Co-selection of antibiotic and metal resistance

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There is growing concern that metal contamination functions as a selective agent in the proliferation of antibiotic resistance. Documented associations between the types and levels of metal contamination and specific patterns of antibiotic resistance suggest that several mechanisms underlie this co-selection process. These co-selection mechanisms include co-resistance (different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals). Indirect but shared regulatory responses to metal and antibiotic exposure such as biofilm induction also represent potential co-selection mechanisms used by prokaryotes. Metal contamination, therefore, represents a long-standing, widespread and recalcitrant selection pressure with both environmental and clinical importance that potentially contributes to the maintenance and spread of antibiotic resistance factors.

Metal contamination: agents of indirect selection
The persistence and proliferation of antibiotic resistance in bacterial pathogens represents a considerable public health concern. Subsequent measures to control the emergence and propagation of antibiotic resistance have encountered limited success, and it persists in spite of the restricted use of several key antibiotics, which indicates that there are components governing the evolution, dissemination and perpetuation of these resistance systems that have yet to be understood. Resistance to antibiotics can be conferred by chromosomal or mobile genetic elements (e.g. plasmids) and achieved using four main strategies: (i) reduction of membrane permeability to antibiotics; (ii) drug inactivation; (iii) rapid efflux of the antibiotic; and (iv) mutation of cellular target(s) [1]. In addition, antibiotic sequestration has also been suggested as a potential resistance strategy. Overall, the structural and functional characteristics of antibiotic resistance share common themes with those of metal resistance (Table 1).

Although bacterial exposure to metals predates human history, anthropogenic-derived sources of metals represent a major source of contamination in the environment. Importantly, a substantial number of reports suggest that metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance [2–4]. This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics [5]. Unlike antibiotics, metals are not subject to degradation and can subsequently represent a long-term selection pressure [5]. Thus, there are concerns regarding the potential of metal contamination to maintain a pool of antibiotic-resistance genes in both natural and clinical settings. In addition to metals, other toxicants are implicated in the co-selection of antibiotic resistance, including quaternary ammonium compounds and anti-fouling agents and detergents [6,7]. However, in this review, we focus on the current body of knowledge regarding the role of metals as a component in co-selection and dissemination of antibiotic resistance, and delineate and discuss some of the potential mechanisms that underlie this process.

Co-resistance as a mechanism for co-selection
Co-resistance occurs when the genes specifying resistant phenotypes are located together on the same genetic element such as a plasmid, transposon or integron [7]. This physical linkage results in the co-selection for other genes located on the same element (Figure 1). It has been known for several decades that metal- and antibiotic-resistance genes are linked, particularly on plasmids, because the evidence for co-resistance as a mechanism of antibiotic–metal co-selection came from studies that used transformation, plasmid curing and plasmid sequencing approaches [8,9]. The genetic linkage of mercury- and antibiotic-resistance traits on plasmids was demonstrated by Summers et al. [3] when mercury resistance was co-transferred with antibiotic resistances in a subset of matings between Enterobacteriaceae and recipients. Ghosh et al. [10] cured plasmids from Salmonella abortus equi strains that were resistant to ampicillin, arsenic, chromium, cadmium and mercury. Upon plasmid removal, the strains became sensitive to these toxicants. The authors also analyzed transformed Escherichia coli DH5α to confirm the plasmid location of resistance
Table 1. Well-characterized examples of shared structural and functional characteristics of prokaryotic antibiotic- and metal-resistance systems

<table>
<thead>
<tr>
<th>Resistance mechanism</th>
<th>Metal ions</th>
<th>Antibiotics</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduction in permeability</td>
<td>As, Cu, Zn, Mn, Co, Ag</td>
<td>Cip, Tet, Chlor, ß-lactams</td>
<td>[68,69]</td>
</tr>
<tr>
<td>Drug and metal alteration</td>
<td>As, Hg</td>
<td>ß-lactams, Chlor</td>
<td>[70,71]</td>
</tr>
<tr>
<td>Drug and metal efflux</td>
<td>Cu, Co, Zn, Cd, Ni, As</td>
<td>Tet, Chlor, ß-lactams</td>
<td>[72,73]</td>
</tr>
<tr>
<td>Alteration of cellular target(s)</td>
<td>Hg, Zn, Cu</td>
<td>Cip, ß-lactams, Trim, Rif</td>
<td>[74,75]</td>
</tr>
<tr>
<td>Drug and metal sequestration</td>
<td>Zn, Cd, Cu</td>
<td>CouA</td>
<td>[76,77]</td>
</tr>
</tbody>
</table>

*a* Abbreviations: Chlor, chloramphenicol; Cip, ciprofloxacin; CouA, coumermycin A; Rif, rifampicin; Tet, tetracycline; Trim, trimethoprim.

*b* Includes reduction of membrane permeability to metals and antibiotics.

*c* Includes drug and metal inactivation and modification.

*d* Includes rapid efflux of the metal and antibiotic.

*e* Includes alteration of a cellular component to lower its sensitivity to the toxic metal and antibiotic.

*f* Includes drug and metal sequestration.

Figures and diagrams are not rendered as text.
Plasmid sequencing in conjunction with bioinformatic and phenotypic analysis offers additional support for co-resistance, with numerous reports in the literature of plasmids that contain both antibiotic- and metal-resistance determinants. For example, plasmids that originate from sewage treatment plants contain genes that encode resistance to mercury [21,22], chromate [23] and tellurite [24], along with multiple antibiotic-resistance genes. Sequence analyses indicate that many of these elements are flanked with insertion sequences and transposase sequences. Presumably, the plasmids have accrued these resistance determinants through multiple recombination events [25]. Additionally, genomic sequencing of the pathogen Salmonella enterica serovar Typhi CT18 revealed the presence of a conjugative plasmid (pHCM1) that confers resistance to trimethoprim (dfr1b), sulfonamide (sulII), chloramphenicol (catI), ampicillin (bla) and streptomycin (strAB) and contains a mercury-resistance operon [26] (Figure 1).

It must be noted, however, that metal-resistance plasmids predate the anthropogenic use of antibiotics. Mindlin et al. [27] suggested that the emergence of integron-carrying transposons that contain both antibiotic-resistance and mercury-resistance determinants is a relatively recent phenomenon because permafrost-derived Pseudomonas (~15 000–40 000 years old) contained closely related transposons found in many present-day bacteria, albeit devoid of antibiotic-resistance cassettes [27]. In addition, the early characterization of R-plasmids from pre-antibiotic era culture collections of Enterobacteriaceae provided no evidence of transferable antibiotic resistance [28]. Thus, the recent acquisition of antibiotic-resistance determinants on pre-existing R-plasmids must be evaluated within this context. Of interest is the precise timescale at which pre-existing metal-resistance elements acquired antibiotic-resistance genes and whether this predated the widespread usage of antibiotics. Because of the importance of this association, large-scale genomic sequencing of these mobile genetic systems involved in horizontal gene transfer is necessary and should provide further insights into the ubiquity of environmental metal–antibiotic co-selection.

**Cross-resistance as a mechanism of co-selection**

A second potential mechanism involved in the selection and proliferation of antibiotic resistance is cross-resistance, which can occur when different antimicrobial agents attack the same target, initiate a common pathway to cell death or share a common route of access to their respective targets. The end result is the same: the development of resistance to one antibacterial agent is accompanied by resistance to another agent [7] (Figure 1). Cross-resistance can manifest itself through efflux of structurally dissimilar compounds using the same mechanism. For example, the multiple-drug resistance (MDR) pump in Listeria monocytogenes can export metals in addition to antibiotics [29]. Recent characterization of the MexGHI–OpmD efflux pump of Pseudomonas aeruginosa showed that the presence of the entire pump operon in trans resulted in increased resistance to vanadate, tetracyclin and clavulanic acid compared with mutants that lack MexGHI–OpmD [30]. Mutational analysis of a membrane-bound DsbA–DsbB disulfide bond formation system in Burkholderia cepacia followed by phenotypic analysis suggested that the DsbA–DsbB system is involved in the formation of a metal-efflux system and a multi-drug resistance system [31]. Mutants without a functional DsbA–DsbB system were less resistant to a range of antibiotics and metals, including β-lactams, kanamycin, erythromycin, novobiocin, ofloxacin, sodium dodecyl sulfate, cadmium and zinc. Hernandez et al. [32] screened Enterobacteriaceae from contaminated oil refinery soils for isolates that could accumulate metals and found that two strains, Escherichia hermannii and Enterobacter cloacae, were markedly more antibiotic resistant when these isolates were grown in the presence of vanadate (compared with controls). Although the precise mechanisms underlying this co-resistance were not elucidated, the authors suggested that the MDR phenotype stimulated by vanadate addition was facilitated by membrane-bound efflux systems in these bacteria [32].

**Co-regulation as a mechanism of resistance**

A range of transcriptional and translational responses to metal or antibiotic exposure can be linked to form a coordinated response to either stress (Figure 1). Several recent post-genomic studies provide insights into co-regulation as a means of maintaining and proliferating antibiotic-resistance determinants. Microarray analysis of chemostat-cultured E. coli strain MG1655 demonstrated that the mdtABC operon was upregulated in response to stress caused by excess zinc [33]. This resistance-nodulation-cell division (RND-type) efflux system (all members of which catalyze substrate efflux through an H+ antiport mechanism) has been implicated in conferring resistance to certain antibiotics, including novobiocin and the bile-salt component deoxycholate [33,34]. Subsequently, the finding that mdt is upregulated in response to zinc at environmentally and clinically relevant concentrations is of some concern. Overexpression analysis of the plasmid-derived E. coli gene robA (a member of the XylS–AraC subfamily of DNA-binding proteins) demonstrated that this gene increased the resistance spectrum in mutants to a range of different antibiotics and metals including silver, cadmium and mercury in addition to tetracycline, chloramphenicol and novobiocin [35]. Perron et al. [36] addressed the issue of metal and antibiotic co-regulatory resistance in the Gram-negative bacterium P. aeruginosa. Isolates exposed to zinc were also found to be resistant to other heavy metals (cadmium and cobalt) and the carbapenem-class antibiotic imipenem. Analysis of the mechanisms that underlie cross-resistance to both zinc and imipenem revealed co-regulation of imipenem influx with heavy metal efflux [36]. The P. aeruginosa two-component sensor protein CzcS was subsequently found to be responsible for resistance to both zinc and imipenem. Conejo et al. [37] found that zinc eluted from silicone latex urinary catheters exerted a negative effect on the expression of OprD2, a membrane porin responsible for carbapenem resistance in P. aeruginosa, which subsequently increased the overall resistance to this class of antibiotic.
Biofilm induction as a mode of co-selection

Numerous reports demonstrate the potential for biofilm phenotypes as effective mechanisms of resistance to both metals and antibiotics. In contrast to some of the other resistance mechanisms previously outlined here (including efflux pumps, modifying enzymes and target mutations), additional mechanisms seem to be responsible for the protection of prokaryotes as a biofilm [38]. Biofilms are considered to be the predominant growth phenotype of bacteria in industrial, clinical and environmental ecosystems [39] and account for a number of recalcitrant infections in clinical settings, including P. aeruginosa, Klebsiella pneumoniae and Staphylococcus epidermidis. Bacteria encased within biofilms exhibit unusual phenotypic traits, and growth within this complex multilayered, cellular matrix-embedded community and its eventual dispersal requires the coordinated expression of an array of genes [40]. The protected environment and close proximity of microbial species within biofilms also represents an ideal environment for lateral gene transfer. A wealth of data suggest that bacteria in biofilms can tolerate levels of antimicrobial agents that are an order of magnitude higher than the minimum inhibitory concentrations (MICs) of genetically equivalent planktonic bacteria [41,42]. Several explanations have been proposed for the enhanced resistance of biofilm-associated cells to both metals and antibiotics. Both metal and antibiotic sequestration in the biofilm matrix and the presence of a small population of ‘persister’ cells might be contributing factors in the time-dependent tolerance of both planktonic cells and biofilms to high concentrations of antimicrobial agents [43]. The addition of either metals or antibiotics to planktonic cells can stimulate the production of extracellular polymeric substances (EPS), which lead to cell adhesion and, ultimately, the formation of a biofilm. Rachid et al. [44] exposed S. epidermidis to sub-inhibitory concentrations of tetracycline and the semisynthetic streptogramin antibiotic quinupristin-dalfopristin and observed a 9-fold to 11-fold enhanced expression of ica, a precursor of polysaccharide intercellular adhesin. Exposure to metals also stimulates EPS production in Archaea (C. Baker-Austin, unpublished), the cyanobacterium Phormidium [45] and Pseudomonas species [46] and has been observed in bacterial biofilms from metal-contaminated activated sludge [47]. Therefore, the co-regulation and cross-resistant role of metals and antibiotics in biofilm development is important if bacteria that are exposed to metals in either environmental or clinical settings induce a biofilm mode of growth and, thus, simultaneously adopt an antibiotic-resistant phenotype.

Co-selection in the environment

Evidence for co-selection of antibiotic and metal resistance in the environment originates from diverse habitats contaminated with a variety of metals, which indicates that co-selection is not limited to a subset of metals, environments or microbial taxonomic groups. The strength of evidence presented by these studies ranges considerably between anecdotal reports of co-resistances to experimental studies that unambiguously implicate metals in antibiotic resistance co-selection.

Co-resistance of antibiotic- and metal-resistance traits

Reports of the co-resistance of antibiotic- and metal-resistance phenotypes exist for clinical and environmental isolates and for bacterial populations and communities [19,48–53]. In an example of co-resistance in clinical isolates, Staphylococcus species were resistant to multiple metals and antibiotics: the most common co-resistance involved chromium, lead and penicillin G [54]. Potential public health concerns for the co-resistance of metal and antibiotic resistances were raised by Pettibone et al. [50] and Pathak and Gopal [55], who observed that bacterial isolates obtained from fish tissue commonly consumed by humans exhibited resistance to multiple metals and antibiotics. Although these studies do not directly address the hypothesis that metal exposure co-selects for antibiotic resistance, they highlight the fact that metal and antibiotic resistances are commonly found within the same bacteria.

Resistance profiles from contaminated and reference settings

Corroborative support for co-selection stems from several studies that directly compare resistance profiles of bacteria collected from contaminated and reference sites. Although a direct assessment of the role of metals in co-selection is often hampered by the presence of other anthropogenic contaminants, these studies do link contaminant exposure with elevated antibiotic resistance. For example, the association of antibiotic- and metal-resistant phenotypes in environmental bacteria was hypothesized as early as 1974, when multiple antibiotic- and metal-resistant E. coli were more prevalent in sludge-contaminated estuarine sediment sites relative to reference locations [56]. Additional studies conducted in marine systems observed elevated metal and antibiotic resistance co-occurring in marine isolates from contaminated sediments compared with reference sites [57–59]. Rasmussen and Sorensen [59] demonstrated an increased occurrence of conjugative plasmids in a contaminated site and showed that tetracycline- and mercury-resistance genes were located on plasmids, thereby providing indirect evidence of co-resistance. In freshwater systems, McArthur and Tuckfield [60] examined spatial patterns of antibiotic and metal resistance in contaminated and reference stream sediments and observed that isolates from industrially contaminated sediments were more resistant to kanamycin and streptomycin than those from a reference site. Furthermore, resistance to streptomycin was positively correlated with sediment mercury concentration.

Bacteria found within drinking water systems are exposed to a variety of metals, which is a potential selective force for metal resistance. To test this hypothesis and to ascertain whether antibiotic-resistant bacteria were co-selected, Calomiris et al. [61] compared resistance profiles for isolates from a raw water intake site with isolates from multiple points along the drinking water system. In addition to the finding that isolates from within the drinking water system were more resistant to zinc, copper and lead than isolates from the raw water system, the authors demonstrated that metal-resistant isolates from within the drinking water system exhibited multiple
antibiotic resistances whereas metal-sensitive isolates did not. Interestingly, this pattern did not hold true for all tested metals because cadmium resistance did not correlate with multiple-antibiotic resistance. The authors also noted that a gradient of metal and antibiotic resistances existed within the water system with isolates further from the source being more resistant than those at a site closer to the water intake.

Co-selection has also been observed in several agricultural-based studies. Huysman et al. [62] observed higher frequencies of resistance to a range of metals and antibiotics (including zinc, cadmium, nickel, cobalt, ampicillin, streptomycin, olaquindox and spiramycin) in copper-resistant isolates when compared with copper-sensitive bacteria isolated from agricultural fields in which copper-contaminated pig manure had been applied [62]. Enterococcus faecium isolates obtained from pigs, broiler chickens, calves and sheep were also tested for resistance to copper and erythromycin and vancomycin [11]. The correlation between copper resistance and antibiotics was important only for isolates obtained from pigs, which might partly be a result of the higher copper exposure in pigs through feed additives compared with other livestock in Denmark.

Recent developments in culture-independent flow cytometry have provided a more quantitative, high-throughput insight of co-selection in the environment. Using this technique, co-selection for resistant bacteria from freshwater aquatic systems that receive metal-laden coal ash effluent has been detailed [5] (M.S. Wright et al., unpublished). Stepanauskas et al. [5] observed elevated tolerances to a range of metals and antibiotics in bacterioplankton collected from ash-settling basin water relative to bacterioplankton in intake water at three coal-fired power plants. Bacterial exposure to metals within the ash-settling basin was most probably the selective agent driving co-resistance [5]. Elevated metal and antibiotic tolerances across a gradient of metal contamination were also demonstrated in multiple stream microhabitats (M.S. Wright et al., unpublished). Sediment bacteria had the highest tolerance values to cadmium, nickel, ampicillin and tetracycline, followed by bacterial tolerance in biofilms, in the water column and from within the digestive tract of the Asiatic clam, Corbicula fluminea. These results suggest that benthic bacteria are likely to be sources of antibiotic- and metal-resistance genes within stream ecosystems.

Experimentally induced co-selection

Experimental studies in which metal exposure is directly manipulated to test for co-selection in bacterial communities are rare. Berg et al. [63] found that soil microbes isolated from a copper-amended field were more resistant to copper and antibiotics than strains isolated from control plots 21 months after copper amendment. Additionally, copper-resistant strains were significantly more resistant to ampicillin and sulphonamide than copper-sensitive isolates, which strengthened the argument that the traits are co-selected. Experimental evidence for co-selection has been demonstrated using microcosms amended with a variety of metals or antibiotics, which resulted in an increased frequency of multiple resistance phenotypes (R. Stepanauskas et al., unpublished). For example, addition of cadmium to microcosms that contained freshwater bacterioplankton resulted in increased resistance not only to this metal but also to nickel, ampicillin and gentamycin (R. Stepanauskas et al., unpublished).

Mercury contained within dental amalgams has been linked to co-selection of antibiotic-resistant bacteria by studies that examined isolates from intestinal and oral bacterial communities [3, 16, 64]. Ampicillin resistance profiles in isolates obtained after dental amalgam installation indicated that resistance to ampicillin increased relative to pre-installation levels [3]. In a subsequent study, resistance to multiple antibiotics was more common in mercury-resistant isolates compared with those that were sensitive to mercury. Furthermore, the number of antibiotics to which isolates were resistant correlated to the harbored mer locus, which is probably a result of the location of the mer operon on an integron-containing transposon such as Tn21 [16]. Other studies have found no clear association between dental amalgam presence and antibiotic resistance [65, 66].

Concluding remarks and future research challenges

It is well established that there is a clear association between heavy antimicrobial consumption within a population and the frequent recovery of antibiotic-resistant bacteria [67]. However, it is apparent that a range of other agents might represent important mechanisms that drive the selection of antibiotic-resistance determinants [4]. These observations have direct public health implications considering that some pathogenic strains of several bacterial genera such as Vibrio, Enterobacter and Pseudomonas have established environmental reservoirs, and resistance genes can be laterally transferred from environmental organisms to human commensals. Toxic metals that are present in biocides used in agricultural settings (such as copper amendments to soil) and antiseptics that contain mercury (i.e. thimerosal) and silver (i.e. silver sulfadiazine) have been used extensively. Clearly, agricultural and clinical settings are two environments in which the proliferation of antibiotic resistance is of particular concern. Unfortunately, the majority of studies that demonstrate co-selection at the population and community level are observational and have relied heavily on culture-dependent phenotypic analysis of isolates, making quantitative analyses of co-selection in microbial communities difficult. In addition, many studies fail to adequately address the role of metal exposure as a mechanism for increasing the incidence and propagation of antibiotic-resistance determinants within a framework of ecological relevance (i.e. distinguishing changes in the population and community structure of microbial populations exposed to metals while simultaneously quantifying the abundance, transfer and retention of antibiotic-resistance systems in these given environments). Current advances in microbial genomics, physiology and biochemistry could provide the basis for the precise determination of important processes involved in metal–antibiotic resistance interactions. Areas of particular interest include the multifunctional properties
Box 1. Major uncertainties and future research opportunities in metal–antibiotic co-selection

(i) What are the dominant mechanisms of co-selection for metal- and antibiotic–resistance at the population and community level? Comprehensive genetic, transcriptional and biochemical studies of diverse microorganisms and extrachromosomal elements that encode multiple resistance factors are needed.

(ii) Do metals maintain a pool of horizontally transferable antibiotic-resistance determinants and, if so, are these genes available to clinically important pathogenic bacteria? Future research requires a simultaneous analysis of the environmental antibiotic-resistance gene pool and the movement of genes to organisms of clinical importance.

(iii) What are the in situ rates of horizontal gene transfer (HGT)? Do metals stimulate HGT of antibiotic–resistance factors? Several novel, culture-independent techniques are now available to study HGT in the environment, including fluorescent reporter genes, quantitative real-time PCR and fluorescent in situ hybridization.

(iv) Do metal–contaminated environments that lack anthropogenic contributions of these resistance systems to the fitness of bacteria in different environmental and clinical settings (Box 1). In addition, there are few studies that link molecular mechanisms of co-selection to population and community level effects. It is necessary to evaluate potential mechanisms at several levels of biological organization to comprehensively assess the role of metal contaminants as a selective force in maintaining and propagating the pool of antibiotic-resistance determinants in the environment (Box 1). Other toxicants have been implicated in the co-selection and propagation of antibiotic resistance, which further obscures the role of metals, particularly in settings where mixtures of contaminants can function additively or synergistically to co-select for antibiotic resistance. Therefore, a detailed delineation of the complex inter-relationships between metals and antibiotics is required to provide a cohesive and rigorous understanding of the persistence and proliferation of antibiotic resistance.

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References

18 Khodolli, G. et al. (2003) Thn5060 from the Siberian permafrost is most closely related to the ancestor of Tn21 prior to integron acquisition. FEMS Microbiol. Lett. 226, 251–255
23 Tauch, A. et al. (2003) The 79 370 bp conjugative plasmid pB4 consists of an IncP-1 β backbone loaded with a chromate resistance transposon, the strA-strB streptomycin resistance gene pair, the oxacillinase gene bla(NPS-1), and a tripartite antibiotic efflux system of the resistance-nodulation-division family. Mol. Genet. Genomics 268, 570–584
30 Harrison, J. et al. (2005) Persister cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environ. Microbiol. 7, 981–989
30 Huysman, F. et al. (1994) Effect of manuring practices and increased copper concentrations on soil microbial populations. Soil Biol. Biochem. 26, 103–110