and QnrS1 (Rodriguez-Martinez et al., 2008). Similarly, those Gram-positive species could also constitute a reservoir for Qnr-like although none of these genes has been yet identified as plasmid-located determinants.

AMINOGLYCOSIDE ACETYLTRANSFERASE AAC(6′)-Ib-cr

The AAC(6′)-Ib-*cr* enzyme is a PMQR determinant that has been discovered from *qnrA*-positive *E. coli* from Shanghai, China (Robicsek et al., 2006). The *aac(6′)-Ib-cr* (for *c*iprofloxacin *r*esistance) gene encodes a variant of the widespread aminoglycoside acetyltransferase AAC(6′)-Ib usually responsible for resistance to kanamycin, tobramycin, and amikacin (Strahilevitz et al., 2009). This variant possesses two substitutions at codons 102 (Trp → Arg) and 179 (Asp → Tyr) compared to the wild-type AAC(6′)-Ib, both mutations seem to be required to confer reduced susceptibility to several FQ molecules (Robicsek et al., 2006). The protein AAC(6′)-Ib-cr is able to acetylate kanamycin, tobramycin, and amikacin, but also ciprofloxacin conferring slightly higher MIC values (twofold to fourfold increase). Nevertheless, it acetylates more efficiently aminoglycosides than ciprofloxacin. Since acetylation occurs at the amino nitrogen on the piperazinyl substituent, only FQs harboring an unsubstituted piperazinyl group (such as ciprofloxacin and norfloxacin) are substrates of AAC(6′)-Ib-cr (Robicsek et al., 2006). Although the *aac(6′)-Ib-cr* gene by itself confers low-level resistance to certain FQs, it may facilitate survival of target-site mutants with a 10-fold increase of their MPC (Cattoir and Nordmann, 2009).

The overall prevalence of *aac(6′)-Ib-cr* may range from 0.4 to up to 34% depending on the studied human clinical strains (Robicsek et al., 2006). This gene has been reported mostly from *E. coli* and *K. pneumoniae* clinical isolates. However, it has also been identified in *Aeromonas* spp. collected in 2006 from feces of zoo animals in Japan (Ahmed et al., 2007). Recently, it has been identified in *Salmonella* spp. recovered from chickens in Japan, and in *E. coli* of poultry origin in Spain or of pig origin in China (Liu et al., 2011; Soufi et al., 2011; Du et al., 2012). Since this gene seems to be geographically widespread, stable over the time, and equally prevalent in ciprofloxacin-susceptible and -resistant strains (Park et al., 2006), its significance remains debatable. Its occurrence could also result from human contamination, as suggested with a study from Gibson et al. (2010) identifying this gene in companion animals.

The *aac(6′)-Ib-cr* gene has been identified as a form a gene cassette into *sul1*-type class 1 integrons, and has been identified both among ESBL-positive and ESBL-negative enterobacterial isolates (Cattoir and Nordmann, 2009). Its occurrence in animals and in the environment is likely frequent, but extensive surveys are still required to better evaluate their prevalence in environmental habitats.

EFFLUX PUMP QepA

Whereas efflux pumps are chromosome-encoded, a novel PMQR determinant, *qepA* (for *q*uinolone *e*fflux *p*ump), has been identified in *E. coli* human clinical isolates from Japan and Belgium (Perichon et al., 2007; Yamane et al., 2007). This gene encodes a 511-amino-acid deduced protein (53 kDa) that shares significant identity with various 14-transmembrane-segment (14-TMS) putative efflux pump belonging to the major facilitator superfamily (MFS) of proton-dependent transporters (Perichon et al., 2007; Yamane et al., 2007). This protein confers significant decreased susceptibility to the hydrophilic quinolones (e.g., norfloxacin, ciprofloxacin, and enrofloxacin) with an 8- to 32-fold increase of MICs as compared to a wild-type susceptibility profile (Yamane et al., 2007). On the opposite, QepA protein does not significantly modify MICs of moderately hydrophilic (e.g., pefloxacin, sparfloxacin, levofloxacin, moxifloxacin) and hydrophobic (e.g., nalidixic acid) quinolones (Perichon et al., 2007; Yamane et al., 2007).

The occurrence of QepA among human clinical isolates seems to be quite limited according to the few studies that have been conducted on this subject worldwide. However, its occurrence in animals might be significant. A study performed on *E. coli* isolates from pigs in China showed that 28 (58.3%) out of 48 16S rRNA methylase RmtB-producing *E. coli* isolates were *qepA*-positive suggesting a strong linkage between *qepA* and *rmtB* genes (Liu et al., 2008). RmtB confers resistance to all aminoglycosides (except streptomycin) by decreasing the affinity of the ribosome for the antibiotic after N7-methylation at the G1405 within the 16S rRNA (Perichon et al., 2007).

Other *E. coli* isolates from pigs in China have been reported as co-expressing the *qepA*, *qnrS2*, and *aac(6′)-Ib-cr* genes (Liu et al., 2008). This co-expression of several PMQR determinants may facilitate the selection of mutants under selective pressure of antimicrobial agents. QepA-producing enterobacterial isolates were also identified from pets in China (Deng et al., 2011). In Nigeria, an *E. coli* strain recovered from chicken co-harbored a *qepA* and a *qnrB* gene (Fortini et al., 2011).

The natural reservoir of *qepA* remains unknown. However, it may be *Actinomycetales* species since QepA had significant amino acid identity with likely membrane transporters of the members of the order of *Actinomycetales* (such as *Streptomyces globisporus*, *Streptomyces coelicolor*, *Nocardia farcinica*, or *Polaromonas* spp.), and its high GC% content (72%) is compatible with this origin.

EFFLUX PUMP OqxAB

The OqxAB multidrug resistance mechanism was initially identified from *E. coli* strains recovered from swine manure (Hansen

et al., 2004). The identified plasmid harbored the *oqxA* and *oqxB* genes that are similar to genes encoding resistance–nodulation–cell-division efflux systems. That plasmid conferred resistance to olaquindox that is a veterinary growth promoter. Then, it was shown to mediate resistance to other molecules, such as chloramphenicol, nalidixic acid, and ciprofloxacin (Hansen et al., 2007). In Denmark, a retrospective study showed that nine out of 156 *E. coli* strains isolated from pigs were positive for the *oqxA* gene (Hansen et al., 2005). Recently, a Chinese study showed that 39% of the *E. coli* isolates recovered from sows, piglets, weaners, and boars in swine farms, and chicken in chicken farms harbored the *oqxAB* gene (Zhao et al., 2010). An OqxAB-positive *E. coli* strain was also identified from a liver sample of a diseased chicken in China (Liu et al., 2008). Interestingly, another Chinese study reported a series of *K. pneumoniae* isolates in which the *oqxAB* genes were actually chromosomally located (Kim et al., 2009).

DISCUSSION

The discovery of a series of PMQR determinants within the last 10 years further raised out a novel issue regarding resistance to quinolones. Indeed, whereas such resistance was supposed to be only vertically transmitted, the occurrence of those PMQR encoding genes show that it may be also horizontally mediated. Noteworthy, and even if the first research interests focused on the impact and relevance of PMQR genes among human clinical isolates, subsequent studies rapidly showed that they were also of main concern in animal and environmental strains. Such observation raises out several questions: are there relationships between quinolones in the environment that are poorly biodegraded and the prevalence of those resistance mechanisms? Are those resistance mechanisms really new and emerging? Which is the extend of the interplay between the situation observed in the environment and the current clinical concerns?

The heavy use of quinolones in animals and in particular in fish farming might likely have played a role in the selection of some resistance mechanisms. This may have impacted the fauna itself, and as a consequence the environment through contamination of aquatic habitats, but that speculation remains debatable. A recent study showed that there was no correlation between the occurrence of FQ-resistant bacteria in aquatic environments and the FQ contamination in Vietnam and Thailand (Takasu et al., 2011). However, the authors designed their study by selecting FQ-resistant bacteria with high level of resistance (more than 16 mg/l), that is not a correct criteria when focusing on PMQR only conferring decreased susceptibility to FQ.

The fact that most if not all PMQR encoding genes originate from bacterial species that are naturally present in the environment, and in particular in the aquatic one, likely suggests that this latter may represent the main source of the problem. This is indeed probable that genetic events leading to the mobilization of the resistance gene from the natural reservoir (the progenitor or the donor) to the recipient (the target plasmid or the target strain) occur in those environments where the donor is numerous.

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Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution?

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Wildlife is normally not exposed to clinically used antimicrobial agents but can acquire antimicrobial resistant bacteria through contact with humans, domesticated animals and the environment, where water polluted with feces seems to be the most important vector. *Escherichia coli*, an ubiquitous commensal bacterial species colonizing the intestinal tract of mammals and birds, is also found in the environment. Extended-spectrum beta-lactamases producing *E. coli* (ESBL-*E. coli*) represent a major problem in human and veterinary medicine, particular in nosocomial infections. Additionally an onset of community-acquired ESBL-*E. coli* infections and an emergence in livestock farming has been observed in recent years, suggesting a successful transmission as well as persistence of ESBL-*E. coli* strains outside clinical settings. Another parallel worldwide phenomenon is the spread of ESBL-*E. coli* into the environment beyond human and domesticated animal populations, and this seems to be directly influenced by antibiotic practice. This might be a collateral consequence of the community-onset of ESBL-*E. coli* infections but can result (a) in a subsequent colonization of wild animal populations which can turn into an infectious source or even a reservoir of ESBL-*E. coli*, (b) in a contribution of wildlife to the spread and transmission of ESBL-*E. coli* into fragile environmental niches, (c) in new putative infection cycles between wildlife, domesticated animals and humans, and (d) in problems in the medical treatment of wildlife. This review aims to summarize the current knowledge on ESBL-*E. coli* in wildlife, in turn underlining the need for more large scale investigations, in particular sentinel studies to monitor the impact of multiresistant bacteria on wildlife.

Keywords: ESBL, wildlife, wild birds, rodents, multiresistance

INTRODUCTION

The mere occurrence of antimicrobial resistance and corresponding resistance genes in the environment is an ancient phenomenon which results from the simple fact that most of the antimicrobial substances currently in use are based on natural precessors produced by soil bacteria like *Streptomycetes* (D'Costa et al., 2011). The function of these precessors of modern day antibiotics was presumably more related to microbial competition for an ecological niche, and thus is very distinct from the "weapon-shield" role they play in clinical settings today (Martinez, 2009a,b; Allen et al., 2010). Nevertheless the increase in non-intrinsic antimicrobial resistance in pathogenic bacteria started after the introduction of antibiotics in medicine some 60 years ago suggesting a correlation between antimicrobial pressure and the emergence of resistance in pathogens (Allen et al., 2010; Bonnedahl, 2011). Although we consider the detection of multidrug resistant pathogens like Extended-spectrum beta-Lactamases producing Gram-negatives in wildlife as a new phenomenon, it could have been anticipated, as antimicrobial resistant bacteria other than intrinsically resistant soil organisms were already found in environmental samples apparently free from any antimicrobial pressure decades ago (Sato et al., 1978; Kanai et al., 1981; Hughes and Datta, 1983; Tsubokura et al., 1995).

While various bacterial species are important in terms of multiresistance and nosocomial infections in human and veterinary medicine, we consider the Gram-positive Methicillin resistant *Staphylococcus aureus* (MRSA) and Extended-spectrum beta-lactamases producing Gram-negative bacteria like *Escherichia coli* (ESBL-*E. coli*) as being key indicator pathogens to trace the evolution of multiresistant bacteria in the environment and wildlife. Both multiresistant organisms also made their way into livestock farming and companion animals (Smet et al., 2010b; Ewers et al., 2011; Wieler et al., 2011). Recent surveillance data on antimicrobial resistance among these organisms in human clinical settings display two major trends. According to the EARS-NET database[1] the prevalence of MRSA has remained on a high but stable level over the last years, whereas that of ESBL-*E. coli* has been on a continuous rise during the last decade. Although the majority of ESBLs are still reported from human clinical isolates (Bradford, 2001; Bonnet, 2004; Pitout, 2010), they are also increasingly recorded in community-acquired bacterial infections. This indicates that ESBL-*E. coli* have made their way out of the clinics, have been successfully transmitted and now persist in the community (Arpin et al., 2005; Pitout et al., 2005; Wieler et al., 2011).

To understand the dynamics of the dispersal of ESBL-*E. coli* into natural environments beyond human and domestic animal

[1] http://ecdc.europa.eu/en/activities/surveillance/EARS-Net/database/Pages/database.aspx

population, it is important to keep in mind the general *E. coli* population as well. *E. coli* is ubiquitous, and asymptomatically colonizes the gut of birds and mammals. Therefore, *E. coli* are found globally, not only in the gut but also in the environment (Wirth et al., 2006; Goldberg et al., 2008; Rwego et al., 2008). The intestinal population of *E. coli* in mammals and birds varies enormously between individuals even of the same species. This is why knowledge on the *E. coli* population of a single species is actually scarce and limited to single studies only, which do not represent the species as a whole (Schierack et al., 2008a,b; Leser and Molbak, 2009) It is however clear, that the use of antimicrobial compounds selects for resistant clones, with one mechanism being horizontal gene transfer between strains (LeClerc et al., 1996).

Although so far it is not clear how ESBL-*E. coli* make their way into the natural environment, they were seen to occur in the environment two decades after the first ESBL-*E. coli* outbreaks in human clinical settings (Kitzis et al., 1988; Bauernfeind et al., 1989; Costa et al., 2006). Simultaneously a community-onset of ESBL-*E. coli* has taken place and one might speculate whether environmental ESBL-*E. coli* are a spill-over form of environmental pollution from highly human influenced settings (Arpin et al., 2005; Pitout et al., 2005; Martinez, 2009a). Interestingly the first reports on ESBL-*E. coli* in wildlife date back shortly after their appearance in livestock farming which could also hint toward a manure driven spread of ESBL-*E. coli* into the environment (Kummerer, 2009). It seems unlikely that pathogens isolated from wildlife have acquired resistance through new parallel mutations in the respective genes. Horizontal transfer of resistance genes from clinical isolates or the intake of already resistant bacteria from human waste, sewage, and domesticated animal manure might be more probable (Kummerer, 2009; Martinez, 2009b).

Escherichia coli from wildlife may thus express a multiresistant phenotype, not due to the nearby use of antimicrobials or antimicrobials in subtherapeutic concentrations in natural environments, but because distant use had caused a multiresistant organism to evolve in the first place which subsequently spread to different ecological niches (O'Brien, 2002). The presence of commensal and pathogenic bacteria in fecal contaminations can be assumed to be a link between settings with regular or even constant antimicrobial pressure (livestock farming, aquaculture, human, and veterinary clinical settings) and the environment, resulting in a constant release of antibiotic-resistant human and animal bacteria into the environment through wastewater or manure (Martinez, 2009b). The detection of antimicrobial resistant bacteria in aquatic environments affected by human and animal wastewater and soil provides evidence for this hypothesis (Kummerer and Henninger, 2003). In this context the common use of antibiotics in aquaculture of fish is also of utmost importance due to possible direct influences on waterbirds (Baquero et al., 2008; Smith, 2008). As intestinal bacteria like *E. coli* can be easily disseminated in different ecosystems through water they are intensively used as indicator species for fecal pollution, but *S. aureus* is also regularly isolated from fecal samples. Therefore they could also be used to track the evolution of antimicrobial resistance into different ecosystems (Van Den Bogaard et al., 2000). Furthermore *E. coli* despite its commensal character is frequently implicated in animal and human infections that require the use of antibiotics which adds public health concerns to the list of implications that arise from the spread of ESBL-*E. coli* into wildlife.

In contrast to studies on the appearance of ESBL-*E. coli* in humans and domesticated animals, their presence in wildlife has been addressed rarely. This review therefore summarizes currently available data on the presence of ESBL-*E. coli* in wildlife, concentrating on birds and rodents. It aims to bring about awareness about the urgent need to gather knowledge on the impact of ESBL-*E. coli* to the microbiota of wild animals and the consequences arising thereof for the environment and public health, acknowledging the zoonotic potential of *E. coli* and its abundance in nature (Allen et al., 2010; Bonnedahl, 2011).

BETA-LACTAM ANTIBIOTICS

The class of beta-lactam antibiotics is among the most important groups of antimicrobial agents in human and veterinary medicine. The chemical substances are in principal identical in both fields of clinical use. Besides the first widely used antimicrobial substance penicillin, other members of this family have gained a similar importance over the last decades, namely the first- to fourth-generation cephalosporins and the beta-lactamase-inhibitors. In the veterinary context the few studies that exist confirm that beta-lactam antimicrobials are the most commonly prescribed antimicrobials in small animals (DANMAP, 2007; SVARM, 2008). In livestock, a decrease in the use of beta-lactam antimicrobials could be observed over the last years (NORM/NORM-VET, 2010), basically due to restrictions in prescription. All beta-lactams interfere with the final stage of peptidoglycan synthesis through acting on penicillin-binding proteins, thereby preventing the bacterial cell wall from forming. The peptidoglycan constitutes a layer between the outer membrane and the cytoplasmic membrane which maintains the cell shape and protects the bacterium against osmotic forces. The most common resistance mechanism of Enterobacteriaceae spp. against beta-lactams is the inactivation of the drug by hydrolytic cleavage of the beta-lactam ring system (Greenwood, 2000).

BETA-LACTAMASES

More than 400 different beta-lactamase enzymes are currently known, sharing the same resistance mechanism but differing in their range of substrates and susceptibility against inhibitory substances[2]. Extended-spectrum beta-lactamases display an extended substrate spectrum, and this has directly influenced a global change in the epidemiology of beta-lactamases since the early 1990s in human medicine and since 2000 in veterinary medicine (Kong et al., 2010; Pitout, 2010; Smet et al., 2010b). The term extended-spectrum determines the ability of ESBLs to hydrolyze a broader spectrum of beta-lactam antimicrobials than the parent beta-lactamases they were originally derived from. While they are capable of inactivating beta-lactam antimicrobials containing an oxyimino-group such as oxyimino-cephalosporins (e.g., ceftazidime, cefotaxime) as well as oxyimino-monobactam (aztreonam), ESBLs are not active against cephamycins and carbapenems. They are usually inhibited by beta-lactamase-inhibitors like clavulanic acid and tazobactam, which marks a difference between

[2]http://www.lahey.org/studies/

ESBL- and AmpC–beta-lactamases producing bacteria (Bradford, 2001). Several different classification schemes for bacterial beta-lactamases have been described, including the system devised by Bush et al. (1995) which is based on the activity of the beta-lactamases against different beta-lactam antimicrobials, and the currently most widely used Ambler system, which divides beta-lactamases into four classes (A, B, C, and D), based on their amino acid sequences (Ambler, 1980). The majority of ESBLs belong to Ambler class A and to the Bush group 2be. ESBLs have been found in a wide range of Gram-negative bacteria, but the vast majority of bacterial hosts belong to the family of Enterobacteriaceae, including *Klebsiella* spp., *E. coli*, *Salmonella enterica*, *Citrobacter* spp., and *Enterobacter* spp. (Bradford, 2001). Four enzyme families, namely TEM (Temoneira) -type beta-lactamases, SHV (Sulfhydryl variable) -type beta-lactamases, CTX (cefotaximase) -M-type beta-lactamases, and OXA (oxacillinase) -type beta-lactamases are currently regarded the most common ESBLs among Enterobacteriaceae spp. TEM-type beta-lactamases are derivatives of TEM-1, which was first demonstrated in 1965 in an *E. coli* isolate from a patient in Athens, Greece, named Temoneira, and of TEM-2 and consist of more than 150 different enzymes. While the majority of TEM beta-lactamases are ESBLs, TEM-1, TEM-2, and TEM-13 are only able to hydrolyse penicillin derivates and thus are not regarded as ESBLs (Livermore, 1995).

Similar to TEM-type enzymes the majority of SHV enzymes are ESBLs. All currently recognized SHV enzymes are derivatives of SHV-1 and SHV-2. Whereas SHV-1 merely confers resistance to broad-spectrum penicillins, SHV-2, which was first described in 1983 in a *Klebsiella ozaenae* strain isolated in Germany, is able to hydrolyse cefotaxime (Gupta, 2007). In contrast to TEM- and SHV-type beta-lactamases, most of the members of the OXA-type beta-lactamase family are not regarded as ESBLs because they do not hydrolyze third generation cephalosporins with the exception of OXA-10, OXA-2, and their derivatives[3]. However, distinct OXA-types (OXA-carbapenemases) play an important role in antimicrobial resistance, e.g., of *Acinetobacter baumannii* (Pfeifer et al., 2010).

Currently regarded as the most important ESBL enzyme family are the CTX-M-type beta-lactamases, named after their ability to hydrolyze cefotaxime. They are supposed to originate from beta-lactamases from *Kluyvera* spp. and currently comprise of more than 70 different CTX-M enzymes divided into five groups depending on their amino acid sequence (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25; Hawkey and Jones, 2009; Pitout, 2010; Naseer and Sundsfjord, 2011). AmpC–beta-lactamases confer resistance to most of the beta-lactam antimicrobials with the exception of methoxy-imino-cephalosporins (cefepime) and carbapenems, while they are not inactivated by beta-lactamase-inhibitors like clavulanic acid.

Within the last years the emergence of carbapenem-hydrolyzing beta-lactamases like NDM-1, KPC, and OXA has threatened the clinical utility of this antibiotic class (Pfeifer et al., 2010, 2011; Poirel et al., 2010; Walsh, 2010). Carbapenemases are beta-lactamases that are active not only against the oxyimino-cephalosporins and cephamycins but also capable of hydrolyzing carbapenems like imipenem or meropenem. These substances often display the "last line of defense" in the treatment of infections with multiresistant Gram-negative pathogens. This group of beta-lactamases is very diverse and can be found in three different β-lactamase classes (class A, B, and D). Detailed information on these enzymes is given in some excellent reviews (Walsh, 2010; Patel and Bonomo, 2011).

ESBLs IN HUMAN AND DOMESTIC ANIMALS

The first nosocomial outbreak of CTX-M-1 type ESBLs was recorded in an intensive care unit in a hospital in Paris, France (Kitzis et al., 1988). Shortly after that, Bauernfeind et al. (1989) reported on a clinical cefotaxime-resistant *E. coli* strain in Germany which produced a CTX-M-1 type beta-lactamase. In the following 10 years several studies reported about an explosive dissemination of ESBLs in human clinical settings worldwide (Bernard et al., 1992; Gniadkowski et al., 1998; Radice et al., 2002; Canton and Coque, 2006). Several review articles provide detailed insight into the occurrence and molecular epidemiology of ESBL producing Enterobacteriaceae in humans and animals (Bradford, 2001; Bonnet, 2004; Canton and Coque, 2006; Livermore et al., 2007; Cantón et al., 2008; Oteo et al., 2010; Pfeifer et al., 2010; Pitout, 2010; Wieler et al., 2011).

Since about 2000, the CTX-M enzymes have formed a rapidly growing family of ESBLs in human clinical and community settings (Bonnet, 2004; Pitout and Laupland, 2008; Mshana et al., 2009), whereas the prevalence of classical ESBL enzymes like TEM or SHV is decreasing (Livermore et al., 2007). With the beginning of the twenty-first century *E. coli* producing CTX-M-15 have emerged and disseminated worldwide as an important cause of both nosocomial and community-onset urinary tract and bloodstream infections in humans (Coque et al., 2008; Hunter et al., 2010; Oteo et al., 2010; Pitout, 2010). A number of molecular epidemiological studies revealed that the sudden worldwide increase of CTX-M-15-producing *E. coli* has been largely influenced by the spread of one single clonal group of strains, namely B2:O25b:H4-ST131-CTX-M-15, across different continents (Nicolas-Chanoine et al., 2008; Rogers et al., 2011). The recent emergence of yet another clonal group, ABD-O1:H6-ST648-CTX-M-15, envisions the potential of just a limited number of clones to spread globally (Doi et al., 2010; Zong and Yu, 2010; Van Der Bij et al., 2011; Wieler et al., 2011). Unraveling the microevolution of strains of these clones in habitats and ecological niches others than human and veterinary clinics offers the chance to understand what leads to persistence of ESBL-*E. coli* in surroundings lacking selective antibiotic pressure.

In the field of veterinary medicine an SHV-12-type beta-lactamase producing *E. coli* was the first clinical ESBL producing bacteria isolated from a dog with recurrent urinary tract infection in Spain in 1998 (Teshager et al., 2000). This was followed by the detection of ESBL producing *E. coli* (mostly TEM and SHV) in dogs from Italy, and Portugal (Feria et al., 2002; Carattoli et al., 2005).Very recently several studies also reported companion animals as hosts for ESBL-*E. coli* harboring CTX-M enzymes, leading to the assumption that CTX-M-type enzymes will dominate the situation in veterinary medicine in the future as well (Vo et al.,

[3]http://www.lahey.org/studies/

2007; Carattoli, 2008; O'Keefe et al., 2010; Smet et al., 2010b). This is exemplified by the emergence of the clonally related group of *E. coli* B2-O25b:H4-ST131-CTX-M-15 in the field of companion animals, as well (Pomba et al., 2009; Ewers et al., 2010, 2011; Biohaz, 2011; Wieler et al., 2011).

Extended-Spectrum beta-lactamases mostly of the TEM, CTX-M, and SHV-type have been frequently demonstrated in the microbiota of food-producing animals which has nicely been reviewed by Smet et al. (2010b). Within the last decade, the number of publications reporting ESBL-*E. coli* isolated from food-producing animals has increased drastically. Noticeably, most ESBL enzymes identified in *E. coli* from livestock are likewise present in bacteria from humans (Smet et al., 2010b).

ESBLs IN WILDLIFE

A HISTORICAL PERSPECTIVE

The first reports on the presence of resistance determinants in *E. coli* from human and animal populations lacking selective antimicrobial pressure date back to the 1960s (Mare, 1968). Antimicrobial resistant *E. coli* isolates originating from wildlife species were reported for the first time at the beginning of the 1980s from Japanese wild birds (Sato et al., 1978; Kanai et al., 1981; Tsubokura et al., 1995) and 5 years later in South African baboons feeding on human refuse (Rolland et al., 1985; Routman et al., 1985). With the new millenium the number of studies describing the occurrence of antimicrobial resistant *E. coli* in wildlife increased significantly (Gilliver et al., 1999; Souza et al., 1999; Sherley et al., 2000; Fallacara et al., 2001; Livermore et al., 2001; Osterblad et al., 2001; Swiecicka et al., 2003; Cole et al., 2005; Lillehaug et al., 2005; Middleton and Ambrose, 2005; Sayah et al., 2005; Skurnik et al., 2006; Dolejska et al., 2007; Literak et al., 2007; Carattoli, 2008; Gionechetti et al., 2008; Ewers et al., 2009; Guenther et al., 2010c).

However, the detection of ESBL-*E. coli* of wildlife origin dates back to 2006 only (Costa et al., 2006). Since then several reports followed (Costa et al., 2008; Poeta et al., 2008, 2009; Bonnedahl et al., 2009, 2010; Dolejska et al., 2009; Literak et al., 2009a,b, 2010; Guenther et al., 2010a,b; Hernandez et al., 2010; Pinto et al., 2010; Radhouani et al., 2010; Simoes et al., 2010; Smet et al., 2010b; Garmyn et al., 2011; Ho et al., 2011; Silva et al., 2011; Sousa et al., 2011; Wallensten et al., 2011).

A GEOGRAPHICAL PERSPECTIVE

Although ESBL-*E. coli* isolates of wildlife origin have only been reported from Europe (Costa et al., 2008; Poeta et al., 2008, 2009; Bonnedahl et al., 2009, 2010; Dolejska et al., 2009; Literak et al., 2009b, 2010; Guenther et al., 2010a,b; Pinto et al., 2010; Radhouani et al., 2010; Simoes et al., 2010; Garmyn et al., 2011; Silva et al., 2011; Sousa et al., 2011; Wallensten et al., 2011), Africa (Literak et al., 2009a), and Asia (Hernandez et al., 2010; Ho et al., 2011) so far, their absence in the Americas, Antarctica, and Australia might simply reflect the different number of studies performed in these continents. As multiresistant *E. coli* have already been reported from the latter continents (Souza et al., 1999; Sherley et al., 2000; Fallacara et al., 2001; Cole et al., 2005; Middleton and Ambrose, 2005; Sayah et al., 2005; Kozak et al., 2009; Silva et al., 2009) one could anticipate ESBL-*E. coli* of wildlife origin to be present as well. Nevertheless data from one continent or region may not act as a suitable baseline for another, and may not correlate with the level of antibiotic use in the regions involved. Besides simple geographical effects like the continent of origin it seems more appropriate to reconsider the type of region where the isolates originate from. Parameters which have been assumed as important criteria include the natural preservation state, livestock, and human density or the remoteness of an area (Allen et al., 2010). The level of resistant bacteria observed in wild animals seems to correlate well with the degree of association with human activity (Skurnik et al., 2006; Allen et al., 2010). Nevertheless, several studies report the occurrence of ESBL-*E. coli* in remote places or preservation areas as well (Hernandez et al., 2010; Pinto et al., 2010) underlining the complexity of the spread of antimicrobial resistance in wild animals. These findings suggest on the one hand an influence of migratory behavior of wild birds for instance into remote areas or on the other hand the omnipresence of human influence in various ecological niches of the planet basically via human feces. Most studies on ESBL-*E. coli* in wildlife originate from Central Europe, an area with high livestock and human density and an assumable frequent interaction of wildlife with human influenced habitats of any kind like livestock farms, landfills, sewage systems, or wastewater treatment facilities, resulting in a higher risk for wildlife acquiring antibiotic-resistant bacteria (Allen et al., 2010). It has previously been shown that gulls shared strains of *E. coli* with isolates cultured from landfills and wastewater treatment plants (Nelson et al., 2008). This underlines the possibility of bacterial exchange between human sewage and birds.

As summarized in **Table 1** the detection rates of ESBL-*E. coli* in different geographical areas ranged from 0.5% in birds of the remote Azores islands in the Atlantic Ocean (Silva et al., 2011) to 32% for birds of the Iberean peninsula (Simoes et al., 2010). However, one should certainly keep in mind differences with regards to host species, sampling schemes, and geographic regions and the limitations that arise from this when interpreting these data. Nevertheless, for Central Europe the number of studies performed is relatively high, and there does not seem to be a difference in the detection rates between agriculturally used lands or urban environments compared to natural preserve areas, since in both types of areas detection rates higher than 20% have been observed (**Table 1**). Only in remote areas like the Azores or the Kamchatka peninsula the rates seem to be lower with approximately 1% ESBL-*E. coli* (Hernandez et al., 2010; Silva et al., 2011), suggesting a possible dilution effect of the pollution of wild animals with ESBL-*E. coli*. Nevertheless our own data from birds of prey from the Mongolian Gobi-Desert, an area among the ones with the lowest human density, revealed ESBL rates which were comparable with the situation in Central Europe (Guenther et al., 2010c).

A HOST SPECIES PERSPECTIVE

General information about the microbiota of wild living birds and rodents is scarce and restricted to single species as hosts of certain pathogens. *E. coli* is a common gastrointestinal but very versatile bacterium, and can be grouped into non-pathogenic (commensal) and pathogenic strains; the latter cause intestinal or extraintestinal diseases in humans and animals (Johnson and Russo, 2002; Wirth et al., 2006). The ubiquitous occurrence of *E. coli* is based on its asymptomatical colonization of the gut of birds and mammals

Table 1 | Presence of extended-spectrum beta-lactamases producing *E. coli* in wildlife in chronological order according to the date of publication.

Reference	Animal species	No. of ESBL producing isolates per total no. (%) of isolates investigated	Detected ESBL types (% in relation to total no. of ESBL)	Country	Year of isolation	MLST (no. of isolates)
Costa et al. (2006)	Bird of prey, Deer, Fox, Owl (all unspecified)	9/56 (16.1)	bla_{TEM-52} (33), $bla_{TEM-52} + bla_{CTX-M-14}$ (11), $bla_{CTX-M-14} + bla_{TEM-1}$ (22), $bla_{CTX-M-1} + bla_{TEM-1}$ (11), bla_{SHV-12} (11), $bla_{CTX-M-14}$ (11)	Portugal	2003–2004	Not specified
Poeta et al. (2008)	Seagulls (*Larus* sp.)	11/57 (19.3)	bla_{TEM-52} (72.7), $bla_{CTX-M-1}$ (9.1), $bla_{CTX-M-14a}$ (9.1), $bla_{CTX-M-32}$ (9.1)	Portugal	2007	Not specified
Dolejska et al. (2009)	Black headed gull (*C. ridibundus*)	7/213 (3.2)	$bla_{CTX-M-1}$ (14.2), $bla_{CTX-M-15}$ (28.6), bla_{SHV-2} (14.2), bla_{SHV-12} (28.6), unknown (14.2)	Czech Republic	2005	Not specified
Bonnedahl et al. (2009)	Yellow legged gull (*L. michahellis*)	16/180 (8.8)	$bla_{CTX-M-1}$ (43.8), $bla_{CTX-M-1} + bla_{TEM-1}$ (6.6), $bla_{CTX-M-15} + bla_{TEM-1}$ (6.6), bla_{TEM-1} (31.3), $bla_{SHV} + bla_{TEM-1}$ (12.5)	France	2008	ST1199, ST533, ST1140 (2), ST156, ST90, ST1142, ST681 (2), ST1134, ST1143 (2), ST1135, ST1144, ST746, ST351
Poeta et al. (2009)	Wild boar (*S. scrofa*)	8/77 (10.3)	$bla_{CTX-M-1}$ (75), $bla_{CTX-M-1} + bla_{TEM-1}$ (25)	Portugal	2005–2007	Not specified
Literak et al. (2009a)	Brown rat (*R. rattus*)	1/37 (2.3)	$bla_{CTX-M-15}$ (100)	Senegal	2007	Not specified
Hernandez et al. (2010)	Glaucous winged gull (*L. glaucescens*)	4/532 (0.8)	$bla_{CTX-M-14}$ (50), $bla_{CTX-M-15}$ (50)	Russia	2007	ST131, ST609 (2), ST746
Bonnedahl et al. (2010)	Black headed gull (*C. ridibundus*)	3/83 (3.6)	$bla_{CTX-M-14}$ (66), $bla_{CTX-M-15}$ (33)	Sweden	2008	ST1646, ST1340, ST1647
Literak et al. (2010)	Mallard duck (*A. platyrhynchos*), Herring gull (*L. argentatus*)	9/83 (10.8)	$bla_{CTX-M-1}$ (66), $bla_{CTX-M-9} + bla_{TEM-1b}$ (11), $bla_{CTX-M-15} + bla_{OXA-1}$ (11), bla_{SHV-12} (11)	Poland	2008–2009	Not specified
Pinto et al. (2010)	Buzzard (*B. buteo*), Barn owl (*T. alba*), Tawny owl (*S. aluco*), Booted eagle (*A. pennata*), Montagu's harrier (*C. pygargus*), Black kite (*M. migrans*), Black vulture (*C. atratus*), Bonelli's eagle (*A. fasciata*), Eurasian eagle owl (*B. bubo*), Raven (*C. corax*)	32/119 (26.9)	$bla_{CTX-M-1}$ (40.5), $bla_{CTX-M-1} + bla_{TEM-1}$ (43.8), $bla_{CTX-M-1} + bla_{TEM-20}$ (3.1), bla_{SHV-5} (3.1), $bla_{SHV-5} + bla_{TEM-1}$ (6.3), $bla_{SHV-5} + bla_{TEM-20}$ (3.3)	Portugal	2008	Not specified
Radhouani et al. (2010)	Buzzards (*B. buteo*)	5/33 (15.2)	$bla_{CTX-M-32} + bla_{TEM-1}$ (70), $bla_{CTX-M-1} + bla_{TEM-1}$ (30)	Portugal	2007–2008	Not specified

(Continued)

Table 1 | Continued

Reference	Animal species	No. of ESBL producing isolates per total no. (%) of isolates investigated	Detected ESBL types (% in relation to total no. of ESBL)	Country	Year of isolation	MLST (no. of isolates)
Simoes et al. (2010)	Seagulls (*Larus* sp.)	45/139 (32)	$bla_{CTX-M-1}$ (18), $bla_{CTX-M-9}$ (9), $bla_{CTX-M-15}$ (39), $bla_{CTX-M-32}$ (34)	Portugal	2007–2008	ST1284 (4), ST131 (4), ST224 (3), ST453, ST86, ST205, ST359, ST165, ST69, ST1152, ST405, ST559, ST1163, ST10, ST58, ST156, ST155, ST297, ST43, ST58, ST156
Wallensten et al. (2011)	Seagulls (*Larus* sp.)	18/194 (9.2)	$bla_{CTX-M-1} + bla_{TEM-1}$ (50), $bla_{CTX-M-1} + bla_{TEM-1}$ (38.8), $bla_{CTX-M-14} + bla_{TEM-1}$ (5.5), bla_{SHV-12} (5.5)	Sweden	2010	Not specified
Silva et al. (2011)	Black cap (*S. atricapilla*)	1/220 (0.45)	$bla_{CTX-M-14} + bla_{SHV-12}$ (100)	Azores/ Portugal	2006–2010	Not specified
Sousa et al. (2011)	Gilthead sea bream (*S. aurata*)	5/118 (4.2)	bla_{TEM-52} (40), bla_{SHV-12} (60)	Atlantic ocean/ Portugal	2007	Not specified
Ho et al. (2011)	Rodents (unspecified)	19/456 (4.2)	$bla_{CTX-M-9}$ (42.6), $bla_{CTX-M-1}$ (47.4)	China	2008–2010	Not specified
Guenther et al. (2010b)	Norway rat (*R. norvegicus*)	1/220 (0.5)	$bla_{CTX-M-9}$	Germany	2009	ST131
Guenther et al. (2010a)	Eurasian blackbird (*T. merula*), White fronted goose (*A. albifrons*), Rock pigeon (*C. livia*)	4/172 (2.3)	$bla_{CTX-M-15}$ (100)	Germany	2009	ST648
Literak et al. (2009b)	Wild boars (*S. scrofa*)	5/293(2)	$bla_{CTX-M-1} + bla_{TEM-1}$ (20), $bla_{CTX-M-1}$ (60), $bla_{TEM-52b}$ (20)	Czech Republic	2009	Not specified
Garmyn et al. (2011)	Wild geese (*B. canadensis*, *A. anser*)	2/396 (0.5)	bla_{TEM-52} (50), bla_{SHV-12} (50)	Belgium	2010	ST1079, ST1844

and in turn the resulting spread into the environment (Rwego et al., 2008; Schierack et al., 2008a). The degree of colonization varies a lot between different bird species (Gordon and Cowling, 2003) and the same has been shown for rodents and small mammals (Swiecicka et al., 2003; Guenther et al., 2010c). *E. coli* is most likely to be isolated from omnivore birds and mammals (Gordon and Cowling, 2003). More than 30 wild animal species have been found shedding ESBL-*E. coli* and most of them were birds or rodents (**Table 1**). The occurrence of ESBL-*E. coli* is therefore clearly influenced by the host spectrum of *E. coli* and furthermore by the degree of synanthropic behavior shown by the host animal species. In other words, animals living in urbanized areas are more likely to carry *E. coli* than animals living in remote areas (Allen et al., 2010; Bonnedahl, 2011).

Other basic questions concerning the occurrence of ESBL-*E. coli* in fecal samples are still unsolved. Future studies should therefore address the nature of the abundance of these multiresistant strains in feces to clarify if they are just shedded in short terms, present transient, or long term colonizations of the gut of the animals asymptomatically or even are persistent infections.

When reviewing the current literature it appears that wild birds could be the main wildlife hosts for ESBL-*E. coli*. This impression is created because most of the studies were carried out on wild birds; however, taking into account studies involving other wildlife animals such as deer, small ruminants, small and large predators, lagomorphs, reptiles, and amphibians (Costa et al., 2006; Literak et al., 2009b) an insignificant number of ESBL-*E. coli* was observed.

Due to their diversity in ecological niches, and their ease in picking up human and environmental bacteria, wild birds might act as mirrors of human activities. Within the heterogeneous class of birds two groups seem to be in the focus of ESBL carriage in wildlife: waterfowl/water related species (Poeta et al., 2008; Bonnedahl et al., 2009, 2010; Dolejska et al., 2009; Guenther et al., 2010b; Hernandez et al., 2010; Literak et al., 2010; Simoes et al., 2010; Garmyn et al., 2011; Wallensten et al., 2011) and birds of prey (Costa et al., 2006; Pinto et al., 2010; Radhouani et al., 2010; detailed species information is given in **Table 1**). Other groups

of birds like passerines (Guenther et al., 2010a; Silva et al., 2011) seem to carry ESBL-*E. coli* less often or sometimes not at all (Silva et al., 2010). This finding might be influenced by differences in the composition of the microbiota and the harboring of *E. coli* within these diverse avian species. In a recent study we were able to show that if *E. coli* could be isolated from different bird species, multiresistant *E. coli* clones originated from birds of prey or waterfowl (Guenther et al., 2010d). Other avian hosts reported were Owls (Costa et al., 2006; Pinto et al., 2010) and Ravens (Pinto et al., 2010), birds which display a feeding behavior comparable to birds of prey.

While the dominance of waterfowl within the avian host spectrum of ESBL-*E. coli* can be explained by fecal pollution of water by human or livestock sources, the transmission scenarios for the other main group – birds of prey – seem to be more complex. As birds of prey are on top of the food chain they could accumulate ESBL-*E. coli* from their typical prey, like mice and shrews. Due to their synantropic behavior, they are presumably more often in contact with humans or livestock. Indeed for mice it has been shown that proximity to livestock farming increases the carriage rates of multiresistant *E. coli* (Kozak et al., 2009). Although rodents have earlier been in the focus of research on ESBL in wildlife (Gilliver et al., 1999; Kozak et al., 2009; Literak et al., 2009b; Guenther et al., 2010c), to the best of our knowledge ESBL-*E. coli* have not yet been detected in rodents with the exception of urban rats (Guenther et al., 2010b; Ho et al., 2011).

Our own data from Germany revealed a very low abundance for multiresistant *E. coli* in rodent and shrews, indicating that other groups of prey and transmission routes between the human influenced ecosphere and birds of prey might be possible (Guenther et al., 2010c). Furthermore, many of the bird species which have been tested positive for ESBL-*E. coli* carriage display migration behavior which provides a possible mechanism for the establishment of new endemic foci over great distances from where a multiresistant microorganism was first acquired (Bonnedahl, 2011).

As mentioned above another important host of ESBL-*E. coli* seems to be a group of rodents namely Norway rats and Black rats with reports on ESBL producing isolates from different continents (Literak et al., 2009a; Guenther et al., 2010b; Ho et al., 2011). This synantropic species can easily pick up human waste and often interacts with human feces in the sewage system in urban environments and can therefore easily acquire multiresistant bacteria. Interestingly wild boars have also been reported as host s of ESBL-*E. coli* in Central Europe, which might reflect their omnivorous feeding behavior (Literak et al., 2009b; Poeta et al., 2009). Other mammals found to be positive hosts of ESBL producing bacteria were deer and foxes (Costa et al., 2006). Very recently there has been a report on a marine fish, the Gilthead Sea bream (Sousa et al., 2011) as a carrier of ESBL-*E. coli*, indicating a dissemination of ESBL-*E. coli* into the Atlantic ocean.

A PERSPECTIVE ON ESBL ENZYMES

In parallel to the current situation in human and veterinary medicine the type of extended-spectrum beta-lactamases found in wild animals are clearly dominated by the $bla_{\text{CTX-M}}$ gene-family. With the exception of one study (Sousa et al., 2011) all wildlife studies identified the $bla_{\text{CTX-M}}$ genes as the main ESBL enzyme. In 35% of the studies different SHV enzymes were additionally detected. As shown in **Table 1**, most of the studies reported $bla_{\text{CTX-M-1}}$, followed by $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-14}}$, $bla_{\text{CTX-M-32}}$, and $bla_{\text{CTX-M-9}}$. Only occasionally $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-13}}$, $bla_{\text{CTX-M-55}}$, and $bla_{\text{CTX-M-65}}$ were detected (**Table 1**). Besides the $bla_{\text{CTX-M}}$-type family only $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-52}}$ were also often detected. Other less prevalent ESBL genes were $bla_{\text{OXA-1}}$, $bla_{\text{SHV-5}}$, and $bla_{\text{TEM-20}}$. The spectrum of the different enzyme types found in wild animals is very narrow compared to clinical isolates of human and veterinary origin (Pitout, 2010; Smet et al., 2010b). While the reason for this is unknown, we offer the following hypotheses: these findings might simply reflect the small number of studies performed on wildlife so far, or they could indicate that certain types of beta-lactamases are more successful in the environment, for example due to co-selection of other non-resistance genes accompanied by these beta-lactamases. Another explanation could be that the types of ESBLs found in wild animals simply reflect the ones that are most prevalent in human and veterinary clinics and in livestock farming, such as $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-15}}$ (Pitout, 2010; Smet et al., 2010b). This could lead to the assumption that the situation we are observing in wild animals is just presenting spill-over effects from clinics and livestock farming. If this was true, future studies presumably should find a rise in those pandemic CTX-M-15 types, exemplified by the clonal *E. coli* lineages of ST131 and ST648. Several studies observed a similarity in the overall resistance profiles of wild animal isolates with human or veterinarian clinical isolates which supports this hypothesis (Costa et al., 2008; Literak et al., 2009b; Guenther et al., 2010d). The most prevalent non-ESBL resistant phenotype in wildlife *E. coli* is resistance to streptomycin, ampicillin, and tetracycline. This pattern is also very common in human and livestock populations in Europe (Van Den Bogaard et al., 2000; Guerra et al., 2003). As mentioned above the types of ESBL genes are basically the same in human, livestock, and wildlife, which strengthens the hypothesis that wildlife isolates resemble those found in animal and human patients. However, as the dissemination of ESBL genes is highly driven by horizontal gene transfer through plasmids, the occurrence of identical ESBL genes could also be based on the spread of ESBL-plasmids which are randomly distributed in the environment. In summary, unraveling the basis of the ESBL-*E. coli* spill-over into wildlife needs to include both a characterization of the clonal nature of bacterial strains isolated from different hosts as well as an accurate identification of the ESBL genes and their episomal or chromosomal localization.

A ZOONOTIC POTENTIAL PERSPECTIVE

Regarding the basic question of the zoonotic potential of *E. coli* it is useful to address the population genetics of this bacterial species. Besides comparative whole genome analysis of bacteria, this can also be done by other approaches like Multi-locus sequence typing (MLST)[4]. Although it is based on a small set of marker genes only, this method seems to reflect the microevolution of the *E. coli* core genome. In general, MLST analysis revealed the existence of strains belonging to identical sequence types (STs) and

[4]www.mlst.net

being isolated from different hosts rather being the rule than the exception. This indicates a common phylogeny and therefore a zoonotic potential for most strains analyzed so far (Wirth et al., 2006). Indeed, several research groups found clusters of *E. coli* causing systemic infections in birds, and urinary tract infection and neonatal meningitis in humans, which are genetically so similar, that a zoonotic potential is foreseen (Johnson and Russo, 2002; Ewers et al., 2007; Moulin-Schouleur et al., 2007). So far the number of ESBL-*E. coli* from wildlife in that global data base is rather limited. However, if the same clusters of *E. coli* can cause disease in humans and domesticated birds, their transmission scenarios become important and such routes of transfer indeed are a plausible transfer mechanism of ESBL-*E. coli* from humans to wild birds and vice versa.

To answer the question about the similarity of human clinical, livestock, companion animal, and wildlife ESBL-*E. coli* isolates we need to gain insight into the clonal relatedness of isolates from all these groups. Initial MLST paired with pulsed field gel electrophoresis (PFGE) is an ideal tool to reveal clonal relatedness or even clonal identity of epidemiologically unrelated isolates. This attempt has been put forward by a small number of studies on wild birds, all clearly pointing out that similar STs or clonal groups are present in humans, domestic animals, and wild birds (Bonnedahl et al., 2009, 2010; Guenther et al., 2010a; Hernandez et al., 2010; Simoes et al., 2010). Overall up to 35 different STs have been detected in wild avian ESBL-*E. coli* (**Table 1**; **Figure 1**). Although some of the "avian" STs appeared twice in different wildlife studies like ST746 (Bonnedahl et al., 2009; Hernandez et al., 2010) and have not been detected in human clinical samples yet, the majority of the STs found in avian ESBL-*E. coli*, such as ST131, ST10, ST90, ST648, or ST69, are also present in human clinical isolates.

Figure 1 shows a minimum spanning tree (MSTree) displaying human, domestic animal, and wildlife ESBL–STs based on data of the MLST database[5] as of October 2011 and the current literature on human and animal ESBL-*E. coli* isolates providing MLST data (Minarini et al., 2007; Yumuk et al., 2008; Blanco et al., 2009; Hrabak et al., 2009; Naseer et al., 2009; Oteo et al., 2009; Sidjabat et al., 2009; Suzuki et al., 2009; Valverde et al., 2009; Coelho et al., 2010; Doi et al., 2010; Peirano et al., 2010; Smet et al., 2010a; Zong and Yu, 2010; Ben Slama et al., 2011; Djamdjian et al., 2011; Leverstein-Van Hall et al., 2011; Mshana et al., 2011; Van Der Bij et al., 2011; Woerther et al., 2011). In the MSTree each circle represents a ST and the size of the circle is proportional to the number of ESBL-*E. coli* isolates belonging to this ST. Here is becomes remarkably clear that ESBL-*E. coli* of wildlife, domestic animal, and human origin share identical STs suggesting an interspecies transmission of phylogenetically related multiresistant strains. This hypothesis is further strengthened by the detection of the worldwide emerging clonal group of *E. coli* specified as B2-O25b:H4-ST131 in human (Nicolas-Chanoine et al., 2008), veterinary clinical settings (Ewers et al., 2010), and in a Glaucous winged gull in Kamchatka (Hernandez et al., 2010).

In another study (Bonnedahl et al., 2010) in south Sweden ESBL-*E. coli* of several new STs were detected, including ST1646

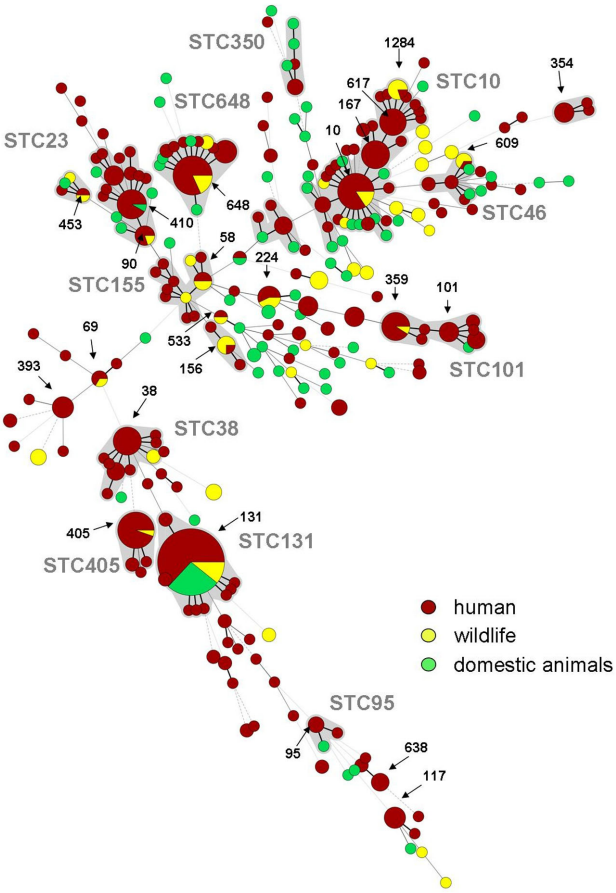

FIGURE 1 | Minimum spanning tree (MSTree) of human, domestic animals, and wildlife sequence types known for the production of ESBLs based on data of the MLST database (http://mlst.ucc.ie/mlst/dbs/Ecoli; *n* = 288 isolates identifiable as ESBLs, October 2011), previously published articles with human clinical background (Minarini et al., 2007; Yumuk et al., 2008; Blanco et al., 2009; Hrabak et al., 2009; Naseer et al., 2009; Oteo et al., 2009; Suzuki et al., 2009; Valverde et al., 2009; Coelho et al., 2010; Cortes et al., 2010; Peirano et al., 2010; Smet et al., 2010a; Zong and Yu, 2010; Ben Slama et al., 2011; Djamdjian et al., 2011; Leverstein-Van Hall et al., 2011; Mshana et al., 2011; Van Der Bij et al., 2011; Woerther et al., 2011) and data on wildlife given in Table 1. Red: human isolates, Green: domestic animals, Yellow: wildlife, Gray underplayed: Sequence type complexes, calculated with Bionumerics 6.6 (Applied Maths, Belgium).

which is closely related to ST648 previously also found in wild birds in Germany (Guenther et al., 2010a) and in humans in Africa, Asia, and the United States (Doi et al., 2010; Zong and Yu, 2010). Interestingly, ST648 has been detected as one of the STs associated with the carriage of the newly emerging carbapenemase NDM-1 which underlines unforeseeable consequences of the entry of certain multiresistant clones into wild bird populations (Mushtaq et al., 2011).

As mentioned above there are currently three studies that provide evidence for the frequent occurrence of ESBL-*E. coli* in rats (Literak et al., 2009a; Guenther et al., 2010b; Ho et al., 2011). However, comparative data on the clonal relatedness of these isolates

[5]http://mlst.ucc.ie/mlst/dbs/Ecoli

and those of human or domestic animals as assessed by MLST or PFGE is limited to a single study only (Guenther et al., 2010b). Here an ESBL-*E. coli* from a rat belonging to the pandemic clone B2-O25b:H4-ST131 was detected, which might point toward a direct transmission from human feces to the rat in an urban sewage system.

CONCLUSION

The current data on ESBL-*E. coli* in wild animals reveals that carriage of these multiresistant strains is widespread in at least some wild populations like waterfowl, birds of prey, and rodents, even though these have never been exposed continuously to antibiotics. This clearly undermines the presumption that resistance will decline with the absence of antibiotic treatment alone. It underlines the very complex nature of the spread of antimicrobial resistance which has been also already pointed out for non-beta-lactam resistance in human populations in remote areas in South America (Pallecchi et al., 2007; Bartoloni et al., 2009).

The origins of resistance and the selection mechanisms responsible for maintaining high prevalence of resistance are largely unknown and therefore need to be addressed more soundly (Gilliver et al., 1999). The common occurrence of ESBL-*E. coli* in wildlife, especially in avian hosts, has several implications. Firstly, wildlife has the potential to serve as an environmental reservoir and melting pot of bacterial resistance. Secondly by taking into account the zoonotic potential of *E. coli* and the concomitant observation that ESBL-*E. coli* of wildlife origin are basically the same than the ones found in clinical isolates they additionally have the potential to re-infect human populations. Bird's feces are omnipresent in urban and rural settings and smear infections of humans by avian droppings should not be underestimated. Many bird species, including those that were already identified as carriers of ESBL-*E. coli*, display considerable mobility, often involving the crossing of continents. In the same way the phenomenon of bird migration creates the potential for the establishment of new endemic foci of disease along their routes like it has been seen for the West Nile Virus in the USA (Reed et al., 2003), antimicrobial resistant bacteria might also be carried over long distances by avian hosts. Bird migration could therefore contribute to the dissemination of resistance over the globe as has previously been observed for human travelers (Peirano et al., 2011). One might think that this is of minor impact compared to human travel but we have to keep in mind that in contrast to the human population there is no sewage system for bird feces, and droppings are therefore directly exposed to the environment as well as to human and animal population.

Avian mobility and spread of ESBL-*E. coli* might also have unpredictable consequences through possible interactions of these birds with environmental bacterial ecosystems in remote areas reached mainly by migrating birds. We have to keep in mind that the role of antimicrobial substances or their ancestors in natural ecosystems like soil differs considerably from their antinfective function in clinical settings. This in turn means that the crosstalk of bacterial communities in fragile ecosystems could be highly influenced by the entry of multiresistant bacteria (Allen et al., 2010).

Future studies should address whether ESBL-*E. coli* can persist in the environment or circulate in animals for long periods and may thus be disseminated by wildlife and other vectors. The pandemic spread of certain ESBL-*E. coli* lineages into the environment highlights the complexity of dissemination of antimicrobial drug resistance. As previously suggested, thorough spatial and temporal studies of antimicrobial drug resistance in different natural habitats are warranted (Gilliver et al., 1999; Hernandez et al., 2010) to fully understand the importance of wildlife as a source of antimicrobial resistance.

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Origin and evolution of antibiotic resistance: the common mechanisms of emergence and spread in water bodies

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The environment, and especially freshwater, constitutes a reactor where the evolution and the rise of new resistances occur. In water bodies such as waste water effluents, lakes, and rivers or streams, bacteria from different sources, e.g., urban, industrial, and agricultural waste, probably selected by intensive antibiotic usage, are collected and mixed with environmental species. This may cause two effects on the development of antibiotic resistances: first, the contamination of water by antibiotics or other pollutants lead to the rise of resistances due to selection processes, for instance, of strains over-expressing broad range defensive mechanisms, such as efflux pumps. Second, since environmental species are provided with intrinsic antibiotic resistance mechanisms, the mixture with allochthonous species is likely to cause genetic exchange. In this context, the role of phages and integrons for the spread of resistance mechanisms appears significant. Allochthonous species could acquire new resistances from environmental donors and introduce the newly acquired resistance mechanisms into the clinics. This is illustrated by clinically relevant resistance mechanisms, such as the fluoroquinolones resistance genes *qnr*. Freshwater appears to play an important role in the emergence and in the spread of antibiotic resistances, highlighting the necessity for strategies of water quality improvement. We assume that further knowledge is needed to better understand the role of the environment as reservoir of antibiotic resistances and to elucidate the link between environmental pollution by anthropogenic pressures and emergence of antibiotic resistances. Only an integrated vision of these two aspects can provide elements to assess the risk of spread of antibiotic resistances via water bodies and suggest, in this context, solutions for this urgent health issue.

Keywords: water, freshwater, antibiotic resistance, environment, gene transfer

INTRODUCTION

Evolution of bacterial antibiotic resistances, and its spread and emergence, represent one of the most threatening health care problems with worldwide proportions (Hawkey, 2008). The rise of new resistances and of multi-drug resistances urgently asks for a better understanding of the factors and hot spots involved in its diffusion and development. All the known antibiotic resistance mechanisms, acquired by opportunistic and pathogenic bacteria, evolve by means of Darwinian forces, i.e., mutations occurring in pre-existing genes of the bacterial chromosome positively selected by environmental forces (Gullberg et al., 2011; Zhang et al., 2011). Mutations within the chromosome can be responsible for the decreased affinity of antibiotics to their targets. Furthermore, some resistance mechanisms (e.g., efflux pumps, chromosomal AmpC β-lactamases) are finely regulated in their expression and at a basal level confer a naturally reduced susceptibility to the drugs. Mutations in the genomic architectures regulating such mechanisms result in their over-expression and high level of antibiotic resistance (Jacoby, 2009; Coyne et al., 2011). However, adaptation to the selective pressure of antibiotics accelerates acquisition of antibiotic resistance genes by lateral transfer from donor species (Wiedenbeck and Cohan, 2011). Aminov (2011) has reviewed the role of horizontal gene transfer mechanisms in environmental microbiota. Although many more studies are necessary to completely understand the role of horizontal gene transfer in the environment, experimental evidences have demonstrated that transduction has an important role in genetic exchanges among environmental microbiota, especially in freshwater. Horizontal gene transfer events are responsible for the acquisition of heterologous resistance mechanisms among species and from antibiotic producers to commensal and pathogen bacteria. Hospitals, human community, farms, aquacultures, and agriculture are reactors where the usage of antibiotics selects for resistant bacteria and promotes the gene exchange. Recently, much more attention has focused on the role of the environment and of connected ecological habitats, water bodies such as rivers, streams, waste water effluents, and lakes, that have been suggested to be important in facilitating the transport and transfer of the antibiotic resistance genes (Aminov and Mackie, 2007; Baquero et al., 2008). Low-cost pharmaceuticals, preventative medication with broad spectrum antibiotics together with the overuse of those drugs contribute significantly to the emergence of bacterial drug resistances (Depledge, 2011). The combination of all these factors together with an inadequate waste-management

of the pharmaceuticals seem to be responsible for the alarming pollution of the environmental habitats such as agricultural soils and rivers, which probably contribute to the selection of antibiotic resistant bacteria and speed up the emergence of new resistances. Furthermore, rivers often receive bacteria from different sources, e.g., waste water treatment plants or water originating from urban effluent, industrial, or agricultural activities, thus constituting potential compartments where environmental, human, and/or animal related bacteria can coexist, at least temporally (Baquero et al., 2008). This mixing can result in two main risks: (i) many environmental bacterial species are provided with intrinsic antibiotic resistance genes, constituting part of the so-called resistome. These bacteria represent a reservoir of drug resistance mechanisms and may act as donors for human related bacteria which, in turn, could introduce new acquired resistance mechanisms in the clinics (Wright, 2010); (ii) due to the intensive usage of antibiotics in medicine, agriculture, and aquaculture, human or animal related bacteria are more likely to be selected for antibiotic resistances within polluted environments directly by the presence of antibiotics and indirectly through co-selection by other pollutants (Martinez, 2009). Thus, when antibiotic resistant bacteria contaminate rivers, their resistance mechanisms can spread in the environment through bacteria, and/or mobile genetic elements. The localization of antibiotic resistance genes on diverse genetic structures such as integrons, which are platforms for gene aggregation, and mobile genetic elements (e.g., transposons and plasmids), together with the presence of phages, enhance their spread, influencing the course of their evolution (Wright et al., 2008). In particular, integrons are supposed to have a crucial role in the development of multi-drug resistances (Cambray et al., 2010). Recent studies suggest that the spread of resistant bacteria in natural fresh water systems can reach drinking water supplies and thus enter the human food chain (Walsh et al., 2011). These factors lead to an unlimited spread of antibiotic resistances and indicate that water sanitation or a better management of the respective water quality is crucial for a better control of the spread of antibiotic resistances.

This review aims to describe the current knowledge on the origins of antibiotic resistances mechanisms and environmental reservoirs of antibiotic resistances. Mechanisms, originating and spreading in bacterial populations naturally occurring in the water habitats, will be highlighted. The consequences of horizontal gene transfer by transduction and gene recombination events mediated by integrons in water habitats will be underlined. The **Table 1** summarizes the mechanisms of resistances cited in the review.

MUTATIONS AND THE RISE OF ANTIBIOTIC RESISTANCES IN FRESH WATER HABITATS

Mutations in environmental habitats occur frequently and usually depend on evolutionary or demographic factors such as population size etc. Generally, the rate of mutations can be increased due to anthropogenic impacts. However, especially antibiotics in the environment are more likely to select for specific mutations within bacteria. We summarize which mutations are most relevant in the context of antibiotic resistance in water habitats and might therefore be selected to a higher frequency due to the presence of antibiotics in water bodies.

In the clinics it has often been observed that the onset of spontaneous mutations in chromosomal bacterial genes may lead to the emergence of resistances affecting from one antibiotic to several drug classes of antibiotics. For instance, different point mutations in ribosomal proteins confer aminoglycosides, tetracyclines, and macrolides resistance; or in the RNA polymerase confer rifampicin resistance. To the best of our knowledge, these mechanisms have never been reported in bacteria of environmental origin, most likely because they have been overlooked.

Mutations in the penicillin binding proteins (PBPs) can lead to a decreased affinity for β-lactams drugs establishing bacterial resistance (Lambert, 2005). This mechanism has been extensively reported from clinical species. Although no direct reports exist from environmental sources, bacterial species such as *Enterococcus faecium* and *Proteus mirabilis* have been described to resist to β-lactams by mutations in PBP5 and PBP2 respectively. *E. faecium* has been frequently reported as contaminant of water body and *P. mirabilis* has been described as shuttle species between human or animal guts and water bodies (Sosa et al., 2006). The risk associated with the spread of organisms harboring such mechanisms is probably low. However, investigations to understand the link between PBP modification and fitness in terms of survival in water could yield significant information, to better assess the risk of spread for these species and antibiotic resistances. Probably, the role of mutations in the propagation and emergence of antibiotic resistant bacteria is minor compared to the acquisition of heterologous determinants. However, it seems that also very low concentrations of antibiotics can select for less susceptible bacteria (Gullberg et al., 2011). In environmental habitats this could result in an ecological imbalance with a higher prevalence of resistant microorganisms.

CHROMOSOMALLY ENCODED CEPHALOSPORINASE

Bacteria, especially Gram-negative, can also resist to β-lactams by the production of hydrolytic enzymes, β-lactamases. Detection of antibiotic resistant bacteria, in freshwater, harboring β-lactamases has been reported from several geographical areas. Clinical occurrence of β-lactamases encoding genes has been extensively reviewed (Bonnet, 2004; Pitout et al., 2005; Coque et al., 2008; Bush, 2010) and recently much attention to their propagation in the environment has been paid (see hereafter the section on acquired β-lactamases). According to Ambler (1980) β-lactamases are classified in four classes, simplistically ranging from A to D. Bush and Jacoby have proposed a categorization of the β-lactamases according to their hydrolytic and inhibitory profiles. For an exhaustive knowledge of β-lactamases nomenclature and biochemical characteristics, we suggest the recent review by Bush and Fisher (2011). Jacoby (2009) has reviewed the distribution, origins, and enzymatic action of AmpC β-lactamases, belonging to the class C. The author has observed that *amp*C genes are located on the chromosome of bacteria belonging to different and phylogenetically distant species. *amp*C genes are widely distributed in bacterial species of environmental origins. Water borne species such as *Aeromonas* spp., *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Ochrobactrum anthropi*, as well as several Enterobacteriaceae, commonly found in water habitats, like *Enterobacter* spp., *Morganella morganii*, and *Hafnia alvei* harbor chromosomal *amp*C genes. Typically, *amp*C genes are regulated

Table 1 | Overview of some mechanisms of bacterial antibiotic resistances occurring in water habitats.

Antibiotic	Resistance mechanism	Host	Source
β-Lactams	PBP2 mutations	*Proteus mirabilis*	–[a]
	PBP5 mutations	*Enterococcus faecium*	–[a]
	*amp*C regulators mutations	Gram-negative species	–[a]
	*amp*C promoter region mutations	*Escherichia coli*	Recreational beaches, drinking water
	Acquired AmpC	*E. coli*	Recreational beaches, drinking water, river, biofilm of water supplies
	Acquired CTX-M	*E. coli*	River, sediment, birds
	Acquired KPC	*Klebsiella pneumoniae*	Hospital waste water effluent
	Acquired VIM	*Brevundimonas diminuta*, *Rhizobium radiobacter*, *Pseudomonas monteilii*, *Pseudomonas aeruginosa*, *Ochrobactrum anthropi*, *Enterobacter ludwigii*, *Pseudomonas pseudoalcaligenes*	Hospital waste water effluent
	Acquired IMP	*Pseudomonas fluorescens*	Waste water
	Acquired OXA-23	*Acinetobacter baumannii*	River, hospital waste water effluent
	Acquired OXA-48	*Serratia marcescens*	River
	Acquired NDM-1	*P. aeruginosa*, *Achromobacter* spp., *Kingella denitrificans*	Tap water
Fluoroquinolones	QRDR (quinolones resistance determining region) mutations	*P. aeruginosa*	Hospital and urban waste water effluent
		Aeromonas spp., *E. coli*	River and lake, urban effluent
	QnrS	*Aeromonas allosaccarophila*	Lake
		E. coli	River
	QnrS2	*Aeromonas punctata*, *Aeromonas media*	Lake
	QnrVC4	*A. punctata*	Waste water effluent
	QepA efflux	Metagenome	River sediment, water from farm environment
	OqxAB efflux	*E. coli*	Farm water
Vancomycin	modification of the peptidoglycan	*Enterococci* spp.	Waste water effluents, biofilm
Chloramphenicol and florfenicol	FloR efflux	Gram-negative species *Aeromonas bestiarium*	Aquacultures streams
Tetracyclines	Tet efflux	Several species	Farms, sediment
MDR[b]	Over-expression of RND efflux pumps	Gram-negative	–[a]

[a] *Observed in clinics but likely occurring in environmental and water habitats.*

[b] *MDR, multi-drug resistance.*

and their expression is induced in the presence of β-lactams. The regulation of *amp*C expression is quite complex and has been reviewed for *P. aeruginosa* by Lister et al. (2009). Mutations in the transcription factor AmpR, a LysR-type transcriptional regulator, in the inner membrane permease AmpG, or in the cytosolic amidase AmpD, have been found to confer a constitutive expression of the cephalosporinase gene, even in the absence of antibiotics.

Mataseje et al. (2009) described the over-expression of *amp*C, by mutations in the promoter region, in *Escherichia coli* strains isolated from recreational beaches and drinking water (**Table 1**). As for other β-lactamases, chromosomal cephalosporinases have been described to evolve by point mutation, hydrolyzing a broader spectrum of β-lactams (Jacoby, 2009). Of particular concern is the plasmidic location of several *amp*C genes, which likely originated from the chromosomal cephalosporinase of environmental species. Details will be discussed in the section on acquired β-lactamases. AmpC enzymes are indistinguishable from the D-peptidases, involved in the cell wall biosynthesis (Jacoby, 2009). Thus, these enzymes probably adapted to confer β-lactams resistance from the natural physiological function, likely by gene duplication and mutation events (Sandegren and Andersson, 2009). Genes encoding AmpC enzymes are largely distributed on the chromosomes of many bacterial species of environmental origins. The intrinsic function of AmpC remains unknown, but the conservation of this enzyme in several unrelated species and the complex regulation of its structural gene highly suggests a physiological role. Deciphering this role could provide useful information on the evolutionary processes and driving forces that have lead to the selection of β-lactamases.

DNA GYRASE AND TOPO-ISOMERASE

Generally, mutations in the quinolone resistance determining region (QRDR) of *gyr*A, *gyr*B, *par*C, and *par*E genes coding for the bacterial DNA gyrase and the topo-isomerase IV respectively, are responsible for the onset of bacterial resistance to fluoroquinolones. This mechanism is known to occur in water

environments. Schwartz et al. (2006) have detected ciprofloxacin resistant *P. aeruginosa* in six different treatment plants from four cities in Germany receiving the waste water from hospitals and cities. Molecular investigations demonstrated the occurrence of mutations in *gyr*A and *par*C genes. Further, the study demonstrated the spread of the ciprofloxacin resistant *P. aeruginosa* also in the waste water receiving river (**Table 1**). Alcaide et al. (2010) have reported about the *gyr*A and *par*C mutation conferring fluoroquinolones resistances in a variety of *Aeromonas* spp. isolated from freshwater. The authors found that the mutations in *gyr*A and *par*C, which are responsible for fluoroquinolones resistance, in recently described *Aeromonas* spp. such as *Aeromonas media*, *Aeromonas veronii*, and *Aeromonas popoffi* are similar to the one described in *Aeromonas hydrophila*, *Aeromonas sobria*, *Aeromonas caviae*, and *Aeromonas salmonicida*. In Portugal, Figueira et al. (2011) reported about mutations in *gyr*A and *par*C mostly linked to *Aeromonas punctata* and *A. media* isolated from an urban effluent. The same authors have recently characterized *E. coli* strains, isolated from a waste water effluent, that harbored mutations in *gyr*A and *par*C genes, likely responsible for the observed ciprofloxacin resistance (**Table 1**).

EFFLUX PUMPS

The role of efflux pumps in conferring antibiotic resistance and multi-drug resistances in bacteria has been extensively studied and reviewed (Poole, 2004; Piddock, 2006; Martinez, 2009; Nikaido and Pages, 2011). Efflux systems conferring drug resistance typically belong to five main families: the ATP-binding cassette (ABC) transporter, the major facilitator superfamily (MFS), the small multi-drug resistance (SMR), the multi-drug and toxic-compound extrusion (MATE), and the resistance nodulation division (RND) families, the latter present only in Gram-negative bacteria and chromosomally located. The structural genes for these systems can be located on transferable genetic elements and constitute the main acquired mechanisms for drug resistance (e.g., the Tet and the CmlA/FloR efflux systems families for tetracycline and chloramphenicol resistance, respectively). However, bacteria are intrinsically provided with chromosomally encoded efflux systems that are believed to participate in the cell homeostasy, by extruding endo and/or exogenous toxic compounds, heavy metals, virulence factors, quorum sensing signal, etc. In Gram-negative bacteria, RND systems exhibit a wide substrate spectrum, which usually includes drugs of different classes. Nikaido and Pages (2011) have recently reviewed the role of these efflux pumps in a wide range of pathogenic and opportunistic bacterial species such as *E. coli*, *Klebsiella pneumoniae*, *Enterobacter* spp., *P. aeruginosa*, *Acinetobacter baumannii*, and the emergent opportunistic *Stenotrophomonas maltophilia*. Typically, the expression of RND efflux pumps is finely regulated by a dedicated regulator (Coyne et al., 2011). A more complex regulation network, linking efflux to membrane permeability and other cellular functions, is likely to occur in these bacteria, as described for the *mar* regulon in *E. coli* (reviewed by Grkovic et al., 2002). Some RND efflux genes are not expressed in absence of inducing signal, whereas others exhibit a basal level of expression, and therefore contribute to intrinsic resistance (Coyne et al., 2011). Point mutations in a regulator or in the promoter sequence of RND efflux genes can be responsible for their over-expression and, in turn, for enhanced resistance. Similarly, the acquisition of an insertion sequence, carrying a strong and constitutive promoter, upstream of the regulator or the promoter sequence of RND efflux genes, can also mediate their over-expression and cause drug resistance. These systems have been mostly studied in the context of antibiotic resistance; therefore only little information concerning the natural and physiological mechanisms inducing the expression of RND efflux genes exist. Recently, studies have identified the role of oxidative and nitrosative stress in the activation of MexXY and MexEF–OprN, respectively (Fetar et al., 2011; Fraud and Poole, 2011). These stress signals are likely to occur in the environment and might represent natural inducers of the efflux systems expression. The natural role of efflux systems has been extensively reviewed by Martinez et al. (2009) who concluded that the intrinsic role of efflux in the bacterial physiology has lead to the conservation of the genes coding for efflux pumps among species of the same genus. For example, if the over-expression of *mdf*A confers MDR to *E. coli*, a basal expression is involved in the $Na^+(K^+)/H^+$ antiport, that allows the pH homeostasis of the cell (Lewinson and Bibi, 2001). Efflux pumps conferring resistance to antibiotics, such as the AcrAB–TolC from *Salmonella* spp. has also been shown to efflux bile salts, therefore conferring a selective advantage which allowed colonizing and surviving in human or animal intestines (Lacroix et al., 1996). Mosqueda and Ramos (2000) described the contribution of efflux pumps in the cellular extrusion of toluene, an organic solvent, in *Pseudomonas putida*. This species, able to grow on the liquid interface of water and toluene and to survive in highly contaminated environments, extrudes the solvent by the TtgABC pump. The genes coding for this RND efflux pump usually exhibit a basal expression level but are induced by the presence of toluene in the medium. In water sediment, Groh et al. (2007) demonstrated that a MexF-like pump from *Shewanella oneidensis*, further than contributing to resistance to tetracycline and chloramphenicol, confers an increased fitness in anoxic environments. The underlying mechanism is unclear but could involve the extrusion of toxic compounds. A well documented role, for some efflux pumps, is their involvement in the cell to cell communication. This function has been demonstrated for MexAB–OprM in *P. aeruginosa* (Evans et al., 1998), BpeAB–OprB in *Burkholderia pseudomallei* (Chan and Chua, 2005) and AcrAB–TolC in Enterobacteriaceae (Rahmati et al., 2002). These RND pumps, further than extruding homoserine lactones, are also able to confer MDR. Moreover, several reports have shown that efflux pumps, notably from the RND family, are involved in mechanisms leading to bacterial virulence. For example, Piddock (2006) highlighted the crucial role of efflux pumps in extruding abiotic substances such as flavonoids during plant colonization and in establishing virulence. In antibiotic producing bacteria, efflux pumps play a crucial role as a self defense mechanism by extruding the bioactive secondary metabolites. For instance, an efflux-mediated self-resistance has been developed in the oxytetracycline-producing *Streptomyces rimosus* (Petkovic et al., 2006). Bacteria living in the same habitat, being exposed to the produced antibiotics, could either adapt their intrinsic mechanisms, e.g., by the over-expression of an efflux pump, or acquire by horizontal gene transfer the resistance mechanism from the producers. The first option would require a point mutation to

over-express a pre-existing efflux system able to pump out the toxic compound, whereas the second pathway would involve the mobilization and transfer of the gene coding for the self-protecting mechanism. Thus, efflux pumps had an ecological role much before they conferred drug resistances in clinics, as they constitute a selective advantage in presence of competing microorganisms. The massive usage of these drugs has further selected optimized mechanisms and enhanced their spread. The role of mobile and mobilizing genetic elements, such as insertion sequences, integrons, transposons, and plasmids, were critical for a successful and rapid spread. Nikaido and Pages (2011) have observed that the rise of resistance due to efflux pumps mechanisms in clinics is tightly linked to the sub-inhibitory concentration of the antibiotics during clinical therapies. Consequently, the appearance of this kind of resistance favors the emergence of other mechanisms such as reduced membrane permeability to drugs, increase of point mutation in the drug target genes or activation of enzymatic resistance mechanisms. It would be of interest to investigate this aspect of resistance development in environmental habitats, where the concentration of antibiotics varies dependent on the degree of pollution and where other selective forces are present. Especially, heavy metals, naturally present in the soil, and solvents produced as consequences of metabolic activities, have been demonstrated to be substrates of several efflux pumps conferring multi-drug resistance (Silver and Phung, 1996; Moken et al., 1997). Concerning heavy metals, pumps have the additional role to defend bacteria from a toxic excess and to maintain the proper intra-cellular concentration for co-factors and enzymes (Teitzel et al., 2006). The presence of these compounds in freshwater could therefore select for the over-expression of an intrinsic efflux pump. Some heavy metals efflux genes, notably from the SMR family, are located on R plasmids containing antibiotic resistance genes, and heavy metals may favor the co-selection of these two features. In the environment, maintenance and propagation of antibiotic resistance genes might have been promoted by heavy metals selection (Martinez et al., 2009). Moreover, a causal relationship between pollution of the water environment by antibiotics or other pollutants agents and the selection of bacteria expressing or over-expressing efflux pumps appears conceivable. Hernandez et al. (2011a) have recently demonstrated *in vitro* how triclosan, a detergent antibiotic used in cosmetic, binds the regulator SmeT of the SmeDEF pump in *S. maltophilia*, leading to the over-expression of the pump and consequent multi-drug resistance. This observation is of major concern since *S. maltophilia* is an aquatic species that can be responsible for nosocomial infection.

Until now, it remains unclear how the efflux pumps contribute to the emergence of resistant bacteria in the environment. It has been demonstrated that an efflux pump over-expression could be coupled with a reduced bacterial fitness. However, this is not a general rule. Sanchez et al. (2002) investigated the fitness of two *P. aeruginosa* mutants over-expressing the MexAB–OprM and MexCD–OprJ efflux pumps, both conferring multi-drug resistance. The authors demonstrated *in vitro* that the MexAB–OprM over-expressing mutant showed a significantly decreased survival in water compared to the wild type strain, while no significant differences were observed for the second efflux pump mutant. In addition, the production of biofilm in both mutants was not affected if not promoted in the MexCD–OprJ mutant. Production of biofilm implies a higher probability of survival in natural water ecosystem and would thus constitute a beneficial characteristic. Selection in polluted environments of opportunistic species such as *P. aeruginosa*, *S. maltophilia*, or *A. baumannii*, over-expressing efflux systems could contribute to the spread of these bacteria and their introduction into clinics. It would be interesting to focus on the above described mechanisms also in water environments, to gain a better understanding of their physiological function and their role in the emergence of bacterial drug resistance.

ACQUISITION OF GENES IN WATER HABITATS AND DEVELOPMENT OF ANTIBIOTIC RESISTANCES

Acquisition of heterologous genes by lateral transfer largely facilitate the adaptive evolution of bacteria, especially under strong selective pressures. Transfer of exogenous DNA in bacteria may be mediated by plasmids, phages, transposons, genomic islands, or captation of free DNA by transformation. Sengelov and Sorensen (1998) have found that in environments such as bulk water, plasmid transfer from a donor to a recipient cell occur, even at a low frequency. Taylor et al. (2011) have observed that several factors could, not only influence, but also promote gene transfer among bacteria in water environment. One such factor is filter feeding organisms that collect bacteria belonging to different species and concentrate them at high density in a reduced space, facilitating gene exchange. Biofilm matrix in water habitats also creates favorable conditions both for plasmid exchange and transformation process (Molin and Tolker-Nielsen, 2003). Interestingly, Meibom et al. (2005) have demonstrated how chitin present in the crustacean exoskeletons is able to activate the competence status of *Vibrio cholerae*, and thus enhance transformation by acquisition of exogenous DNA. Although they are not classified as mobile genetic elements, integrons are platforms for genes aggregation, and thus contribute to MDR development. Furthermore, the abundance of integrons in bacterial communities of water habitats seems to be associated with the degree of water bodies' pollution (Wright et al., 2008). Many findings support the crucial role of genetic transfer in water habitats mediated by phages (Ripp and Miller, 1995).

INTEGRONS

Several studies have highlighted the crucial role of integrons, particularly class 1 integrons, in the evolution of antibiotic resistances in clinics (Cambray et al., 2010). Indeed, class 1 integrons are not only platforms for genes aggregation, leading to the establishment of multi-drug resistance, but their localization on mobile genetic elements such as plasmids and transposons favor the spread of several genes in a unique transfer event. Recently, studies on environmental microbial communities have demonstrated that integrons of class 1 are largely present in the environment. Gillings et al. (2008) have provided evidences that the clinical class 1 integrons originated from environmental bacterial communities. The authors observed that class 1 integrons isolated from environmental samples do not carry any antibiotic resistance gene and harbored the *qac* gene cassettes, which is responsible for the bacterial resistance to quaternary ammoniums by efflux. Clinical class 1 integrons would have arisen from environmental ones by integration on a *Tn*402-like transposon, which then

disseminated in human commensals and pathogens. The presence of the *qac* gene has conferred a selective advantage to adapt in clinical environments, where bacteria are often challenged by disinfectants. The establishment of class 1 integrons in clinical strains has later on enabled the acquisition of antibiotic resistances positively selected by the usage of drugs. This hypothesis is also supported by the fact that clinical class 1 integrons demonstrated similar structures among them, in terms of integrases and recombination site, inferring a common ancestor. Gaze et al. (2005) have demonstrated how pollution of water bodies and their sediments with quaternary ammonium compounds, directly select for bacteria harboring *qac*E gene cassettes, located on the class 1 integrons. Furthermore, evidence of selection of bacteria harboring class 1 integrons in water bodies contaminated by industrial waste has been provided by Wright et al. (2008). The authors demonstrated that the contamination of freshwater with heavy metals correlated positively with a higher abundance of class 1 integrons in the bacterial community. More recently, Gaze et al. (2011) showed in sewage sludge and pig slurry that the prevalence of class 1 integrons and of *qac* genes was higher in bacteria exposed to detergents and/or antibiotic residues. All these studies demonstrate that pollution of water bodies with different agents increases the risk of selection and spread of integron structures. These genetic structures may be acquired by bacterial species that play role as shuttle between environment and clinics, constituting gene vectors for further dissemination in nosocomial bacteria.

PHAGES

Phages are major constituents of environmental ecosystems, in particular freshwater (Weinbauer, 2004; Srinivasiah et al., 2008). Their abundance is usually higher than bacterial abundance and, since a significant fraction of the prokaryotic community is infected with phages in aquatic systems, phages are likely to play an important role in horizontal gene transfer. Parsley et al. (2010) have proven the presence of β-lactamases genes in the viral metagenome of an activated sludge, confirming that transduction events may be responsible for the propagation of antibiotic resistance genes in these environments. Interestingly, Colomer-Lluch et al. (2011) demonstrated the presence of bla_{TEM} and $bla_{\text{CTX-M}}$, the most common genes conferring β-lactams resistance in Enterobacteriaceae, and *mec*A, responsible for methicillin resistance in *Staphylococcus aureus*, in phage DNA isolated from a waste water treatment plant and the natural water of the receiving river.

The presence of *mec*A in the phage fraction of natural freshwater is of great sanitary concern because of the threat represented by methicillin resistant *Staphylococcus aureus* (MRSA) infections, both in hospitals and communities (Campanile et al., 2011). This finding is also of interest for the understanding of the propagation of this gene. *mec*A codes for a protein with a low affinity to penicillin (PBP2a), conferring methicillin resistance. This gene is located on a mobile genomic element, the staphylococcal cassette chromosome (SCC*mec*), and has been reported only from the *Staphylococcus* genus from clinics. Baba et al. (2009) have characterized a methicillin resistance gene complex, $mecIRA_m$, which could be the progenitor of SCC*mec* observed in clinical MRSA, from a strain of *Macrococcus caseolyticus* (closely related to *S. aureus*), isolated from animal meat. Interestingly, Tsubakishita et al. (2010) found a *mec*A gene in *S. fleurettii* chromosomally located and not associated to the SCC*mec* element. Thus, the authors advanced the hypothesis that *S. fleurettii*, an animal related species, is the progenitor of this resistance mechanism. The *mec*A gene has been reported rarely from natural water, but Schwartz et al. (2003) detected *mec*A in hospital waste waters. Later, Bockelmann et al. (2009) have reported the sporadic presence of *mec*A in a ground water recharge system. Kassem et al. (2008) described the presence of the *mec*A gene in 18 *Proteus vulgaris*, four *M. morganii*, and three *Enterococcus faecalis* isolated from surface water. A ca. 250 bp-sequence of *mec*A from one representative isolate of *P. vulgaris*, *M. morganii*, and *E. faecalis* was found to exhibit 100% similarity with the *S. aureus mec*A gene. However, this result, which is the first report of *MecA* in non-staphylococcal organisms, has never been confirmed by other studies or investigated further. Acquisition by transduction of heterologous genes, particularly of antibiotic resistance genes, might represent an important mechanism of horizontal gene transfer in water bodies. Considering the high concentration of phages in such environments (Weinbauer, 2004; Srinivasiah et al., 2008), transduction constitutes probably one of the main gene transfer mechanisms and of genome evolution for bacteria in water habitats. More studies are needed to understand the impact of phage communities on bacterial evolution and antibiotic resistance spread within the water bodies.

ORIGINS OF ACQUIRED ANTIBIOTIC RESISTANCE MECHANISMS

Recently, D'Costa et al. (2011) have reported a metagenomic analysis of the Beringian permafrost, which is 30,000 years old. They showed molecular evidences of the ancient origins of antibiotic resistances, detecting β-lactamases genes, *van*X-like, component of the vancomycin resistance operon, and *tet*M, coding for a protein protecting the ribosomal target from tetracycline. Sequence analysis revealed that the β-lactamases genes recovered from the permafrost demonstrated an amino-acid homology (53–84%) to known β-lactamases from β-lactams producing *Streptomyces*. The *tet*M sequences revealed a high similarity to the genes coding for the ribosomal protection protein of actinomycetes. The *van*X sequence showed a similarity to the *van*X gene recovered in pathogenic vancomycin resistant enterococci (VRE) and to the *van*X gene from *Amycolatopsis orientalis*. This environmental species, belonging to the actinobacteria phylum, is a natural producer of vancomycin, and very likely the progenitor of the *van* genes operons, responsible for resistance to vancomycin. The integration of the *van* operons on transposons and on conjugative plasmids has enhanced their spread (Courvalin, 2006). Reports of VRE in freshwater have been provided by several authors (Talebi et al., 2008; Lata et al., 2009; Luczkiewicz et al., 2010). Interestingly, Schwartz et al. (2003) detected *van*A genes in the biofilm of drinking water supplies, in the absence of enterococci, demonstrating the lateral transfer of this gene. Notably, the progenitors of these resistance genes are soil bacteria thus most likely, a shuttle has been responsible for the introduction of these genes into the commensal bacterial community and afterward into the pathogenic species.

FLUOROQUINOLONES RESISTANCE BY TARGET PROTECTION

Resistance mechanisms originating from bacterial population of water bodies are less well documented than from soil organisms. However, the significance of water bodies as natural source for resistance mechanisms is similar compared to the soil. For example, a well known example is provided by the acquired fluoroquinolones resistance genes of the *qnr* family. *qnr* genes encode proteins binding the bacterial DNA gyrase, thus preventing the interaction of the antibiotic with its target. Generally, the presence of these acquired genes does not confer a high level of fluoroquinolones resistance, but provides a selective advantage in the presence of these drugs, even at low concentrations (Rodriguez-Martinez et al., 2011). Further, this protecting mechanism and the associated low level resistance may favor the emergence of strains with higher resistances to fluoroquinolones by mutations in the QRDR, quinolones resistance determining region, and/or by overexpressing efflux systems. Several aquatic bacterial species have been proposed as progenitors for these genes families. Poirel et al. (2005b) reported evidences that the *qnr*A gene located on plasmids and found in clinical isolates of fluoroquinolones resistant Enterobacteriaceae, is derived from the chromosome of *Shewanella algae*, a bacterial species present in marine and freshwater. The authors advanced the hypothesis that the gene jumped from the environmental species to Enterobacteriaceae probably under pressure of antibiotic usage. Beaber et al. (2004) have demonstrated that the presence of fluoroquinolones induces the SOS bacterial repair system, which in turn promotes horizontal gene transfer. Poirel et al. (2005a) conducted further investigations in order to understand the origin of this antibiotic resistance mechanism. Their study highlighted that the chromosomes of water borne bacteria, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, and *Photobacterium profundum* harbored *qnr*-like genes with homology (40–67% identity) to the plasmidic *qnr*A, *qnr*B, and *qnr*S genes described in clinical Enterobacteriaceae.

Interestingly, *qnr*A has been observed frequently associated with the insertion sequence IS*CR1*, a genetic element able to mobilize adjacent genes. Toleman et al. (2006) hypothesized that the IS*CR1* mediated mobilization of *qnr*A, as well as a further localization on a class 1 integron to form a so-called complex integron structure. The authors formulated that this complex integron structure is responsible for the successful dissemination of *qnr*A gene. Arsène and Leclercq (2007) investigated the intrinsic resistance of *E. faecalis* to fluoroquinolones and found that this species is provided with a chromosomal *qnr*-like gene, which contributes to resistance against fluoroquinolones. Soon afterward, Sánchez et al. (2008) discovered that the aquatic bacterium *S. maltophilia* is a sink of *qnr* genes and the chromosomally located *Sm*qnr gene identified in this species is able to confer resistance to fluoroquinolones in heterologous species. In 2010, Velasco et al. (2010) reported *qnr*-like genes from *Serratia marcescens*, an environmental species. These genes, called *Sma*qnr, were largely present in the chromosome of the genus. Recently, Jacoby et al. (2011) have highlighted that the *Citrobacter* spp. chromosome constitutes a reservoir for the *qnr*B fluoroquinolones resistance gene. The presence of *qnr* genes on the chromosome of phylogenetically distant bacterial species (*Shewanella*, *Stenotrophomonas*, *Vibrio*, *Enterococcus*, *Serratia*, *Citrobacter*), suggests an ancestral role of this antibiotic resistance mechanism. Hernandez et al. (2011b) postulated a regulatory role for the Qnr proteins. Indeed, by interacting with the DNA gyrase, Qnr may protect the DNA gyrase against toxic DNA substances and indirectly modulate gene expression in response to environmental changes. Moreover, a beneficial role of these protecting mechanisms has been shown for *qnr*A3, which confers a fitness advantage to the bacteria, favoring its dissemination. The fitness advantage was found abolished when *qnr*A3 was carried by large multi-drug resistance plasmids (Michon et al., 2011). The activation of *qnr*B expression by the SOS-response system could also have an implication in the conservation of such mechanism. As ciprofloxacin induces the SOS-response system, it activates its corresponding resistance mechanisms (Da Re et al., 2009). Several studies have reported *qnr* genes in heterologous species from water habitats. Cattoir et al. (2008) recovered from the Seine River *A. punctata* and *A. media* harboring *qnr*S2. Similarly, Picao et al. (2008) detected *qnr*S genes in *Aeromonas allosaccarophila* from the Lugano Lake, in Switzerland. A *qnr*VC4 allele was isolated from aquatic environments in *A. punctata* by Xia et al. (2010). All these reports demonstrate that the *Aeromonas* genus represents a reservoir for fluoroquinolones resistance mediated by Qnr. Our own studies characterized a *qnr*S determinant in *E. coli* belonging to ST131 isolated from freshwater of a Ukrainian River (Lupo et al., submitted). Similarly, Dhanji et al. (2011) isolated *E. coli* strains belonging to ST131 harboring a *qnr*S allele from the Thames River (**Table 1**). These findings reflect a spread of these resistance mechanisms by geographical and clonal means and highlight the potential of rivers in the dissemination of international resistant clones.

ACQUIRED EFFLUX MECHANISMS

Another acquired fluoroquinolones resistance mechanisms is represented by efflux mechanisms. The *qep*A gene, initially characterized on a conjugative plasmid from a clinical isolate of *E. coli* (Perichon et al., 2007), encodes a MFS efflux pump. It has been recently recovered from the metagenome of river sediments impacted by improperly managed urban waste waters (Cummings et al., 2011). Environmental reports of this gene are rare; however, Deng et al. (2011) have highlighted the possible spread of this gene by animal and human related bacterial strains in water compartments. Similarly, the detection of the OqxAB efflux pump, conferring resistance to fluoroquinolones, olaquindox, and chloramphenicol, remains rare in environmental samples. The *oqx*AB, found on a conjugative plasmid in *E. coli* strains, represents the only example of transferable RND efflux pumps, so far (Hansen et al., 2004). Recently, Zhao et al. (2010) have reported an *E. coli* strain, isolated from a water pond in a farm environment, harboring the *oqx*AB gene (**Table 1**).

Resistance by acquired efflux mechanisms to other drug classes than fluoroquinolones has been extensively reported in the literature (Poole, 2004; Piddock, 2006; Nikaido and Pages, 2011). Studies conducted in water habitats such as aquaculture, impacted by anthropogenic activities, and notably by the application of antibiotics, demonstrated the risk of selection of acquired efflux pumps. Fernandez-Alarcon et al. (2010) reported the presence of different Gram-negative species from aquacultures in Chile expressing the *flo*R gene, which codes for a chloramphenicol and florfenicol

exporter, drugs intensively used in veterinary medicine. Alarmingly, those strains resistant to florfenicol also demonstrated a multi-drug resistance, suggesting a process of co-selection. Gordon et al. (2008) characterized the *flo*R gene, in *Aeromonas bestiarum* strains from freshwater streams in France, located on a 25-kb-plasmid harboring also the tetracycline efflux gene *tet*(Y), *str*B-*str*A, conferring resistance to streptomycin, and *sul*2 conferring resistance to sulfonamides (**Table 1**). Interestingly, this plasmid contained sequences with high nucleotide homologies to other genetic elements recognized in different aquatic bacterial species such as *V. cholerae* and *Photobacterium damselae*. This demonstrated the contribution of horizontal gene transfer in the spread of these resistances in aquatic habitats. Furthermore, genes encoding tetracycline efflux mechanisms have been found to circulate between farm environments and ground water (Aminov et al., 2002). Propagation of tetracycline resistance genes, by efflux or by ribosomal protection, has been linked to the extensive usage of this drug class in animal feeding, and although the usage of this antibiotic has been restricted, tetracycline resistance genes seem to persist in the food chain and in the environment.

THE ENZYMATIC β-LACTAMS RESISTANCE

Resistance to β-lactams has spread worldwide. The low toxicity of these molecules and the broad spectrum of action of some of them make β-lactams the most prescribed antibiotic drug class and propagation of resistance constitutes therefore a major clinical concern. Studies have highlighted that the rise of the bacterial resistance against β-lactams is related to the usage of the drug in clinics, both because of selection of resistant bacteria and by promoting the mobilization of the genes responsible for such resistances (Bush and Fisher, 2011). Similarly, the presence of antibiotics in water environments could promote the selection of antibiotic resistant strains. Detecting and measuring the concentration of antibiotics or intermediary products from their metabolization and degradation in water medium is difficult, mainly because of the lack of standardized methods (Pérez-Parada et al., 2011). However, different studies described analytical methods to investigate pollution of freshwater by antibiotic compounds (Bailon-Perez et al., 2009; Ibanez et al., 2009) and antibiotics, including β-lactams, have been found to contaminate significantly several rivers (Pei et al., 2006; Jiang et al., 2011; Yang et al., 2011). A recent report from Pérez-Parada et al. (2011) has demonstrated the presence of compounds derived from amoxicillin in river effluent water. Although a selection due to these compounds has not been demonstrated, a corresponding risk cannot be excluded.

The most prevalent mechanism of β-lactams resistance in Gram-negative bacteria has been, for a long time, the enzymatic inactivation mediated by penicillinases such as TEM, SHV, and the extended spectrum β-lactamases (ESBLs) derived from these families (Coque et al., 2008). In the last decade, bla_{TEM} and bla_{SHV} genes have become less frequently detected in clinics and have been replaced by the more recently described $bla_{\text{CTX-M}}$ (Bonnet, 2004). CTX-M enzymes represent a special concern in clinics due to the extended spectrum of action and to its global, successful spread that has occurred in bacteria responsible for nosocomial and community acquired infections (Pitout et al., 2005). In 1963, bla_{TEM} has been reported for the first time, located on a plasmid. All the currently known bla_{TEM} genes have been documented to derive from the first characterized allele (Barlow and Hall, 2002). However, the origin of this mechanism has not been elucidated until now. The *K. pneumoniae* chromosome is thought to be the origin of bla_{SHV}, even if the physiological role of this mechanism remains unknown (Haeggman et al., 2004). CTX-M enzymes have been extensively investigated in clinics and more recently reported from environmental samples. Presence of $bla_{\text{CTX-M}}$ in bacteria from freshwater (Dhanji et al., 2011; Lupo et al., submitted), water sediment (Lu et al., 2010), or water-associated birds (Randall et al., 2011) constitutes further reservoirs and shuttles for these resistance determinants (**Table 1**). Based on aminoacidic homology, the $bla_{\text{CTX-M}}$ genes are sorted in four groups: $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-9}}$ (Pitout et al., 2005). The progenitor of each gene group has been found located on the chromosome of *Kluyvera* spp., of the Enterobacteriaceae family. Mobilization events from the ancestor genes have given rise to the clinically relevant mechanisms. In detail, $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$ derived from *Kluyvera ascorbata* (Humeniuk et al., 2002; Rodriguez et al., 2004), $bla_{\text{CTX-M-8}}$ and $bla_{\text{CTX-M-9}}$ from *Kluyvera georgiana* (Poirel et al., 2002; Canton and Coque, 2006). The *Kluyvera* genus seems to be a sink of $bla_{\text{CTX-M}}$. Indeed, *Kluyvera cryocrescens* harbors a chromosomal β-lactamase, KLUC-1, which shares ca. 85% identity with CTX-M-1 (Bonnet, 2004). To the best of our knowledge, KLUC-1 has not been encountered in clinical isolates, but this species represents a reservoir of a new potential clinical ESBL. Although *Kluyvera* spp. are considered environmental bacteria and have been found also in water, elucidating the natural habitat of this species may help to evaluate the risk of the propagation of their β-lactamases. The CTX-M enzymes have been extensively investigated because of the clinical consequences that their spread has caused. However, many class A β-lactamases are chromosomally located in several members of Enterobacteriaceae and could constitute, if integrated on mobile elements, future mechanisms emerging in clinics. Bellais et al. (2001) discovered a chromosomal β-lactamase in *Rahnella aquatilis* (RAHN-1), which had similarities to $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$; Arakawa et al. (1989) characterized KOXY from *Klebsiella oxytoca*; Perilli et al. (1991) MAL-1 in *Citrobacter diversus*; Peduzzi et al. (1994) CUM-A in *P. vulgaris*; Liassine et al. (2002) HUG-A from *Proteus penneri*; Peduzzi et al. (1997) SFO-1 from *Serratia fonticola*; Seoane and Garcia Lobo (1991) YENT from *Yersinia enterocolitica*; Vimont et al. (2002) ERP-1 from *Erwinia persicina*; Walckenaer et al. (2004) PLA-1 and ORN-1A from *Raoultella planticola* and *Raoultella ornithinolytica*, respectively. The above mentioned list provides only some examples: Bush and Fisher (2011) have reviewed that almost 600 class A β-lactamases naturally occur and have been reported in 2011. Worryingly, mechanisms exhibiting a spectrum of activity extended to carbapenems are emerging in clinics (Rossolini, 2005; Queenan and Bush, 2007). VIM, IMP, KPC, some OXA, and the newly described NDM-1 represent examples of these enzymes. The emergence of KPC (*K. pneumoniae* carbapenemase) was described in 2001 (Yigit et al., 2001) and this enzyme has been found to spread worldwide and among several bacterial species such as Enterobacteriaceae, *P. aeruginosa*, and *A. baumannii* (Bush and Fisher, 2011). The crucial molecular vector of its spread has been recognized by Naas et al. (2008), who characterized the location of bla_{KPC} gene on

a Tn-*3-like* transposon, the Tn*4401*, probably responsible for the original mobilization of this gene. The transposon contains several sequences encoding transposases or insertion sequences derived from environmental bacterial species, but the ancestral host of this enzyme has not been identified, so far. Recently Chagas et al. (2011) have detected *K. pneumoniae* producing KPC in an effluent receiving hospital waste water, highlighting an environmental vector for the dissemination of these enzymes (**Table 1**). VIM enzymes have been rarely reported from environmental isolates. Scotta et al. (2011) isolated *Brevundimonas diminuta*, *Rhizobium radiobacter*, *Pseudomonas monteilii*, *P. aeruginosa*, *O. anthropi*, and *Enterobacter ludwigii* strains producing VIM enzymes, again from an effluent receiving the waste water of a hospital. Previously, Quinteira et al. (2005) isolated a strain of *Pseudomonas pseudoalcaligenes* harboring bla_{VIM} from a hospital wastewater effluent (**Table 1**). Probably, the presence of VIM producer species in the environment is due to nosocomial selective conditions and contamination by wastewater from hospitals. However, the detection of bla_{VIM} in different environmental species from freshwater highlights the potential of water as a reservoir for these genes and as a vector facilitating their spread. Concerning IMP enzymes, so far, a unique report has been provided by Pellegrini et al. (2009), in a strain of *P. fluorescens* recovered from waste water (**Table 1**). A carbapenemase activity is also exhibited by several class D β-lactamases, among which the families of OXA-23, OXA-40, OXA-58, and OXA-51 are associated to *A. baumannii* (Poirel et al., 2010). This opportunistic pathogen, provided with an intrinsic but silent $bla_{\text{OXA51-like}}$ gene, is widely distributed in nature. The origin of $bla_{\text{OXA-40}}$ and $bla_{\text{OXA-58-like}}$ genes remains unknown but Poirel et al. (2008) have characterized a $bla_{\text{OXA-23-like}}$ chromosomally located in *Acinetobacter radioresistens*, suggesting that this species is the progenitor for OXA-23. Moreover, *A. baumannii* isolates carrying $bla_{\text{OXA-23}}$ have been detected in river (Girlich et al., 2010) and wastewater from hospitals (Ferreira et al., 2011, **Table 1**). OXA-48 represents another class D carbapenemase that dramatically spreads among Enterobacteriaceae. This latter enzyme is supposed to originate from the chromosome of the water borne species *S. oneidensis* (Poirel et al., 2004). Recently, *S. marcescens* strains harboring $bla_{\text{OXA-48}}$ have been isolated from a river in Morocco (Potron et al., 2011), demonstrating the risks for their dissemination in water habitats (**Table 1**). The recent emergence and dramatic spread of NDM-1 enzyme in clinical isolates of *A. baumannii* and Enterobacteriaceae, has focused major attentions. Usually, strains harboring this broad spectrum carbapenemase gene demonstrate a multi-drug resistant phenotype and a wide set of virulence genes (Walsh et al., 2011). The carriage of bacteria harboring $bla_{\text{NDM-1}}$ by healthy individuals has lead researchers to investigate the source of this gene. Walsh et al. (2011) recently demonstrated the presence of different bacterial species (*P. aeruginosa*, *Achromobacter* spp., and *Kingella denitrificans*) harboring $bla_{\text{NDM-1}}$ in tap water used as drinking water in India (**Table 1**). This finding is closing the transmission circle and explains the fast and successful dissemination of this gene. Several genes encoding carbapenemase enzymes have been found chromosomally located in bacterial species of environmental origin and water related, for instance the *sme* gene on the chromosome of *S. marcescens*, and the *sfc* gene on the *S. fonticola* chromosome (Naas and Nordmann, 1994; Henriques et al., 2004). The water borne *S. maltophilia* also harbors a gene coding for the L1 carbapenemase. Avison et al. (2001) have elucidated that this gene is located on a plasmid-like element considered intrinsic to *S. maltophilia*.

Class C β-lactamases located on plasmids (CMY, MIR, DHA, and ACT) have been found worldwide from several sources (Jacoby, 2009). Water borne bacteria, such as *A. hydrophila*, *M. morganii*, *H. alvei*, and shuttle species between water and gut such as *Citrobacter freundii*, *Enterobacter asburiae*, have been proposed to be the progenitors of the most commonly encountered plasmidic *amp*C genes detected in clinical isolates. These genes have been reported from Canadian and Korean water bodies (Kim et al., 2008; Mataseje et al., 2009). Schwartz et al. (2003) detected *amp*C in waste, surface, and drinking water biofilms (**Table 1**). The presence of antibiotic resistance genes in biofilm matrices, especially in those located in drinking water supplies is of particular concern. Indeed, such biofilm matrices can be a long lasting source of antibiotic resistance genes that can directly spread via the food chain.

CONCLUSION

The emergence of antibiotic resistance is the consequence of a complex interaction of factors involved in the evolution and spread of resistance mechanisms. The over-usage of antibiotics in clinics has been believed to be the principal element involved in the rise of new resistances. Recently, many more evidences suggest that environmental habitats especially water bodies such as rivers and streams are ideal vectors for the antibiotic resistance dissemination. Here, the propagation of bacteria harboring antibiotic resistance genes can occur spatially along the river. Furthermore, the dispersion of these bacteria in the environment favors the interaction with the autochthonous microbiota, creating new scenarios for the evolution of antibiotic resistances.

In strong contrast to clinics, there are no data available on the epidemiology of antibiotic resistances in the environment, especially for geographically based data. This in turn makes extremely difficult to make any predictions on the risk of spread and emergence of new antibiotic resistances. For this reason we assume that a better knowledge on the environmental reservoir of resistances is fundamental to predict the emergences of new resistances of clinical concern.

It should be noticed that the pollution of water can select antibiotic resistant bacteria. This process, involving notably co-selection events, could cause an ecological imbalance leading to the dominance of resistant bacteria and global disturbance of the ecosystems. This latter point highlights the necessity of collecting information and data on the status of the sampling sites when performing a study on antibiotic resistance in freshwater. The development of antibiotic resistances occurs very likely naturally in the environment, but factors like pollution, especially of water bodies, could force the speed of its evolution. A good status of water quality could limit this phenomenon. Despite this, the status of the surface water quality in many countries is still poor. We argue for the implementation of feasible methodologies to characterize quality parameters and detect antibiotic resistance in water bodies, and thus to establish adapted and pragmatic measures to improve water resources. Amelioration of water status is

of major concern: it can contribute to a direct and local decreased risk for the health of populations living in the vicinity of the freshwater, and lead to more global effects by avoiding that water bodies could constitute reactors for antibiotic resistance emergence and evolution.

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