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Aminov, Rustam; Mackie, Roderick I.

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MINIREVIEW

Evolution and ecology of antibiotic resistance genes

Rustam I. Aminov[1] & Roderick I. Mackie[2]

[1]Rowett Research Institute, Greenburn Road, Aberdeen, UK; and [2]Department of Animal Sciences, Institute for Genomic Biology, University of Illinois, Urbana, IL, USA

Correspondence: Rustam I. Aminov, Rowett Research Institute, Greenburn Road, Aberdeen AB21 9SB, UK. Tel.: +44 1224 716643; fax: +44 1224 716687; e-mail: r.aminov@rowett.ac.uk

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Abstract

A new perspective on the topic of antibiotic resistance is beginning to emerge based on a broader evolutionary and ecological understanding rather than from the traditional boundaries of clinical research of antibiotic-resistant bacterial pathogens. Phylogenetic insights into the evolution and diversity of several antibiotic resistance genes suggest that at least some of these genes have a long evolutionary history of diversification that began well before the 'antibiotic era'. Besides, there is no indication that lateral gene transfer from antibiotic-producing bacteria has played any significant role in shaping the pool of antibiotic resistance genes in clinically relevant and commensal bacteria. Most likely, the primary antibiotic resistance gene pool originated and diversified within the environmental bacterial communities, from which the genes were mobilized and penetrated into taxonomically and ecologically distant bacterial populations, including pathogens. Dissemination and penetration of antibiotic resistance genes from antibiotic producers were less significant and essentially limited to other high G+C bacteria. Besides direct selection by antibiotics, there is a number of other factors that may contribute to dissemination and maintenance of antibiotic resistance genes in bacterial populations.

Introduction

The issue of antibiotic resistance has received considerable attention due to the problem of the emergence and rapid expansion of antibiotic-resistant pathogenic bacteria. The common perception, however, is that the problem is seen as exclusively associated with the use and overuse/misuse of antibiotics in humans and animals. This is true for the clonal dissemination of pathogenic bacteria with resistance mechanisms based on altering target molecules due to mutational events and strong positive selection of mutants. However, the majority of antibiotic resistances are most likely cases of acquired resistance, through the lateral transfer of antibiotic resistance genes from other ecologically and taxonomically distant bacteria. This raises several questions about the evolution and ecology of antibiotic resistance genes. First, what is the original reservoir of antibiotic resistance genes from which they are continuously mobilized? Are these the antibiotic-producing bacteria or their DNA-contaminating antibiotic preparations? Alternatively, are these soil bacteria that were not producing antibiotics but because of exposure to the antibiotics in soil they developed a diversified pool of antibiotic resistance genes? Can we reconstruct the chain of events that eventually led to the acquisition of antibiotic resistance genes by pathogenic bacteria with the help of phylogenetic analysis? In the final part, the factors that may play a role in dissemination and maintenance of antibiotic resistance genes in bacterial populations in the absence of apparent selection by antibiotics are reviewed.

DNA in antibiotics as the source of antibiotic resistance genes

It was reported more than three decades ago that aminoglycoside-inactivating enzymes in actinomycetes are similar to those present in clinical isolates of antibiotic-resistant bacteria (Benveniste & Davies, 1973). Subsequently, it was reported that many antibiotic preparations contained an amount of DNA visible on agarose gels and that this DNA could be transformed into enterobacteria and vibrios resulting in antibiotic-resistant clones (Chakrabarty *et al.*, 1990). Later, Webb & Davies (1993) confirmed the presence antibiotic resistance gene sequences in a number of antibiotic

preparations employed for human and animal use. They hypothesized that the presence of DNA encoding drug resistance in antibiotic preparations has been a factor in the rapid development of multiple antibiotic resistance due to the uptake of this DNA by bacteria. Hybridization data have also indicated that some mycobacteria may actually carry the resistance genes originally described in streptomycetes (Pang *et al.*, 1994). Recently, evaluation of the residual DNA with a more sensitive fluorescence detection technique demonstrated once more that many antibiotic preparations, of both research and clinical grade, contain detectable amounts of DNA (Woegerbauer *et al.*, 2005). Moreover, in most cases the DNA is specific for the antibiotic-producing strain and contains the corresponding resistance genes. Attempts to demonstrate *in vivo* transformation by these antibiotic preparations, however, were unsuccessful (Woegerbauer *et al.*, 2005), suggesting that the residual DNA in antibiotics is unlikely to be a major force in the lateral transfer of antibiotic resistance genes in clinical or community settings.

Phylogeny of antibiotic resistance genes

The past horizontal gene transfer events between taxonomically distant bacteria are probably difficult to reproduce experimentally because it is not known which bacteria and what kind of genetic mechanisms were involved, what were the environmental conditions at the time of transfer, and simply because the transfer frequencies may be too low to reproduce in a laboratory scale. Fortunately, it is possible to conduct a retrospective analysis using phylogenetic tools in order to test the hypothesis of the past lateral gene transfer. The phylogenetic approach was applied to analyze the evolutionary history of antibiotic resistance genes, encoding ribosomal protection proteins (RPP) that function as alternative elongation factors and confer resistance to tetracyclines (Connell *et al.*, 2002, 2003). Phylogenetic analysis strongly supported a scenario of early branching and long independent diversification of eight (at that time) clusters of RPPs well before the modern 'antibiotic era' (Aminov *et al.*, 2001). No evidence for transfer of antibiotic resistance genes from the antibiotic producing strains to pathogenic or commensal bacteria was found. Recently, this analysis was repeated with a more comprehensive dataset and the results are presented in Fig. 1. This analysis confirmed, once more, the monophyletic origin of the *tet* genes, with an early branching event separating them from the other group of elongation factors, EF-G, encoded by the *fusA* genes (Fig. 1). The rate of nucleotide substitutions in the *tet* cluster is *c.* two times higher than it is in the *fusA* cluster. In addition, the next branching event, also supported by a 100% confidence value, separated the genes from the streptomycetes and *Agrobacterium tumefaciens* (cluster 2) from the rest of commensal and pathogenic bacteria (cluster 1).

The sequence of the antibiotic producer, *Streptomyces rimosus*, in the second cluster is not only very distant from the sequences in cluster 1 but also distant from the sequences in cluster 2, which includes taxonomically related streptomycetes. Extremely long-branch lengths in this cluster possibly warrant a separate designation for the sequences. The sequence of *A. tumefaciens*, the soil/plant pathogen bacterium, at though is clustered with the sequences of streptomycetes it is very divergent and does not reflect a horizontal gene transfer event. Although not confirmed by gene cloning and sequencing, there are indications that some *tet* genes from streptomycetes, in particular, those encoding RPPs, can be detected in mycobacteria (Pang *et al.*, 1994). In general, the genes in this cluster demonstrate much lower diversity than in cluster 1 and distribution of the genes is limited to high G+C bacteria, possibly reflecting the codon usage preferences.

The use of a metagenomic approach allowed the recovery of an additional diversity of the *tet* genes from uncultivated microbiota and some of the sequences in Fig. 1 that are interspersed with the genes from commensal and pathogenic bacteria are of environmental origin. The suggestion that these sequences may actually belong to as-yet unidentified antibiotic producers in the environment is not well supported since at least some sequences may be environmental contaminants of animal origin (pig manure applied to soil, see GenBank accession numbers AY265739–AY265741) and G+C content of these genes (36.7–47.7%) is much lower than that of antibiotic-producing streptomycetes (70–72%). Alternatively, especially in the case of AY265740 and AY265741 sequences from the soil metagenome (Fig. 2), these genes may represent the missing link of the *tet*(W) ancestors in soil microbiota, from which the gene was possibly acquired by commensal and pathogenic bacteria.

Is it possible that this scenario is an exception and peculiar to this particular RPP gene family, while the others may demonstrate the phylogenetic traces of migration from antibiotic-producing bacteria to the human and animal commensal and pathogenic bacteria? To answer this question, phylogenetic analyses were performed with several other families of antibiotic resistance genes. Because of space constraints, only the results of three additional analyses, of the *erm* and *qnr* genes and of the *vanHAX* cluster, are presented in this review (Figs 2–4).

The *erm* gene family encodes enzymes that catalyze *S*-adenosyl-L-methionine-dependent methylation of the specific adenine residue in the 23S rRNA gene molecule and this methylation sterically protects ribosomes from binding clinically important macrolide, lincosamide and streptogramin B antibiotics, thus ameliorating inhibition of

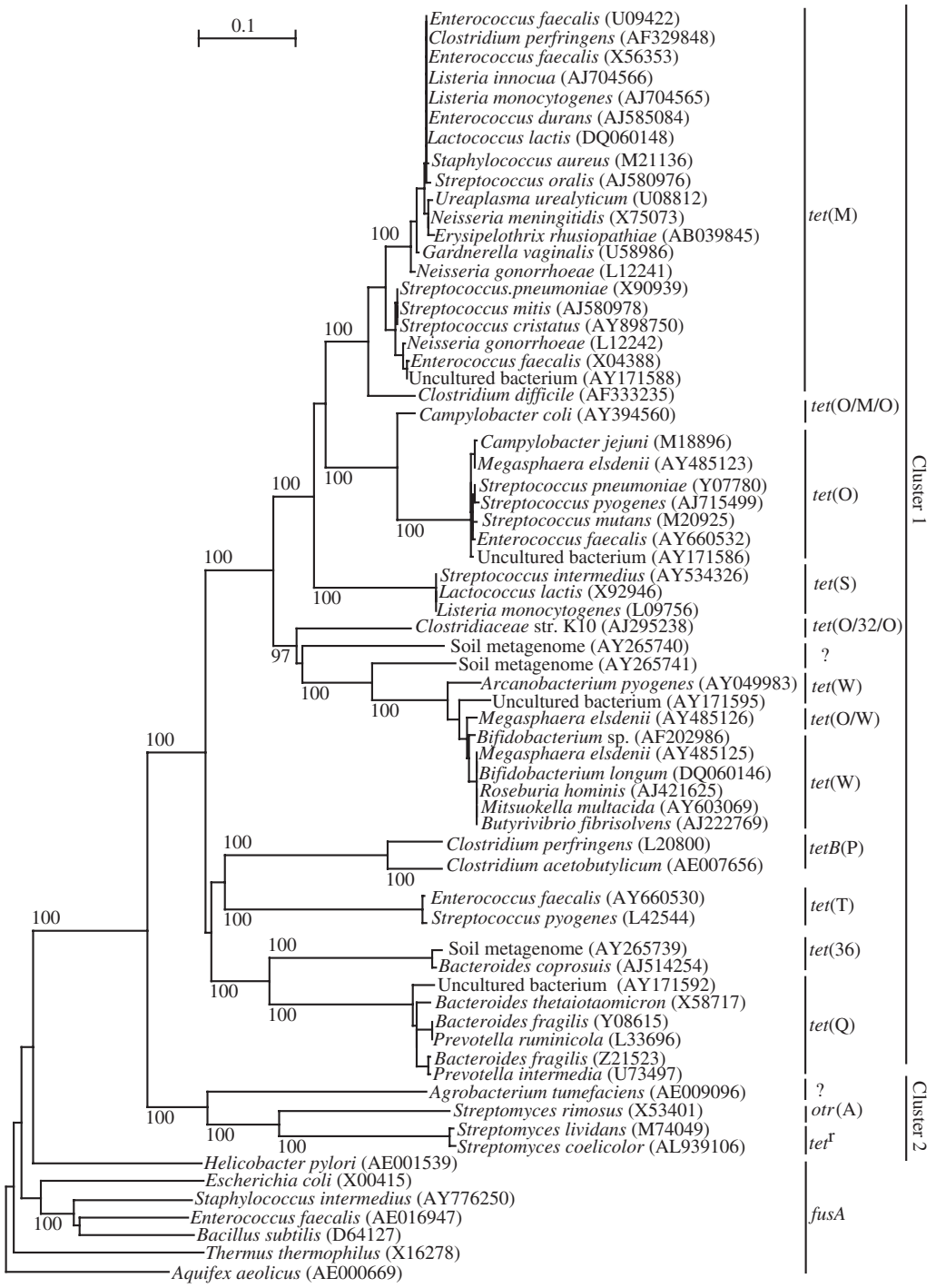

Fig. 1. Phylogenetic analysis of tetracycline resistance genes encoding ribosomal protection proteins performed with neighbor-joining method. The sequence of the *Aquifex aeolicus fusA* gene for translation elongation factor EF-G is used as the outgroup for rooting the tree. Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parenthesis. For the nomenclature of tetracycline resistance genes see http://faculty.washington.edu/marilynr/.

protein biosynthesis. Once again, there is no indication of gene exchange between the commensal and pathogenic bacteria (cluster 1 in Fig. 2) and the group clustered with antibiotic producers (cluster 2 in Fig. 2). Moreover, these two clusters evolved independently and it is not possible to reconstruct the evolutionary scenario of the *erm* gene

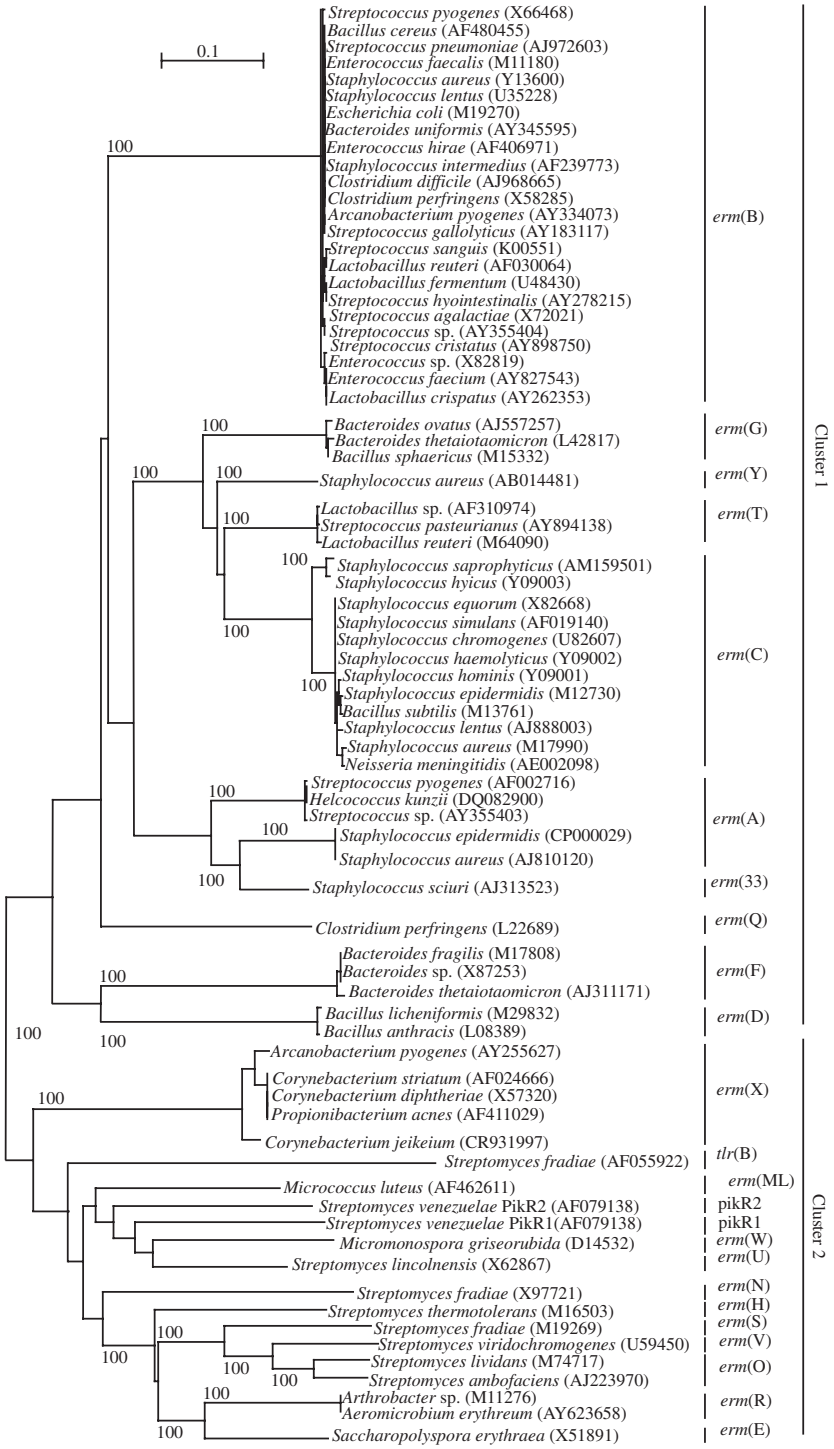

Fig. 2. Unrooted phylogenetic tree of the macrolide resistance genes encoding methylases constructed using the neighbor-joining method. Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parenthesis. Although the sequences AJ810120 and CP000029 and the sequences AF002716, DQ082900, and AY355403 form separate clades, they are all defined as *erm*(A) on the basis of $> 80\%$ amino-acid sequence similarity (http://faculty.washington.edu/marilynr/).

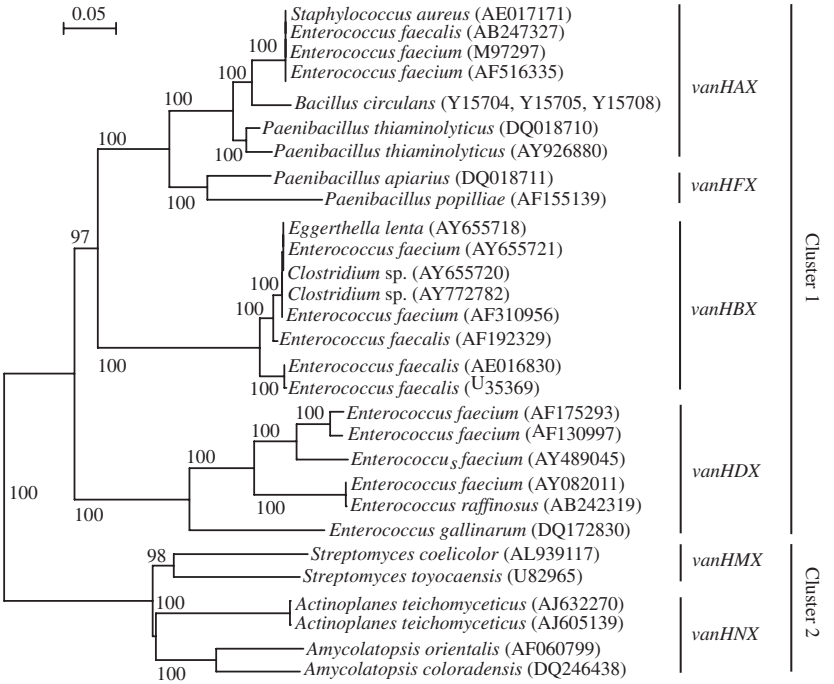

Fig. 3. Unrooted phylogenetic tree of the *vanHAX* cluster of genes encoding D-lactate dehydrogenase, D-Ala-D-Lac ligase and D,D-dipeptidase constructed using the neighbor-joining method. Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parentheses.

duplication and diversification as in the case of the *tet* genes discussed above. Antibiotic producers in cluster 2 are exemplified by sequences from *Streptomyces fradiae*, *Streptomyces venezualae*, *Micromonospora griseorubida*, *Streptomyces lincolnensis*, *Streptomyces ambofaciens*, *Streptomyces thermotolerans*, *Aeromicrobium erythreum*, and *Saccharopolyspora erythrea* and none of these sequences are clustered with the human and animal-associated bacteria to suggest the horizontal gene transfer event. The cluster, however, does contain a group of bacteria of medical and veterinary importance, which carry the *erm*(X) gene but, surprisingly, this subcluster has branched very early in the evolution of cluster 2 genes and, therefore, is more ancient than the resistance genes evolved in antibiotic producers (Fig. 2).

Until the late 1980s, the glycopeptide antibiotic vancomycin was considered as the drug of last resort for treatment of diseases caused by gram-positive bacteria such as enterococci, MRSA, *Streptococcus pneumoniae*, and *Clostridium difficile* (Cunha, 1995). The emergence and extremely rapid dissemination of VRE and a recent arrival of a high-level vancomycin-resistant *Staphylococcus aureus* (Weigel *et al.*, 2003) make the phylogenetic reconstruction of this scenario a particularly valuable example of antibiotic resistance gene evolution. The mechanism of high-level vancomycin resistance, widespread in enterococci and now acquired by *S. aureus*, is target modification, which includes the alternative peptidoglycan synthesis pathway terminating in D-alanyl-D-lactate instead of D-alanyl-D-alanine (Walsh *et al.*, 1996). In the core of this resistance mechanism is the combined action of three enzymes, VanH, VanA, and VanX, the first of which supply the substrate by converting pyruvate into D-lactate (D-lactate dehydrogenase), the second makes the D-Ala-D-Lac ester (D-Ala-D-Lac ligase), and the third enzyme hydrolyzes the D-Ala-D-Ala produced by the endogenous cell wall synthesis machinery (D,D-dipeptidase). The gene order in the *vanHAX* cluster is well conserved and the second similar variant, which is widespread in enterococci and other intestinal bacteria, includes *vanB* instead of *vanA* and therefore can be called the *vanHBX* cluster. Interestingly, the same basic cluster structure is preserved in glycopeptide-producing organisms as well (Marshall *et al.*, 1998). A number of other vancomycin resistance operons was analyzed and if was found that this cluster may also harbor the *vanF* and *vanD* genes instead of *vanA*, with the same gene order and, accordingly, these *van* clusters can be called as *vanHFX* and *vanHDX*. Initially, separate phylogenetic analyses were performed for *vanH*, *vanA* (including other ligase homologs), and *vanX* and all the three genes produced trees with very similar topology and bootstrap support, suggesting that the cluster behaved

as a single evolutionary unit. The only exception was a less stable location of sequences residing in the *Paenibacillus* species, possibly reflecting recombination events (data not shown). Thus, for the phylogenetic analysis, the data set of concatenated ORFs comprising *vanH, vanA* and its homologs, and *vanX* was used to increase the sensitivity and reliability of analysis. With a 100% bootstrap support, the resulting tree displayed a very early branching event, leading to the formation of two large clusters, one consisting of VRE and other vancomycin-resistant intestinal and soil bacteria (cluster 1) and the second consisting the glycopeptide-producing bacteria and streptomycetes (cluster 2) (Fig. 3). No evidence of exchange of vancomycin resistance genes between the two clusters can be detected. The second important conclusion from this analysis is that the scenario of evolution and dissemination of the *vanHAX* gene cluster (Fig. 3), which was eventually acquired by *S. aureus* (Weigel *et al.*, 2003) can be actually reconstructed. Most likely, the ancestral gene cluster has evolved in soil bacteria such as *Paenibacillus*, where it diversified into several lineages, with the presently known representatives *vanHFX* and *vanHAX* (Fig. 3). Subsequently, the latter cluster was acquired by *Bacillus circulans*, the bacterium that is ubiquitous in various environments, including soil and clinical settings, thus serving as a shuttle between the ecosystems. Interestingly, the *van* operon in a clinical isolate of *B. circulans* is chromosomally encoded and it is not the part of a *Tn*1546-like structure (Ligozzi *et al.*, 1998). Further acquisition of the *vanA* operon by enterococci was probably through its integration into *Tn*1546, which allowed extremely rapid dissemination, including *S. aureus*. This scenario, with the involvement of low G+C gram-positive soil bacteria but not antibiotic-producers in the acquisition of the *vanHAX* cluster by enterococci, is also supported by heterologous expression studies, showing that the gene cluster from *Paenibacillus* species but not from *Amycolatopsis coloradensis* is expressed in eneterococci conferring high-level vancomycin resistance (Hasman *et al.*, 2006). The *vanA* and related genes in soil are widespread and diverse (Guardabassi & Agerso, 2006), thus serving as the glycopeptide resistance gene pool, readily available for mobilization by bacteria in other ecosystems, in particular of the gastrointestinal tract. The other two branches of the tree, with *vanHBX* and *vanHDX*, do not have the precursor genes residing in soil bacteria; however, this may reflect a limited diversity of the genes characterized to date. In fact, the soil 'antibiotic resistome' harbors a great diversity of different antibiotic resistance genes and systematic study of this gene pool is essential for prediction of possible emergence of clinically important resistant bacteria (D'Costa *et al.*, 2006).

Probably the best-documented support of the view of the ancient evolution and diversification of antibiotic resistance genes comes from the phylogenetic analysis of β-lactamases. β-lactams are the most widely used antibiotics and resistance to β-lactams is a severe threat because they have low toxicity and are used to treat a broad range of infections (Livermore, 1996). The primary resistance mechanism is enzymatic inactivation through the cleavage of the β-lactam ring by β-lactamases. These enzymes are represented by two unrelated groups, the serine β-lactamases, with an active-site serine, and metallo-β-lactamases, which require a bivalent metal ion to catalyze the hydrolysis (Bush, 1998). Both groups are very ancient and the classes within the groups are diversified to the extent that all traces of homology between the classes at the sequence level are lost (Hall & Barlow, 2004; Garau *et al.*, 2005). Structure-based phylogeny, however, was capable of reconstructing the evolution of both β-lactamase groups and establish that these ancient enzymes originated more than two billion years ago, with some serine β-lactamases being on plasmids for millions of years, well before the modern use of antibiotics (Hall & Barlow, 2004; Garau *et al.*, 2005). A recent work on evolutionary history of β-lactamase genes in *Klebsiella oxytoca* has suggested that these genes were evolving over 100 million years in this host, without concomitant evolution of the antimicrobial resistance phenotype, and the phylogenies of β-lactamase and house-keeping genes are highly congruent in this organism (Fevre *et al.*, 2005). Limited phylogenetic analysis of class A β-lactamases, with the inclusion of sequences from antibiotic producers such as *Amycolatopsis lactamdurans* and streptomycetes as well as from the environmental bacteria, essentially confirmed these findings and also indicated very limited penetration of the β-lactamase genes from the former group into pathogenic and commensal bacteria (data not shown). In an environmental study, molecular analysis of β-lactamases in a metagenomic library from the cold-seep sediments also demonstrated that most of the diversity of these enzymes is not the result of recent evolution but that of ancient evolution (Song *et al.*, 2005). Given the ancient origin and wide distribution of β-lactamase genes, it is not surprising that the resistance genes are rapidly picked up from the environmental reservoirs and disseminated into pathogenic microbiota following the introduction of novel β-lactam antibiotics into clinical and agricultural use.

Another independent phylogenetic analysis of RPP, *erm* and some other antibiotic resistance gene families also arrived at the same conclusion, i.e. there is no evidence for transfer of antibiotic resistance genes from antibiotic producers to human- and animal-associated bacteria (Lau *et al.*, 2004). However, all these examples, including the authors' and those described in the literature, dealt with the resistance to the naturally occurring or partially modified natural antibacterial compounds. An interesting question to ask would be how bacteria may cope with xenobiotic drugs that are usually not encountered in nature and, therefore, no

natural reservoirs of corresponding resistance genes exist in the environment. In this regard, resistance to quinolones, synthetic compounds, is a good example to follow the history of resistance development. The first quinolone, nalidixic acid, was discovered in 1962 (Lescher *et al.*, 1962) and since then many other derivatives were synthesized and introduced into clinical practice (Ball, 2000). This group is one of the most important antibiotics, widely used for treatment of bacterial infections in both human and veterinary medicine. Antibacterial effect of quinolones is due to formation of DNA gyrase–quinolone–DNA ternary complex that arrests replication fork progression (Hiasa & Shea, 2000). Until recently, the most common mechanisms of quinolone resistance encountered were the two types of chromosomal mutations leading either to target alterations (mutations in DNA gyrase and topoisomerase IV) or to alterations in drug permeability (mutations that increase expression of endogenous multidrug efflux pumps, alter outer membrane diffusion channels, or both) (Hooper, 1999). Thus, the initial resistance to quinolones was characterized by vertical inheritance and clonal dissemination of resistant strains. In 1998, however, the first confirmed case of plasmid-mediated quionolone resistance was published, based on an earlier 1994 isolate of *K. pneumoniae* (Martinez-Martinez *et al.*, 1998). The resistance gene, termed *qnr*, was coding for a 218-aa protein belonging to the pentapeptide repeat family, with sequence homology with the immunity protein McbG (Tran & Jacoby, 2002). It was shown that Qnr binds to DNA gyrase in the early stages of gyrase–DNA complex formation and, by lowering gyrase binding to DNA, Qnr may reduce the amount of holoenzyme-DNA targets for quinolone inhibition (Tran *et al.*, 2005). The worldwide emergence of the *qnr* genes among pathogens and the presence of homologous genes in environmental bacteria (Robicsek *et al.*, 2006) may represent a valuable example for phylogenetic reconstruction of how the presently resistant bacteria found the ways to coupe with a xenobiotic that they have never encountered before in natural settings.

The genes encoding the proteins of the pentapeptide repeat family are widespread in nature and are frequently encountered in genomes of environmental bacteria (Fig. 4). Interestingly, in addition to the well-defined *qnr* genes such as *qnrA, qnrB*, and *qnrC*, there are a number of homologous genes in the environmental bacteria (*Photobacterium profundum, Vibrio vulnificus*, and *Vibrio parahaemolyticus*) that confer decreased susceptibility to quinolones once they are cloned in *Escherichia coli* (Poirel *et al.*, 2005b; Saga *et al.*, 2005 Fig. 4). In the *qnrA* gene cluster, the genes in one subcluster are almost identical with genes in the environmental (*Shewanella algae*) and pathogenic (*K. pneumoniae, Salmonella enteriditis*, and *Proteus mirabilis*) bacteria (Poirel *et al.*, 2005a, b), while no confirmed cases of occurrence of *qnrS* in environmental bacteria exist in the literature. However, the sister clade of *qnrS* does include environmental bacteria such as photobacteria and *Psychromonas* sp. (Fig. 4). A 100% identity of sequences within the two *qnrS* subclusters suggests very recent lateral gene transfer events, while the diversification of *qnrA* and especially of *qnrB* was probably going on for some time. The most heterogeneous *qnrB* genes diversified from a common ancestor shared with other species of environmental bacteria, *Stenotrophomonas maltophila* and *Aeromonas hydrophila* (Fig. 4). Interestingly, the large cluster unifying *qnrA, qnrB, qnrS, qnrVV*, and related sequences (cluster 1) is rooted with the *E. coli* sequence (X07875) encoding the immunity protein McbG, which protects DNA replication from the action of microcin B17 (Garrido *et al.*, 1988) (Fig. 4). The target of microcin B17 is also DNA gyrase but the mechanism of inhibitory action is different from that of quinolones (Pierrat & Maxwell, 2005). Another large cluster of genes encoding the proteins of the pentapeptide repeat family (cluster 2, Fig. 4) has evolved independently of cluster 1 and includes a diverse group of genes residing in the chromosomes of environmental bacteria and mycobacteria. Some of them displayed reduced susceptibility to quinolones once cloned in *E. coli* (Montero *et al.*, 2001; Hegde *et al.*, 2005; Saga *et al.*, 2005), suggesting that the genes in this cluster as well may serve as the environmental reservoirs of quinolone resistance genes.

While the functional role of antibiotic resistance genes in the soil antibiotic resistome is evident (protection against the antibiotics synthesized by the producers), their presence and function in bacteria from other ecological niches with no or very limited exposure to the antibiotic-producing soil microbiota is more challenging to explain. The most plausible explanation for this phenomenon is that these genes might have served some other metabolic functions rather than providing antibiotic resistance in the preantibiotic era. The antibiotics target the vital cellular processes such as the integrity of the cell wall and various biosynthetic processes that are vital for the cell and there should be mechanisms supporting and protecting the basic cellular needs. Methylation exerted by Dim1p, the eukaryotic ortholog of bacterial methylases, for example, is essential, probably due to its function as a quality-control mechanism in ribosome synthesis (Lafontaine *et al.*, 1998). Likewise, the bacterial ortholog may have been involved in a similar function in the bacterial cell before assuming the role of protecting ribosomes against the binding of macrolides at the later stage of evolution. The metallo-β-lactamase group of enzymes are highly divergent and, in addition to the hydrolysis of the β-lactam ring, are involved in various basic cellular processes such as hydrolysis, DNA repair and uptake, RNA processing and can be identified in all three domains of life (Garau *et al.*, 2005). The pentapeptide repeat protein family has more than 500

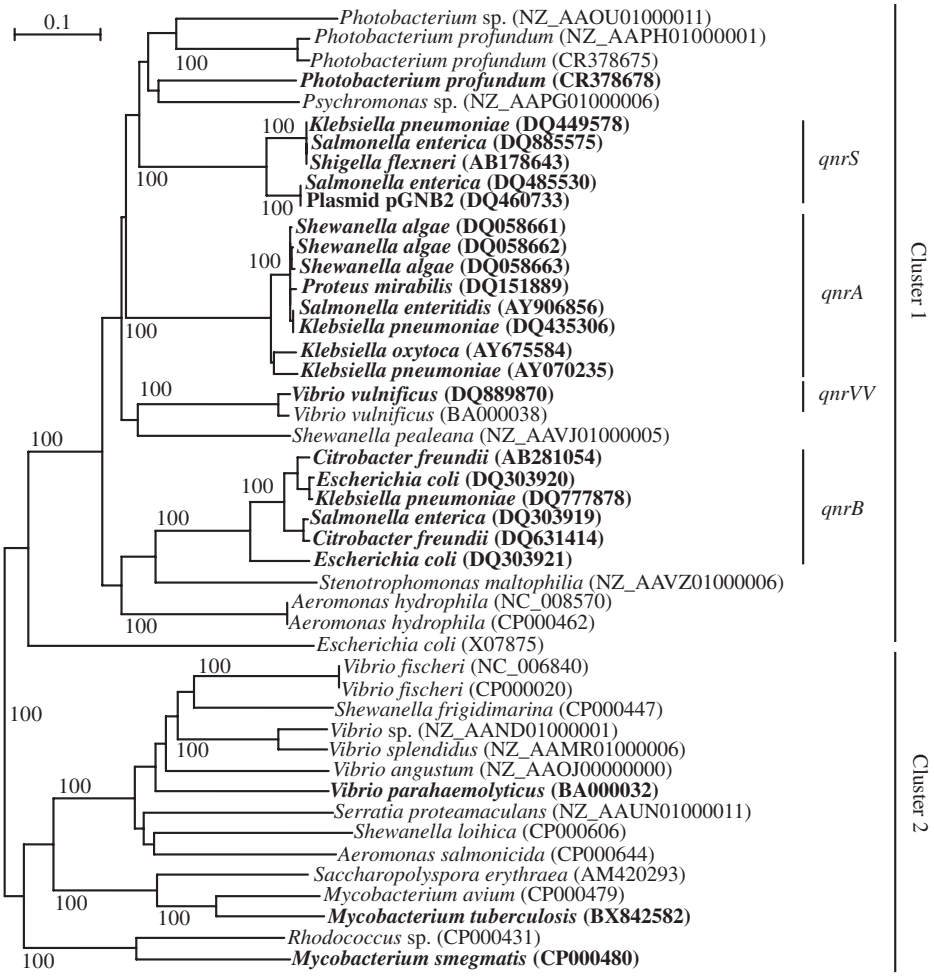

Fig. 4. Unrooted phylogenetic tree of the *qnr* genes encoding plasmid-mediated resistance to quinolones and related sequences from GenBank constructed using the neighbor-joining method. Bootstrap values (%), based on 1000 reiterations, are shown as interior node labels and the scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parentheses. Sequences with 100% nucleotide sequence identity such as ten sequences identical to DQ885575 and AB178643 among the *qnrS* genes, are omitted from the analysis. Cases with confirmed reduced susceptibility to quinolones are shown in bold.

members in all three domains of life (Vetting *et al*., 2006) but very few of them were characterized functionally. The three-dimensional structure of the MfpA protein from *Mycobacterium tuberculosis* is remarkably similar to DNA in terms of size, shape, and electrostatic properties and these properties may explain its inhibitory effect on DNA gyrase and quinolone resistance (Hegde *et al*., 2005). It was suggested that the original function of MfpA was to provide DNA topological assistance when needed, but maintain a condensed chromosome and prevent undesired topological changes during periods of replicative senescence (Hegde *et al*., 2005).

On the other hand, phylogenetic analysis suggests that the rapid movement of antibiotic-resistant genes to taxonomically divergent commensal and pathogenic bacteria is a recent evolutionary event on the phylogenetic scale and it is most probably attributed to the accelerated horizontal transfers within bacteria in the 'antibiotic era'. The *tet*(M) gene, for example, can be found in gram-positive and gram-negative bacteria (Fig. 1) and is known to be transferred by transposon *Tn*916. The *tet*(W) gene is virtually identical in *Megaspaera elsdenii*, *Bifidobacterium longum, Roseburia hominis, Mitsuokella multacida*, and *Butyrivibrio fibrisolvens*, thus suggesting the scenario of the very recent lateral gene exchange events between the human and porcine guts, the rumen, and a probiotic bacterium (Barbosa *et al*., 1999; Kazimierczak *et al*., 2006). The genetic mechanisms responsible for the movement of *tet*(W) between different gut

ecosystems are presently unknown but presumably this is an unidentified mobile element. Interestingly, the vast majority of bacteria carrying the *tet* genes are of oral, intestinal, or genital origin and horizontal gene exchange between these as well as transient bacteria is considerable (Salyers *et al.*, 2004). In the case of the *erm* genes the situation is very similar, in particular, *erm*(B), *erm*(C), and *erm*(G) demonstrated the highest penetrance, being represented in gram-positive and gram-negative bacteria, in commensal and pathogenic bacteria as well as in environmental bacteria (Fig. 2). The retrospective analysis of the *erm* and *tet* genes in *Bacteroides* and gut species suggested that these genes have been disseminated rapidly among the human populations, in hospitals, and in the community, over the past three decades (Shoemaker *et al.*, 2001), during the period of time, which essentially coincides with the modern 'antibiotic era'. Molecular analysis of human, porcine, and poultry *Enterococcus faecium* isolates and their *erm*(B) genes also suggests that horizontal exchange of antibiotic resistance genes is more important in dissemination of antibiotic resistance than the direct transmission of resistant strains (De Leener *et al.*, 2005).

From the clinical point of view, the most important event is the entry of antibiotic resistance genes into pathogenic bacteria. Analysis of the archived clinical isolates for genes encoding tetracycline and macrolide resistance suggests that this may happen within several years from the beginning of the clinical use of antibiotics (Atkinson *et al.*, 1997; Cousin *et al.*, 2003). In the case of vancomycin resistance, the first clinical isolates of VRE were described only in 1988 and extremely rapid dissemination of high-level vancomycin resistance among gram-positive cocci was also likely due to the location of the *van* operons on mobile elements and to the strong selective pressure imposed by the extensive use of glycopeptides in animals and humans (Courvalin, 2006). Similarly, *qnr*-bearing plasmids are integron/transposon associated and carry multiple antimicrobial resistance determinants, thus facilitating the global dissemination of the *qnr* genes (Li, 2005).

Genetic mechanisms involved in acquisition of antibiotic resistance genes from the environmental antibiotic 'resistome' by commensal and pathogenic bacteria may include (1) transformation by naked DNA, which includes the naturally competent state of some bacteria as well as an environmentally induced competence such as the presence of calcium; (2) conjugative transfer (plasmids, transposons, integrons on plasmids or transposons, mobilization of nonconjugative and NBU elements, integrative and conjugative elements, etc.); (3) transduction. These mechanisms are covered in many excellent reviews elsewhere and the topic will be not discussed in this review. But the ecological aspects of this process are still poorly understood. In the next section, the factors contributing to dissemination and maintenance of antibiotic resistance genes in bacterial populations in the absence of direct antibiotic selection will be discussed.

Factors contributing to dissemination and maintenance of antibiotic resistance

The obvious evolutionary factor contributing to the dissemination of antibiotic resistance genes is the ever-increasing production and consumption of antibiotics for various purposes, from treatment of disease to the questionable practice of feeding them to food-production animals at subtherapeutic levels for growth promotion. The pressure imposed by antibiotics obviously selects for antibiotic resistance-harboring bacteria but the problem will not cease to exist when the pressure is removed, a well-known 'easy-to-get, hard-to-lose phenomenon' (Salyers & Amabile-Cuevas, 1997). Although it is generally thought that the carriage of antibiotic resistance imposes additional metabolic cost for the bacterial cell and the antibiotic resistance genes will be eliminated from the population once the selective pressure is removed, there are many examples demonstrating how the remarkable plasticity of bacteria allows them to ameliorate this cost (Lenski, 1997; Enne *et al.*, 2005; Ramadhan & Hegedus, 2005). Moreover, the acquisition of the antibiotic resistance genotype may actually increase the fitness of certain bacteria in the absence of antibiotic selective pressure thus allowing the rapid emergence and dissemination on a worldwide scale (Enne *et al.*, 2004; Luo *et al.*, 2005). In this case, the dissemination of antibiotic resistance becomes a self-perpetuating process, replacing the antibiotic susceptible genotype in the absence of any antibiotic selective pressure. The amelioration of the fitness cost of antibiotic resistance carriage may be one of the reasons why the antibiotic resistance genes are persistent in the apparent absence of selection imposed by the presence of antibiotics, for example, in wild animals (Gilliver *et al.*, 1999). Therefore, the release of antibiotic resistance genes into the environment becomes a controlling point because in the areas with historically low level of antibiotic usage in agriculture the frequency of antibiotic resistance gene carriage is also very low (Osterblad *et al.*, 2001).

Nevertheless, there are many examples of finding antibiotic resistance genes in apparently antibiotic-free environments and, summarizing with the overview in the previous section, several possibilities can be proposed to explain this phenomenon. First, this could be the low-level pre-existing natural gene pool such as the *erm* and *van* genes in soil microbiota (Figs 2 and 3) or the *qnr* genes in aquatic microbiota (Fig. 4). Another explanation that was discussed earlier in this section involves the previous strong antibiotic-driven selection and 'integration' of antibiotic resistance genes into the bacterial genotype, with compensatory mutations reducing the fitness cost. Then this resistant genotype

may enter other ecosystems and successfully compete with antibiotic susceptible genotypes in the absence of selection by antibiotics, e.g. in the antibiotic-free environment. It is possible to distinguish these two outcomes by sequencing antibiotic resistance genes from the environment followed by integrated phylogenetic analysis involving other similar genes in databases. This approach has the ability to reconstruct the chain of evolutionary events as exemplified by the analyses in this work (Figs 1–4) and to establish the origin of genes found *in situ*, thus helping to identify the indigenous and contaminating sources of antibiotic resistance genes (Koike *et al.*, 2007). Third, there could be coselection of antibiotic resistance genes by other factors such as heavy metals in supposedly antibiotic-free environment, the topic that will be discussed later in this section. And, finally, to what extent the antibiotic-free environment is really antibiotic-free? Veterinary antimicrobials can be found even in groundwater as deep as > 10 m, if the area is impacted by agricultural activity (Batt *et al.*, 2006). In surface water such as a river, the only sampling area where antibiotics were not detected was the pristine site in the mountains before the river had encountered urban or agricultural landscapes (Yang & Carlson, 2003). Thus, a carefully designed analysis of a particular ecosystem for antibiotic resistance genes becomes crucial to exclude the possibility of contamination by antibiotics, even in the areas with light agricultural activity or in sparsely populated regions.

Consequences of the prolonged antibiotic use are not only limited to the amelioration of the resistant genotype/phenotype but also could select for a novel gene variant/recombinant that may confer higher MICs. The mosaic recombinant *tet*(O/W/O) variants (Fig. 1) were first isolated from *Megasphaera esldenii* from swine (Stanton & Humphrey, 2003). Then it appeared to be that the previously characterized *tet*(32) gene from the human commensal bacterium *Clostridium* sp. is also a mosaic recombinant, *tet*(O/32/O) (Stanton *et al.*, 2005). Analysis of the published *tet*(O) gene sequence from *Campylobacter coli* (Batchelor *et al.*, 2004) suggests that this is a double-crossover recombinant and must be described as a *tet*(O/M/O) (R.I. Aminov, unpublished data). Characteristically, for the recombinants, this gene forms a separate deep entity in the phylogenetic tree because of its composite structure as well as other recombinants (Fig. 1). The recombinant variants of these genes appeared to have higher MICs for tetracycline and this could be a reason for the selection and persistence of these mosaic recombinants (Stanton *et al.*, 2004). Thus, the commensal bacteria serve not only as reservoirs of antibiotic resistance genes but also provide a dynamic breeding ground for evolution and generation of novel diversity of antibiotic resistance genes.

The use of antibiotics at low subinhibitory concentrations represents another major route for accelerated horizontal transfer and dissemination of antibiotic resistance genes. It was noted more than two decades ago that subinhibitory concentrations of β-lactams enhance the transfer of tetracycline resistance plasmids in *S. aureus* by up to 1000-fold (Barr *et al.*, 1986). Pregrowth of a donor *Bacteroides* strain on low concentration of tetracycline also seems to accelerate the mobilization of a resident nonconjugative plasmid by chromosomally encoded tetracycline conjugal elements (Valentine *et al.*, 1988). The exposure of donor *Bacteroides* cells to low concentration of tetracycline appeared to be a prerequisite for the excision of the CTnDOT family of conjugative transposons from the chromosome and conjugal transfer of the excised elements; virtually no transfer occurs without the tetracycline induction of donor cells (Stevens *et al.*, 1993; Whittle *et al.*, 2002). Incorporation of tetracycline at subinhibitory concentrations in the mating medium also substantially enhances *Tn*916-mediated conjugal transfer (Showsh & Andrews, 1992). The similar stimulatory effect of tetracycline on conjugation transfer was also demonstrated for the conjugative transposon *Tn*925 (Torres *et al.*, 1991). These *in vitro* results have been reproduced in *in vivo* models as well. In gnotobiotic mice, for example, the presence of a low concentration of tetracycline in drinking water increased the frequency of transfer of conjugative transposon *Tn*1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tracts by about 10-fold (Doucet-Populaire *et al.*, 1991). In gnotobiotic rats, selection for the resistant phenotype was the major factor causing higher numbers of *Tn*916 transconjugants in the presence of tetracycline (Bahl *et al.*, 2004). It can be concluded, therefore, that the enhancement of conjugal transfer of antibiotic resistance-carrying transposons in the presence of subinhibitory concentration of antibiotics is not only an *in vitro* phenomenon, but takes place in the gut ecosystem as well. At present, however, the transfer process has been studied in gnotobiotic animals, which may be not the best representation of conditions occurring in the normal gut. The developments of sensitive and accurate molecular tracking techniques may allow the study of this process in conventional animals.

Many antibiotic resistance genes reside on large self-transmissible genetic elements such as conjugative plasmids and transposons, which have a sufficient capacity to carry multiple genes, including those encoding antibiotic, heavy metal, and biocide resistances. The physical linkage between the R plasmids and resistances to heavy metals was noticed almost 40 years ago (Smith, 1967). Subsequent research by other groups demonstrated that the genetic linkage between antibiotic resistance and mercury resistance in enterobacteria had occurred before the late 1950s in Japan (reviewed in Liebert *et al.*, 1999). This genetic element, *Tn*21, encodes a mercury-resistance operon, transposition functions, and resistances to streptomycin/spectinomycin

and sulphonamides. The resistances genes and other ORFs are carried on an integron, thus allowing to capture antibiotic-resistance cassettes. The interest in possible coselection of antibiotic resistance genes by exposure to mercury ions was sparked by a publication suggesting that the exposure to mercury from dental amalgams increases incidence of multiple antibiotic resistance plasmids in the normal microbiota of nonmedicated subjects (Summers *et al.*, 1993). Despite the intensive follow-up studies on the possible link between the exposure to mercury from dental fillings and antibiotic resistance, the results were controversial (Lorscheider *et al.*, 1995; Osterblad *et al.*, 1995; Edlund *et al.*, 1996). Probably the main reason for the failure to establish a cause and effect relationship in this case was due to very high levels of antibiotic resistance already present in the gut of control groups, hence the contribution of coselection by mercury was difficult to ascertain. Genetic organization of *Tn*21 allows speculating on many possible scenarios of coselection of antibiotic resistance genes by resistances to quaternary ammonium compounds, sulphonamides, mercury, and other antibiotic resistances but this process now can be estimated only retrospectively because of the present wide dissemination of antibiotic resistance genes. *Enterobacteriaceae* strains collected by E.D.G. Murray from 1917 to 1954 indeed contained very few antibiotic- and mercury-resistant bacteria, despite the finding that 25% of the strains carried the conjugative functions (Hughes & Datta, 1983).

In the environment, bacteria in metal-contaminated areas appeared to be more tolerant to metals and antibiotics than in control sites (Stepanauskas *et al.*, 2005; Baker-Austin *et al.*, 2006; Wright *et al.*, 2006). Several *in situ* experiments revealed that metal amendment may select for antibiotic resistance. Treatment of agricultural soils with copper, for example, led to a significantly higher incidence of antibiotic resistance in indigenous soil bacteria (Berg *et al.*, 2005). In experiments with freshwater microcosms, amendment with metal concentrations representative of industry and mining-impacted environments increased the frequency of antibiotic resistance in the microbial communities (Stepanauskas *et al.*, 2006). Genetic mechanisms responsible for the coselection phenomena in the environment are presently poorly understood and the invention of environmental genomic and metagenomic approaches as well as adaptation of genetic tools for *in situ* conditions may help to identify genetic elements involved in this process.

Stress has also been shown to induce the mobility of transposons and insertion sequences (Levy *et al.*, 1993; Ilves *et al.*, 2001). In particular, the SOS response, which is induced by DNA-damaging agents such as mitomycin C and antibiotics such as fluoroquinolones and dihydrofolate reductase inhibitors, increases the rate of horizontal transfer of antibiotic resistance genes more than 300-fold (Beaber *et al.*, 2004). More importantly, the use of the SOS response-inducing antibiotics may coselect other antibiotic resistance genes that are physically linked in a mobile genetic element (Hastings *et al.*, 2004). Molecular mechanisms of stress-induced mutagenesis and lateral gene transfer are still under investigation but the reduced use of SOS-response inducing antibiotics at least in food animals and as growth promoters may help to reduce the spread of antibiotic resistance genes.

Concluding remarks

The rapidly growing number of antibiotic-resistant bacterial pathogens severely undermines the ability to control infectious diseases and currently it is one of the most challenging problems in public health care. Clinical microbiology research of antibiotic resistance has been mostly focused on isolation of pathogens, which treatment by empirical administration of antibiotics failed, with subsequent characterization of phenotypic (MIC) and genotypic antibiotic resistance profiles. Realization of the circumstances under which the microbial world evolves, i.e. the *modus operandi* of microorganisms involves much more extensive lateral gene exchange and recombination processes than previously recognized and that there are no isolated compartments in microbial ecosystems, led to the studies integrating the antibiotic resistance research area within the broader evolutionary and ecology contexts. These studies yielded valuable insights into the processes in microbial communities that eventually result in the emergence, dissemination and fixation of antibiotic resistance genes in human and animal bacterial pathogens. In particular, phylogenetic analyses helped to identify the nonantibiotic-producing environmental bacteria, but not the antibiotic producers, as harboring the readily available, abundant, and diverse pool of antibiotic resistance genes, from which the genes can be transferred to bacteria in the human and animal ecological compartments. Further systematic studies of the antibiotic resistome of the environment are necessary, not only for identification of the ancestors and missing links in the evolution of presently well-characterized antibiotic resistance genes, but, more importantly, for identification of the potential threats for newly introduced antibiotics to serve as an early warning system. Besides the soil antibiotic resistome, there are other antibiotic resistance gene reservoirs, the origin and diversity of which is difficult to explain in terms of possible antibiotic exposure (e.g. aquatic environment). These genes might have served some other needs of the bacterial cell rather than conferring antibiotic resistance *per se* and identification of these functions would be helpful for understanding the evolution of antibiotic resistance.

Another aspect of ecology of antibiotic resistance that recently emerged with the advent of molecular ecology tools in antibiotic resistance studies in the environment is the

realization of the fact that the microbial ecosystems are not isolated and there is extensive gene exchange between different compartments. Antibiotic usage in animals, for example, may lead to dissemination of antibiotic resistance genes into the broader environment (Chee-Sanford *et al*., 2001). Thus, there is also an urgent need to develop environmental genetic tools to evaluate the gene transfer/flow rates within and between the microbial ecosystems as well as to identify genetic elements and bacteria involved. Although the environmental genomics/metagenomics approach partially addresses this issue, it is still descriptive and specialized genetic tools, adapted for the *in situ* use, are required.

Besides the treatment of infections in humans and animals, antibiotics are widely used in a number of other practices such as the prophylactic and growth-promoting use in agriculture, aquaculture, and horticulture. In addition to the direct selection of corresponding antibiotic resistance genes, the extensive use of antibiotics leads to coselection, stimulation of transfer, generation of recombinant versions, fixation, and the increase in frequencies of other antibiotic resistance genes in bacterial populations in many different ecosystems. There are many other, as yet poorly defined, indirect factors that may contribute to dissemination and maintenance of antibiotic resistance genes and further studies are necessary to estimate this contribution. At the same time, the proven direct effects associated with the antibiotic overuse that are mentioned earlier require actions, and the use of antibiotics, limited mainly to treatment of infectious diseases, seems a reasonable alternative to preserve the efficacy of antibiotics. As one of these measures, the use of antibiotics as growth promoters has been phased out in the EU countries (Anadon, 2006).

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