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# Expanding the soil antibiotic resistome: exploring environmental diversity

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Antibiotic resistance has largely been studied in the context of failure of the drugs in clinical settings. There is now growing evidence that bacteria that live in the environment (e.g. the soil) are multi-drug-resistant. Recent functional screens and the growing accumulation of metagenomic databases are revealing an unexpected density of resistance genes in the environment: the antibiotic resistome. This challenges our current understanding of antibiotic resistance and provides both barriers and opportunities for antimicrobial drug discovery.

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## Introduction

It was never anticipated that treatment of infectious diseases would remain a challenge over three quarters of a century following the serendipitous discovery and subsequent clinical implementation of penicillin, an introduction that launched the 'Golden Age' of antibiotics. Yet, despite a diverse arsenal of chemotherapies, bacterial infections continue to be one of the leading causes of morbidity and mortality worldwide, attributed in part to the evolution and dissemination of antibiotic resistance genes.

Resistance has posed numerous clinical challenges. Antibiotic misuse and overprescription, among numerous other factors, have served as a driving force influencing the selection and dissemination of resistance. As a result, a plethora of diverse resistance mechanisms have been identified, and in many cases, multiple resistance mechanisms to a class of antibiotic have emerged in pathogens, compounding the problem [1,2].

It is becoming increasingly evident, however, that environmental forces have greatly impacted the determinants that have emerged clinically. Among the first to be recognized publicly was the impact of the agricultural use of antibiotics as animal growth promoters. Since the 1940s and until the past decade, the extensive use of antimicrobials at subtherapeutic levels has not only been shown to select for resistance to antibacterial agents [3] but also bacterial DNA contamination from crude antibiotic preparations often used in such applications has been found to contain resistance determinants [4]. This use of antibiotics in agriculture has resulted in the spread of strains such as vancomycin-resistant enterococci in both farm animals exposed to antimicrobials and humans in contact with the animals [3,5], and has been directly linked to the development of drug-resistant infections [6–8].

Traditional approaches to antibiotic resistance have involved extensive research of human pathogens, limiting efforts to only clinically identified mechanisms. Considering the growing body of evidence suggesting that clinical resistance is intimately associated with mechanisms found environmentally, there is a clear need to expand the focus to include nonpathogenic organisms in antibiotic research. In doing so, it may be possible to establish strategies to predict resistance before it emerges clinically as well as develop diagnostic techniques and therapeutic strategies to counteract resistance before emergence in pathogens.

This review will examine antibiotic resistance in the soil, the ecosystem where antibiotic synthesis probably originally evolved, as a means of both understanding the origins of clinically relevant mechanisms and rationally predicting mechanisms that may emerge in the future in clinical pathogens. In addition, we will make a case for the importance of expanding the study of resistance to broad environmental locales in order to attain a more comprehensive understanding of the prevalence and diversity of resistance worldwide.

## Antibiotic producing bacteria: a reservoir and putative origin of resistance determinants

Inhabited by up to $10^9$ microorganisms/g [9], the diversity of microbial life concealed within the soil has been explored in the search for new clinical and medicinal applications. Unarguably the most significant application to date has been the implementation of natural product antibiotics, a discovery that has revolutionized our approach to treating infectious diseases. Over 80% of antibiotics in clinical use originate from soil bacteria, either directly as natural products or as their semi-synthetic

derivatives [10]. The *Actinomycete* class of bacteria, in particular, is responsible for the synthesis of the vast majority of these clinically important compounds.

The evolution of sophisticated mechanisms of chemical warfare by soil microorganisms has had numerous implications. First of all, it has required the coevolution of mechanisms of self-protection in antibiotic producers, evidenced by the frequent presence of associated resistance genes in or flanking antibiotic biosynthetic gene clusters [11,12]. Secondly, in a powerful example of natural selection, other prokaryotes that inhabit similar niches have evolved or acquired resistance. This extensive coevolution of antibiotic biosynthesis and resistance suggests a possible origin of many clinical resistance determinants, as numerous mechanisms in soil bacteria and human pathogens are identical.

## Exploring the soil antibiotic resistome

The concept of the soil as a location of antibiotic resistance determinants, particularly those harboured in antibiotic producers as self-protection mechanisms, has been acknowledged for decades. However, mechanistic commonalities between clinical pathogens and soil inhabiting organisms were not shown until the 1970s. In 1973, two molecular mechanisms of aminoglycoside resistance in soil-dwelling actinomycetes from the genus *Streptomyces* were determined to be identical to those in clinical pathogens [13•]. These strains, producers of the aminoglycosides kanamycin and neomycin, were capable of drug modification by acetylation and phosphorylation, respectively as a means of self-protection [13•].

Since then, numerous parallels have been identified between determinants in soil actinomycetes and those in clinically important strains, with respect to both molecular mechanism and protein homology. The most striking example is that of the glycopeptide antibiotic vancomycin, still considered an important clinical drug of last resort. Clinical resistance is mediated by the reprogramming of the drug target, the D-Ala-D-Ala termini of cell wall peptidoglycan, to one with a significantly lower affinity for vancomycin [14]. This is most commonly accomplished by three proteins, encoded by the *vanHAX* cluster of genes. Six years after the mechanism of pathogenic strains was elucidated, it was discovered that not only was this strategy identical to those in glycopeptide producing soil actinomycetes, but primary amino acid sequence homology was also apparent between the associated VanHAX resistance proteins [15•].

Parallels have also been identified in non-antibiotic producing soil bacteria, strains whose determinants have evolved a means other than self-protection. This phenomenon has been observed in both actinomycetes and non-actinomycetes, as evidenced in the case of vancomycin resistance in *Streptomyces coelicolor* and *Paenibacillus* spp., respectively [16•,17•].

In recent years, approaches have been implemented to characterize the diversity and prevalence of resistance in soil bacteria — the soil antibiotic resistome — as an important reservoir of resistance [18]. Riesenfeld *et al.* investigated resistance in the soil, concentrating on unculturable organisms, bacteria that have yet to be characterized and thus underappreciated because of challenging culture conditions [19•]. By creating a functional metagenomic library [20] in which cloned genomic fragments were expressed from DNA isolated directly from soil and selecting for resistance, traditional challenges associated with studying genes of unknown sequence were circumvented. Specifically, these functional analyses revealed novel antibiotic resistance proteins that were previously of unknown function and unrecognizable by sequence alone. Thus, this work not only allowed for the identification of aminoglycoside *N*-acetyltransferases, the *O*-phosphotransferases, and a putative tetracycline efflux pump but also a construct with a novel resistance determinant to the aminoglycoside butirosin [19•]. This work shows the power of the functional metagenomic approach when applied to a search of activity with a highly selectable phenotype such as antibiotic resistance.

Focusing on agriculturally associated resistance, Schmitt *et al.* characterized the diversity of tetracycline resistance determinants in soil [21•]. Using PCR-based approaches, three resistance genes were ubiquitously identified in the soil, and an additional five were found in manure-supplemented soils. This work speaks to the diversity of tetracycline resistance in agricultural soils.

To explore the soil resistome from an evolutionary perspective, D'Costa *et al.* established a systematic approach to characterize resistance in actinomycetes as a means of anticipating new mechanisms of resistance that may emerge clinically in the future [22•]. By constructing a morphologically diverse library of hundreds of spore-forming actinomycetes and screening for resistance to a collection of 21 natural product, semi-synthetic and synthetic antibiotics, this work was the first to attempt to quantify the phenotypic density of resistance in any subset of soil organisms. The phenotypic density of resistance and diversity of the resulting profiles were greater than ever anticipated, with strains resistant to an average of seven to eight antibiotics. In addition, this work identified a wealth of antibiotic inactivating enzymes, including novel mechanisms of resistance to the recently approved antibiotics telithromycin and daptomycin [22•].

As a whole, the study of resistance in soil bacteria is rapidly gaining recognition as an important reservoir from which many clinical parallels can be drawn. Further

studies on a more diverse subset of strains, as well as approaches to study slow-growing strains and those difficult to culture will be important to uncover the true extent of the soil resistome.

## Acquisition of resistance to antibiotics: horizontal gene transfer

Genetic transfer among bacteria probably accounts for much of the spread of resistance. This process, known as horizontal gene transfer (HGT) often confers new metabolic capabilities to the recipient, allowing its adaptation to new ecological niches.

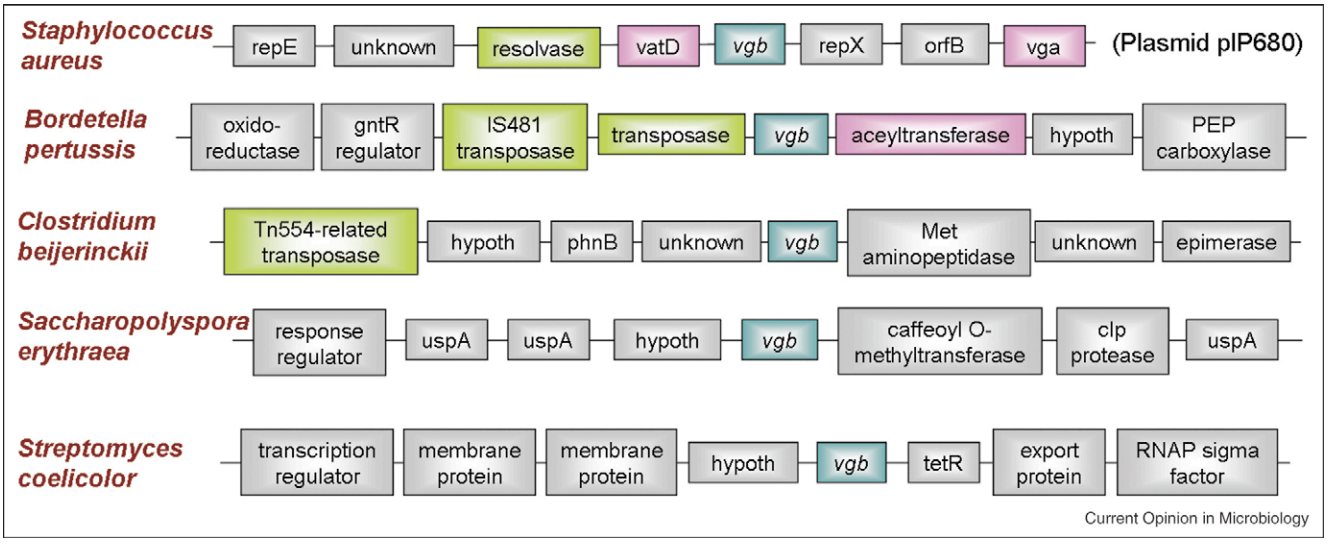
Exogenous DNA is usually acquired by bacteria through transformation, conjugation and transduction. It is then possible to either integrate the new genetic material into the recipient's chromosome or replicate independently. The mobile genetic elements mediating HGT consist of plasmids and transposons and the related gene integrating integrons. Plasmids, circular double-stranded DNA molecules harbouring genetic determinants, are capable of replicating independently of the bacterial chromosome. Transposons are flanked by inverted repeat sequences and encode transposases, enzymes that introduce nicks at the ends of these elements to allow for integration at insertion sequences, sites which are normal constituents of bacterial chromosomes and plasmids. Transposons can carry multiple gene cassettes and participate in gene mobilization within a chromosome. The mobility of a transposon can increase if it cointegrates into a plasmid which is then transmitted to other cells by conjugation or transformation. Integrons are assembly platforms which incorporate genetic material through site-specific recombination and contain within a promoter for expression. An integron-encoded integrase carries out the assembly of tandem genes or gene fragments at the *attI* primary recombination site. Although intergenic rearrangements are possible, integrons are essentially immobile in the chromosome unless associated with a transposon [23]. Evidence of these genetic elements, or their remnants, have been identified in all available prokaryotic genomes [24].

Mobile genetic element-associated transmission of antibiotic resistance determinants is probably responsible for the dispersal of at least some streptogramin B lyases (further discussed in the next section), responsible for antibiotic resistance by means of drug inactivation. As illustrated in Figure 1, analysis of the genetic environment of genes encoding putative lyases (*vgb*) in many instances reveals the presence of mobile elements such as transposases upstream or downstream of the gene of interest. Often in these instances, multiple resistance determinants can accumulate on a mobile element and upon transmission contribute to multi-drug resistance. In this case, *vgb* is often in close proximity to the resistance determinant *vat*, associated with resistance to type A streptogramins. Thus with respect to antibiotic resistance, the presence of mobile genetic elements can play a powerful role in the transmission of resistance between bacterial strains.

## Expanding the resistome: exploring environmental diversity

With respect to environmental resistance to antibiotics, this ability is not simply restricted to soil-dwelling

**Figure 1**



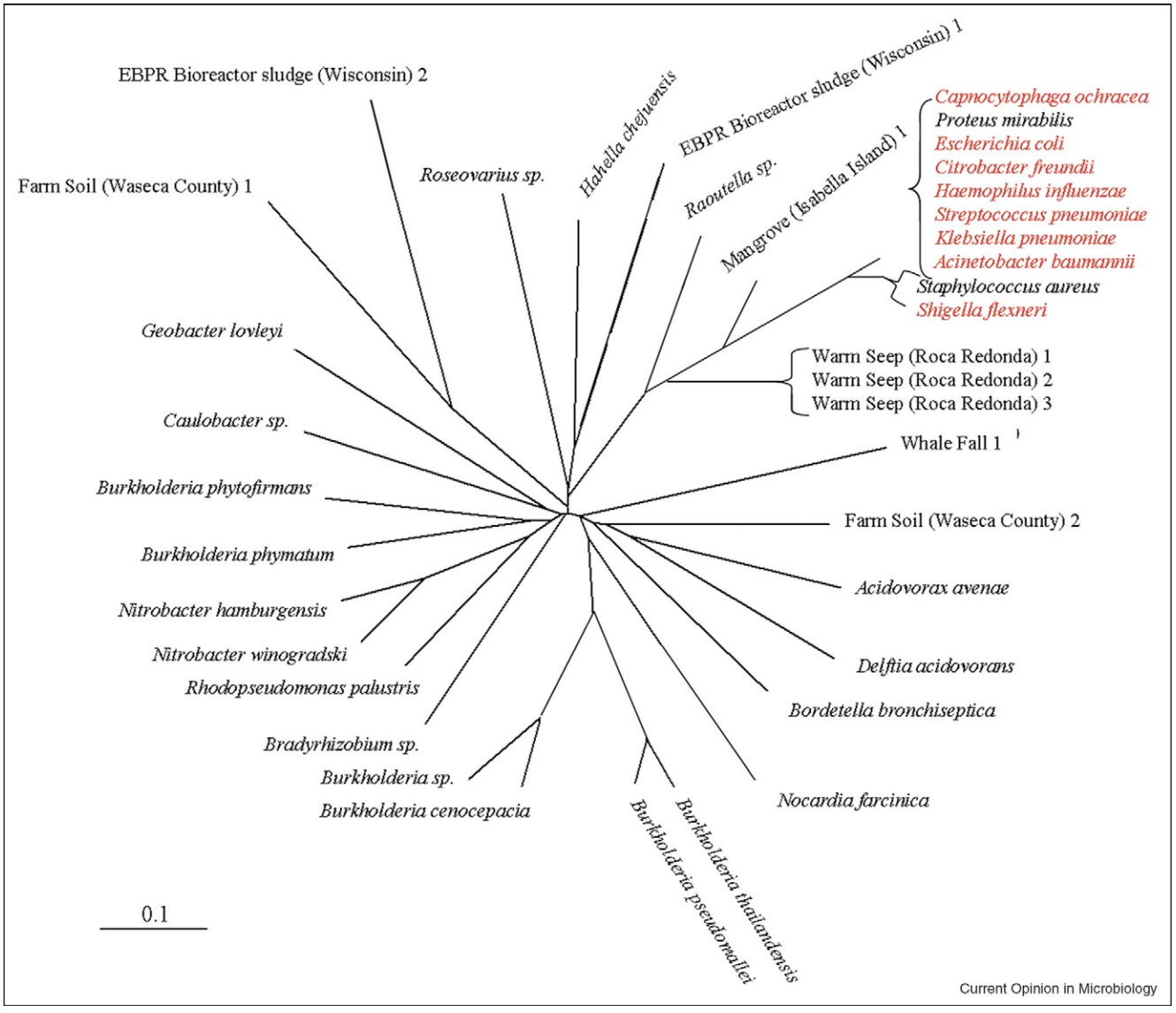

Schematic diagram of *vgb* genetic environment. Putative streptogramin B lyases (*vgb*) are coloured in turquoise, and putative streptogramin A acetyltrasferases are in pink. The presence of putative facilitators of gene mobilization (e.g. transposases) is represented by green boxes. Note that the lengths of the genes represented in the diagram are not proportional to their sizes.

microorganisms. Both phenotypically and genetically, resistance to antibacterials has been extensively documented in genera spanning the entire Bacterial domain from diverse ecosystems [21•,22•,25–32].

The advent of genome sequencing has greatly accelerated our understanding of evolution. With respect to resistance determinants, these efforts have uncovered genes responsible for resistance, cryptic genes that encode resistance but are perhaps insufficiently expressed and thus do not confer the phenotype, as well as those that serve as precursors for resistance determinants. Recent efforts have uncovered a wealth of putative resistance determinants. For example, the recently sequenced genome of the erythromycin producer *Saccharopolyspora erythraea* NRRL23338, a non-pathogenic Gram-positive bacterium resistant to a wide spectrum of antibiotics, is predicted to encode a remarkable number of putative resistance determinants representing approximately 1% of its genome [33].

Furthermore, the field of metagenomics is rapidly expanding our ability to explore the genetic diversity of novel terrestrial and aquatic environments. Metagenomics entails the sequencing of a clone library derived from the total DNA purified from a complex microbial

**Figure 2**



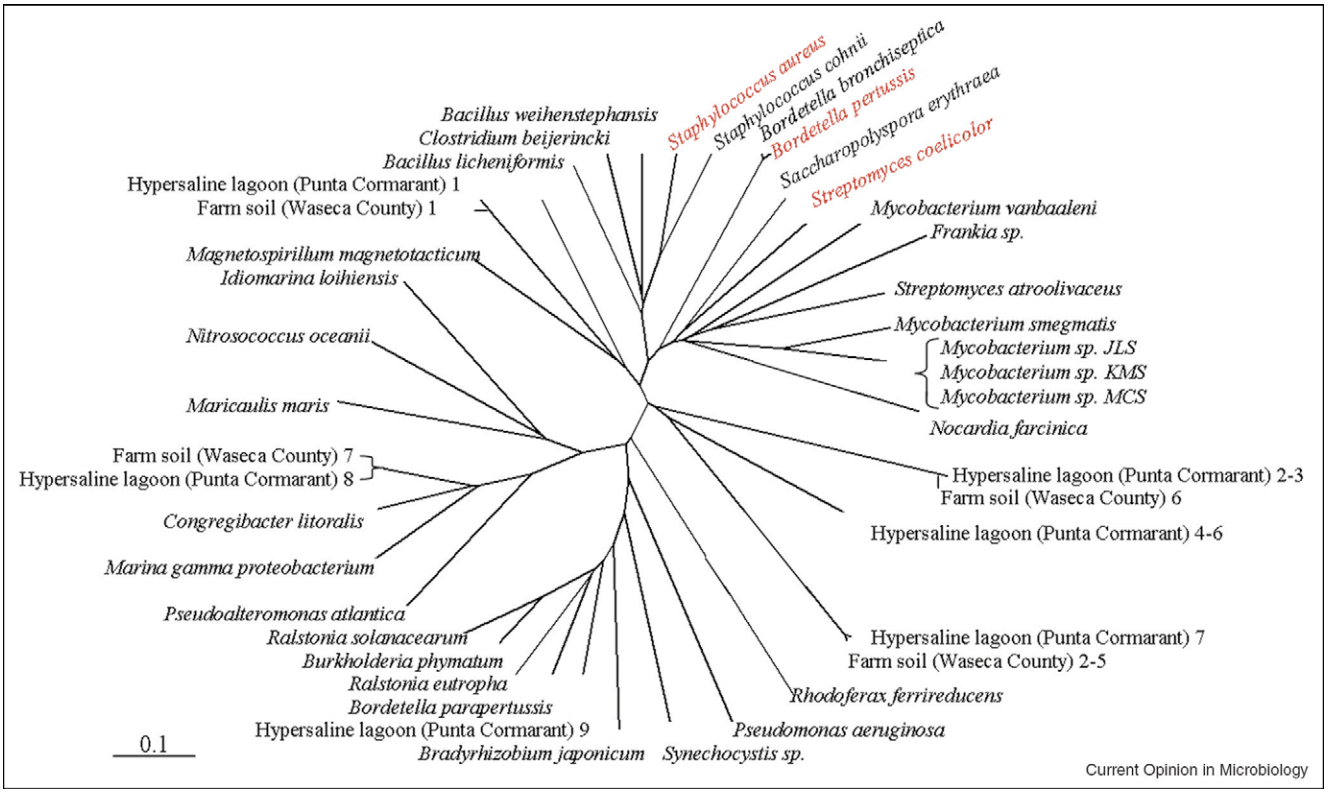

Diversity of TEM β-lactamases. Chromosomally encoded genes, as well as homologous genes from environmental metagenomic analyses are represented above. Enzymes that have been biochemically elucidated to exhibit β-lactam hydrolysis are denoted in red. Note that the scale bar represents 0.1 substitutions per site.

ecosystem [20]. These partial genome fragments are thought to represent the diversity of the community, including strains which cannot be cultured. Several metagenomics projects are ongoing, and their databases are invaluable reservoirs of information for environmental resistance profiling [34•]. Examples of these include the CAMERA database (Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis) based on global ocean sampling expeditions. In addition, 68 new searchable marine microbial genomes are available from the Moore Foundation Marine Microbial Sequencing Project. With respect to environmental sequencing as a whole, the National Centre for Biotechnology Information (NCBI) offers a metagenomics page containing a large number of databases that contain contiguous regions from environmental communities associated with submerged whale carcasses (whale fall), enhanced biological phosphorus removal (EBPR) sludge communities from sites in the US and Australia, farm soil, biofilm microbial communities from acid mine drainage sites, planktonic microbial communities from North Pacific Subtropical Gyre as well as methane-oxidizing archaea from deep-sea sediments. These, as well as other genomics consortia will provide invaluable tools for identifying real and putative bacterial resistance genes in non-clinical and unculturable species.

In order to truly appreciate the diversity and dispersion of the environmental resistome, it is of great value to examine characterized resistance proteins in the context of not only those annotated as putative resistance determinants but also those from previously under-recognized sources of resistance. In addition, it is important to compare proteins of alternate cellular functions from which these determinants probably evolved. Here we discuss three examples illustrating this environmental density, focusing on resistance by means of antibiotic inactivation.

β-Lactams, the first class of natural product antibiotics to be implemented clinically, continue to be among the most extensively prescribed antibacterials in North America [35]. This diverse class that includes both natural products and semi-synthetic derivatives, acts by forming covalent intermediates with active site serine hydroxyl residues of cell wall crosslinking enzymes, effectively titrating them out as inactive complexes [36–39]. The most prevalent mechanism of resistance is enzymatic

**Figure 3**

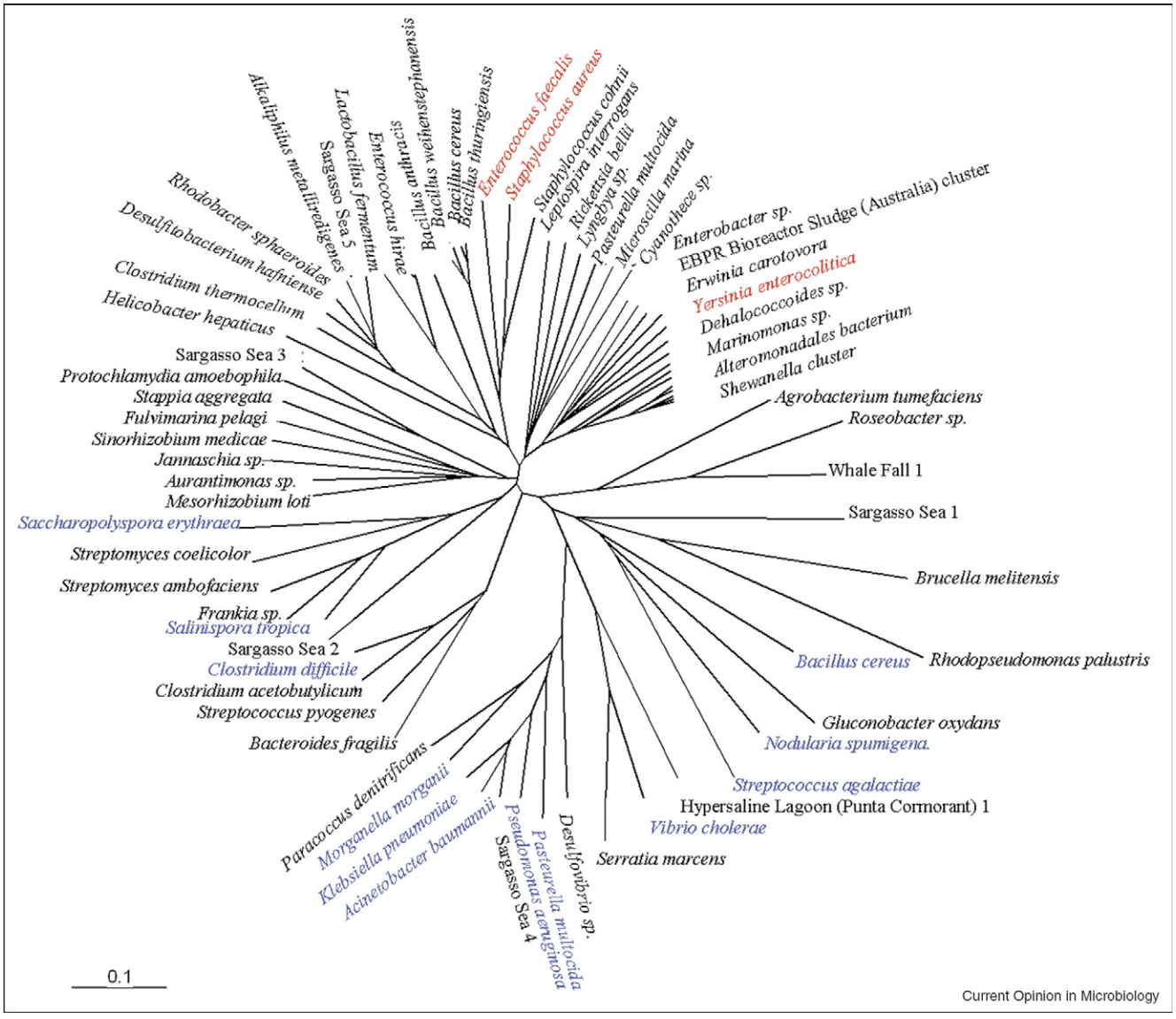



Genetic diversity of different *vgb* genes. Chromosomally encoded genes, as well as homologous genes from environmental metagenomic analyses are included above. Enzymes that have been biochemically elucidated to be type B streptogramin lyases are denoted in red. The scale bar represents 0.1 substitutions per site.

drug inactivation by β-lactamase enzymes which hydrolyze the β-lactam ring essential for antibiotic activity. Two general chemical mechanisms are known: Ser-dependent hydrolysis and metal-dependent hydrolysis. The Ambler class A β-lactamases display sequence homology to penicillin-binding proteins, from which it was thought that these β-lactamases originally evolved.

The class A TEM subset of β-lactamases (Figure 2) are present in the genomes of many soil-dwelling strains, as well as Gram-negatives that are associated with human infectious diseases (e.g. *Burkholderia pseudomallei*, *Bordatella bronchiseptica*, and *Delftia acidovorans*) [40–45]. With respect to genetic background, commonalities in flanking genes are evident in many of these microbes, suggesting evolution from a common ancestor. However, homologs in some of the strains contain integrases upstream, and in *Salmonella enterica*, there is evidence of genetic mobility in nearby regions both upstream and downstream. Comparison to sequences from oceanic metagenomic and environmental databases suggests the presence of these β-lactamases in diverse environmental locations, speaking to the ubiquitous dispersion of this important resistance determinant. Confirmation of β-lactamase activity in these organisms, however, is necessary to fully understand its true diversity.

**Figure 4**




Diversity of *xat* genes. Genes associated with type A streptogramin acetylation are denoted in red, whereas those that acetylate chloramphenicol are in blue. Note that the scale bar represents 0.1 substitutions per site.

Type B streptogramins exhibit activity by binding to and subsequently obstructing the bacterial ribosome exit tunnel, thereby inhibiting translation [46]. Clinical resistance, documented in strains of *Staphylococcus aureus*, can occur by cleavage of the cyclic lactone via an elimination mechanism by the lyase Vgb [47]. This enzyme displays significant sequence homology to putative streptogramin lyases from strains in diverse phyla that include both environmental strains and clinical pathogens (Figure 3). Analysis of the available genetic environment revealed the presence of mobile genetic elements (e.g. transposases and resolvases) in many of the pathogenic strains, illustrating the potential for lateral gene transfer. In addition, searches of environmental genomes and metagenomic databases revealed an abundance of uncharacterized putative lyases, grouping in many phylogenetic clusters.

Collectively, these observations suggest that this mechanism of resistance is not restricted to clinical pathogens, but in fact may be more widespread in the genomes of environmental bacteria than previously anticipated. To date, enzymes in two of the branches have been confirmed to possess streptogramin lyase activity [47], and further studies of lyases representative of other clusters are in progress.

Type A streptogramins, structurally unique from the type B class, are actinomycete natural products or semisynthetic derivatives that bind to the peptidyltransferase centre of the bacterial ribosome, subsequently inhibiting translation [46]. Clinical resistance has been documented in *Staphylococcus* and *Enterococcus* spp. by *O*-acetylation of the antibiotic, as encoded by the *vat* genes [48]. Streptogramin A acetyltransferases have also been identified in environmental isolates, and of particular importance, they have been located in poultry treated with streptogramins as growth promoters [49,50].

Analysis of the Vat(D) subset of streptogramin acetyltransferases reveals homologs in clinically associated pathogens as well as a diverse array of environmental strains (Figure 4) [51]. With respect to sequence, this family of Vat enzymes display homology at both the primary and tertiary level to a subset of chloramphenicol acetyltransferases [52] (collectively known as xenobiotic acetyltransferase *xat* genes), with clustering suggesting divergence from a common ancestor. The *vat(D)* sequence from a number of the clinically relevant strains is harboured on a plasmid, and in other strains, both pathogenic and environmental, the flanking chromosomal environment contain mobile genetic elements. In both plasmid and chromosomal genes, *vat(D)* homologs are commonly found in multi-drug resistance clusters, near genes such as *vgb*, β-lactamases, as well as antibiotic efflux genes. Analysis of other environmental sources suggests the presence of streptogramin acetyltransferases in diverse locations, including aquatic environments. However, further biochemical evidence is required to confirm the activity of these putative resistance enzymes.

## Conclusions

The overwhelming evidence using both functional and *in silico* genomic screening is that environmental organisms harbour a previously underappreciated density of antibiotic resistance genes. This unexpected conclusion should have a paradigm shifting impact on our understanding of the judicious use of antibiotics and the drug discovery process. Furthermore, it raises exciting questions about protein evolution and gene transfer among bacteria. These are early days for studies on the resistome and there are major problems to be tackled. For example:

- What are these genes doing in these bacteria?
- Are they *bone fide* resistance genes or do they have other functions?
- What is the concentration of antibiotics in the environment and is this sufficient to select for resistance?
- What are the triggers that induce HGT in the environment?
- How do these genetic elements make their way into pathogenic bacteria?

These are key questions that emerge from the study of the environmental antibiotic resistome that need resolution in the future.

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