Bacterial antimicrobial metal ion resistance.

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Abstract

Metals such as mercury, arsenic, copper and silver have been used in various forms as antimicrobials for thousands of years, with until recently, little understanding of their mode of action. The discovery of antibiotics and new organic antimicrobial compounds during the twentieth century saw a general decline in the clinical use of antimicrobial metal compounds, with the exception of the rediscovery of the use of silver for burns treatments, and niche uses for other metal compounds. Antibiotics and new antimicrobials were regarded as being safer to the patient and more effective than the metal-based compounds they supplanted.

Bacterial metal ion resistances were first discovered in the second half of the twentieth century. The detailed mechanisms of resistance have now been characterized in a wide range of bacteria. As the use of antimicrobial metals is limited, it is legitimate to ask: are antimicrobial metal resistances in pathogenic and commensal bacteria important now? This review will detail the new, rediscovered and 'never went away' uses of antimicrobial metals; will examine the prevalence and linkage of antimicrobial metal resistance genes to other antimicrobial resistance genes; and will examine the evidence of horizontal transfer of these genes between bacteria. Finally, it will discuss the possible implications of the widespread dissemination of these resistances on re-emergent uses of antimicrobial metals and how this could impact upon the antibiotic resistance problem.

Introduction

Metals and metalloids have had a long empirical history of human usage in medicine or agriculture, as reviewed below; despite problems of host toxicity, or doubts about their efficacy. Even now, a few toxic metal(loid) compounds are still first-line drugs or preferred choice chemotherapeutics or antimicrobials; although the use of most of the previously popular antimicrobial metal(loid)s such as mercury and arsenic/antimony compounds has been reduced or phased out in the past fifty or so years. Other metals such as silver and copper still have limited uses in agriculture and medicine, but are increasingly being included in consumer products, from clothing to computer keyboards, and are being promoted as useful additions to our arsenal of antimicrobials. Against this background of their current usage, it is reasonable to ask: **What is the relevance of antimicrobial metals, and bacterial resistances to them to medical microbiology in the 21st century?**

Any attempt to address this question must be set against the backdrop of widely known problems and opportunities: We are faced with new and emerging opportunistic nosocomial and community acquired pathogens; and increasing epidemic and pandemic multidrug resistant (MDR) pathogens. There is a recognition that the antibiotic discovery pipeline has not delivered significant quantities of new antibiotics in the past few decades, and new formulations and uses for antimicrobial metals as weapons in the antimicrobial armoury are being proposed (Annual Report of the Chief Medical Officer, 2011; Lemire et al., 2013). The recent recommendation by the Chief Medical Officer that antimicrobial resistance should be added into the UK National Security Risk Assessment (https://www.gov.uk/government/news/uk-antimicrobial-resistance-strategy-published--2) provides a timely backdrop to a discussion about resistance to antimicrobials that have been in clinical, non-clinical, and agricultural use for far longer than antibiotics have been.

This review will briefly discuss a wide range of antimicrobial metals but will concentrate on a limited number of the historically most important and most widely used (copper (Cu), silver (Ag), mercury (Hg), arsenic (As) and antimony (Sb)), and the microbial resistances to them. In this article we examine the past and current uses of antimicrobial metals, and the importance of the genetic legacy and dissemination of bacterial resistance to antimicrobial metals in bacteria. In particular we will discuss the genetic elements carrying multiple antimicrobial resistances, both to metals and antibiotics.

Metals in medicine and agriculture - past and present uses

Arguably the most important uses of metals and metalloids in medicine and agriculture have been as biocides and antimicrobials.

Probably the most commonly used toxic metals or metalloids in medicine and agriculture have been: mercury (Hg), copper (Cu), silver (Ag), arsenic (As) and antimony (Sb), and will be dealt with in detail in this review. Other inorganic or organic metal compounds such as lead (Pb) (Lenihan 1988, Trotter, 1990), tin (Sn) (Barnes and Stoner, 1959; Cooney and Wuertz, 1989), zinc (Zn) (Aarestrup and Haasman 2004), bismuth (Bi) (Mahony et al., 1999; Yang and Sun 2007; Ge and Sun 2007), gold (Au) (Novelli et al., 1999; Ray et al., 2007), cerium (Ce) (Garner and Heppell 2005), palladium (Pd) (Ray et al., 2007), tellurite (Te) (Taylor 1999), thallium (Tl) (Kazantzis, 2000) and gallium (Ga) (Chitambar, 2010) have also been investigated or had limited use as antimicrobials. Of these less heavily used antimicrobial metals, zinc, bismuth, and tin are still in common use in consumer products. Although zinc is an essential element required for life and is found in many enzymes, zinc ions can be effective as antimicrobials even at low concentration. Zinc compounds have been described since at least Roman times as an ancient ingredient in eye disease treatment, and zinc tablets were found in a small medical container dating back to 140-130BC retrieved from a Roman shipwreck (Giachi et al., 2013). Current use of some of these metals include the use of zinc oxide as a mild antiseptic most often used topically to protect against

diaper/nappy rash or skin irritation. Zinc compounds are also found in toothpastes (Zinc chloride) and shampoos (zinc pyrithione), and used as a growth promoter/treatment for postweaning diarrhea in animal feeds (Hasman et al., 2006). Stannous fluoride is used in toothpastes, and bismuth subsalicylate is used to treat diarrhea and other digestive system disturbances (Lemire et al., 2013).

In addition, compounds containing gold (Au), platinum (Pt), palladium (Pd), vanadium (V), rhodium (Rh), titanium (Ti), iridium (Ir) and other rare metals have been used recently in medical diagnostics or imaging; as radiotherapeutics; or as anti-arthritis and anti-cancer therapeutics (Abrams and Murrer 1993; Guo and Sadler, 1999; Xin Zhang, and Lippard, 2003; Desoize, 2004).

The medical and agricultural uses of mercury, copper, silver, arsenic and antimony as antimicrobials are discussed in detail below.

Mercury

This element has no known positive role in cellular function and the toxicity to humans of mercury and inorganic mercury compounds have been known since the First Century AD (Lenihan, 1988). The very high levels of toxicity of ethyl and methyl- mercury compounds to humans have been known since they were first synthesized in the laboratory in the mid-19th Century, when two laboratory assistants died several weeks after helping to synthesize dimethylmercury. Even so, organic and inorganic mercury compounds have been widely used in agriculture and medicine. Organic compounds containing mercury were used in agriculture to control plant diseases from the late nineteenth century until the 1970's, with aryl- , aloxyl-, and alkyl- organomercurials becoming widely used in the 1950's, particularly as antifungal seed dressings, but also as pesticides and fungicidal sprays (Huisingh 1974). Antifungal methylmercury cereal seed treatments resulted in death when treated wheat was consumed by humans in Guatemala and Iraq (briefly summarized in Hobman and Silver, 2007), and mortality and reproductive failure of seed eating birds has also been linked to organomercurial seed dressings.

Use of organomercurial seed dressings was discontinued because of these problems.

Inorganic mercury compounds have been used in a variety of medicines: as a laxative, diuretic, and antidepressant, but also to treat sexually transmitted diseases, skin disorders, and as a topical antimicrobial since at least the 15th century, when inorganic salts of mercury or mercury metal were primarily used to treat syphilis- either as an ointment, or fumigant (Hobman and Brown 1997). The effects of the treatment were only slightly less unpleasant than the disease, and probably futile. Mercury metal, (Hg, hydrargyrum); Mercuric chloride (corrosive sublimate; $HgCl_2$), mercurous chloride (calomel; Hg_2Cl_2), and mercury nitrate ($Hg(NO_3)_2$) have been used as the active ingredient in many medical treatments. Included in these uses was the 19th century universal remedy, the blue mass (or blue pill) used for treating everything from tuberculosis to parasites, but most famously in the Royal Navy of the Napoleonic Wars for treating constipation, in conjunction with the black draught. In hindsight, it seems strange that although the toxic effects of mercury on humans had been known since antiquity, mercurous chloride was commonly used in baby teething powders in the Anglo-Saxon World and in "Wurmschokolade" in continental Europe in the early twentieth century. Unfortunately this use of inorganic mercury compounds in these medicines led to Pink disease (acrodynia) in children (Black 1999). The known toxicity of mercuric ion compounds (particularly mercuric chloride) and doubts about their efficacy meant that mercuric chloride in the primary treatment of syphilis was replaced by Salvarsan® (arsphenamine) in the early 20th century, making redundant the aphorism "a night with Venus, a lifetime with mercury" (although mercury or bismuth was sometimes still used as an adjunct treatment to Salvarsan). After World War II antibiotics became the standard treatment for syphilis, but mercury use continued in diuretics (Hall, 1970) antiseptics and in organomercurial antimicrobial compounds in hospitals in the U.K. and America until the early 1970s (Porter et al., 1982) and until the 1990's in over-the-counter antiseptics and ointments (Golden eye ointment used to contain 1-3% mercuric oxide). Ammoniated mercury (NH_4HgCl) was being used to treat psoriasis, ringworm and impetigo in the 1970's (Foye, 1977) and may still be available in some countries. A

variety of organomercurial antimicrobial and antifungal agents such as nitromersol, mercurophen, phenylmercuric borate, phenylmercuric nitrate, and ortho-hydroxyphenylmercuric chloride have been used as disinfectants, preservatives and antiseptics. Even in 2014 over the counter 0.5% v/v chloramphenicol eyedrops bought in the UK contain 0.002% w/v phenylmercuric nitrate as a preservative. One of the most well-known of the organomercurial preservatives is thimerosal/ thiomersal, (Merthiolate™ - sodium ethylmercurithiosalicylate) which has been widely used as a topical antiseptic or preservative, and is still in use in the UK as a preservative.

Mercury containing antimicrobial usage is in decline, and is likely to be eliminated. The use of thiomersal/thimerosal as a vaccine preservative has been subject to vigorous debate, and controversy, and it has been banned in some countries. Other mercury containing disinfectants include merbromin (Mercurochrome) and nitromersol that have been superseded or withdrawn in the U.S. or Europe.

The largest current use of mercury in a healthcare associated role is in dental amalgam, which typically contains 43-54% Hg, 20-35% Ag, 15% Sn, 10% Cu, 2% Zn, depending on formulation (Franke 2007). There has been debate about the safety of mercury amalgam fillings and whether use of them has negative effects on human health or may select for mercuric ion resistant bacteria, although a recent ruling by the U.S. Food and Drugs Administration stated that dental amalgam was safe. In the UK dental amalgam can be used unrestricted, but there are limitations in its use in some other European countries, and bans in place in the Nordic countries.

Copper

Copper is an essential metal to aerobic forms of life, being involved in donating or accepting electrons in redox active enzymes, or in the electron transport chain (Solioz et al., 2010). Copper is also toxic to prokaryotes and eukaryotes at higher cellular concentrations (Gaetke and Chow 2003), and copper (and zinc)

involvement in phagosomal killing of bacteria engulfed by macrophages is being recognized as an important defence mechanism (see German et al., 2013) .

Copper compounds are used as wood preservatives, in antifouling paints, and as molluscicides (Borkow and Gabbay, 2009). In agriculture, copper compounds have been used as an antimicrobial, algicide, pesticide, and antifungal agent and as an animal feed additive. Copper sulphate solutions were used as an antifungal treatment of seed grains in the 18^{th} century. In the late 19^{th} century Bordeaux mixture (copper sulphate and calcium hydroxide) and Burgundy mixture (copper sulphate and sodium carbonate) were widely used to control mildew on grape vines and to control fungal and bacterial disease of seeds or plants (Bremner, 1998). These inorganic antifungal agents are still widely used in plant protection, even in "Organic" agriculture. Copper sulphate is allowed alongside zinc chloride, oxide or sulphate as an additive in animal and poultry feed. In the European Union copper sulphate can be added at up to 250 ppm in piglet feed, but also at 25 ppm in feed for slaughter weight pigs, 20 ppm in broiler chickens and 2 ppm in calves as a growth promoter (Barber et al., 1955) and for postweaning control of diarrhoea (Hasman et al 2006, Sapkota et al., 2007). Alongside copper sulphate, zinc oxide can be added at up to 2500ppm in piglet feed to control post-weaning diarrhoea.

The medical uses of copper and inorganic salts of copper go back at least 4000 years with copper or copper compounds being used as astringents, antiseptics and antifungals, to treat wounds, and to purify and sterilize drinking water, and in contraceptive intrauterine devices, (see Borkow and Gabbay, 2009). Inorganic and organic copper compounds have been used to treat a variety of skin diseases, syphilis, TB and anaemia amongst other maladies (Grass et al., 2011). There is also interest in copper containing wound/ulcer dressings that have been trialled and reported to be effective (Borkow and Gabbay 2009; Borkow et al., 2010). Various laboratory and clinical studies have confirmed that solid copper/ copper alloy surfaces promote rapid killing of Gram-negative and Gram-positive bacteria. Most recently, the use of copper antimicrobial solid surfaces to reduce microbial contamination and transmission of hospital-acquired infections has progressed to clinical trials, with the installation of copper containing surfaces and fixtures in

wards and clinics. Reduction in microbial numbers, and therefore cross contamination has been seen (Casey et al., 2010, Marais et al., 2010, Mikolay et al., 2010). Copper usage in consumer items is perhaps less common than silver, but includes the use of copper oxide impregnated bedding to control house dust mites and socks to treat Athlete's foot (Borkow and Gabbay 2009). Antimicrobial copper surfaces and products may also appear in products available to the domestic market, now that the U.S. EPA has registered copper and copper alloys as public health antimicrobial products.

Silver

There is no known beneficial role for silver in metabolism, and it is highly toxic to bacteria (Nies 1999). We have not been able to find any evidence in the literature for the use of silver compounds as antimicrobials in agriculture, except for the use of silver iodide (AgI) in cloud seeding, but the first use of silver as an antibacterial is reported to have occurred over 2000 years ago in drinking water containers (Silver, 2006), and silver is still widely used in water filters and in other treatments for potable water, or as an algicide for swimming pools.

Medically, silver nitrate, (lunar caustic; $AgNO_3$) was used empirically to treat ulcers and burns in the 17^{th} -19^{th} centuries, and as a cauterizing agent. It was understood in the late 19^{th} century that metallic silver and silver nitrate had antibacterial properties, with metallic silver foil and silver nitrate solutions being used to treat fresh and infected burns and wounds, or silver wire being used to suture surgical wounds. Following the success of arsphenamine (Salvarsan®) in combination with mercury or bismuth salts as a treatment for syphilis (see above and below), Silver arsphenamine (Neo silvol®) by injection into the spine was used in the 1920's as treatment for neurosyphilis. 2% silver nitrate solution has also been used in treating warts and eye infections and as a prophylactic against gonorrhoeal *ophthalmia neonatorum* (Klasen 2000a), and silver metal is a major component of dental amalgam.

The introduction of sulphonamide in the 1930s and antibiotics in the 1940s appears to have led to an almost complete disappearance of interest in the use of silver and silver salts in burn and other treatments, until the 1960's, when Moyer and co-workers looked for antimicrobial agents that prevented invasive burns infections (Moyer et al., 1965). Combinations of 0.5% silver nitrate and Sulphamylon® became popular burns treatments in the mid 1960's and silver sulphadiazine (Flammazine®, Silvadene®)(SSD) was developed shortly after by Fox and co-workers as a burn treatment. SSD is a common treatment for serious burns (reviewed in Klasen 2000b). More recently, silver impregnated dressings and antimicrobial coatings have been used in infection management, stimulation of healing, wound management and treatment of infected wounds, and as antimicrobial coatings in catheters and endotracheal breathing tubes (Silver, 2003; Silver et al., 2006; Chopra 2007, Mijnendonkx et al., 2013).

Silver is generally viewed as a benign metal, and the only widely reported negative health effects of silver to humans have been eschar formation on burns treated by silver, staining or destruction of skin cells when silver nitrate is directly applied for treatment of warts, sometimes elevated silver levels in blood, and the rare argyria and argyrosis in people who self-medicate colloidal silver solutions (Silver 2006). There is some concern about silver and silver nanoparticle toxicity to other (particularly aquatic) organisms (Panyala et al., 2008; Chaloupka et al., 2010), initially based upon the premise that silver nanoparticles were new materials that had not been encountered in nature before, with counter arguments that silver nanoparticles have been produced in colloidal silver preparations for over a century and the majority of approved silver biocides release nanosilver (Nowack et al., 2011). Copper/silver ionization treatments have been used in hospital water supplies and the International Space Station has silver coated water tanks (Van Houdt et al 2012; Mijnendonkx et al., 2013)

One quite noticeable increase in the use of antimicrobial metal products, is the use of silver in consumer and "lifestyle" products. In the past 20 years or so silver-containing plasters, clothes, water filters, personal hygiene and consumer products have appeared worldwide (Silver, 2003; Silver and Phung, 2006; Edwards-Jones,

2009, Minendonkx et al., 2013), and the use of antimicrobial silver nanoparticles in products is also growing (Chaloupka et al., 2010) including examples where they have been integrated into household items such as computer keyboards, washing machine drums, air conditioners and refrigerators.

Arsenic

Arsenic has been used for at least 2000 years as a medicine, cosmetic, tonic, or as a poison. Arsenic trioxide (As_2O_3), (also known as Ratsbane, Inheritance powder or poudre de succession) is colourless and flavourless when put in food or drink and was popular as a rat poison. Prior to the advent of sensitive and accurate chemical tests for arsenic, such as the Marsh test, it is believed that arsenic trioxide was also a popular choice for poisoning people, especially as the symptoms of arsenic poisoning somewhat resemble cholera, and post-mortem toxicology was weak/non-existent. Organic arsenic compounds such as Lewisite (2-chloroethenylarsonous dichloride) and Adamsite (Dibenzo-1-chloro-1,4-arsenine) as well as a range of other organoarsenic halides have also been developed as chemical warfare agents.

Agricultural and non-medical uses of arsenic compounds have included arsenical wood preservatives (particularly chromated copper arsenate-CCA), herbicides, rodenticides, defoliants (Agent Blue used in the Vietnam war was a mixture of dimethylarsenic acid and its sodium salt (Cooksey 2012)), and fungicides. Prior to the introduction of organic pesticides; arsenic compounds such as lead arsenate and Paris green (Copper (II) acetoarsenite) were used as a rodenticides and insecticides. Copper-arsenic and lead-arsenic compounds were used widely as insecticides in orchards from the 1930s to the 1980s, and calcium arsenate and dimethylarsenate were widely used as pesticides (Oremland and Stolz, 2003). Organic arsenic compounds: Carbarsone (4-Carbamoylaminophenylarsonic acid), Nitarsone (4-nitrophenylarsonic acid), and Roxarsone (3-nitro-4-hydroxyphenylarsonic acid) have been used as feed additives for poultry in the United States acting as growth promoters and in controlling coccilobacillosis disease

(Jones 2007). Only very recently (June 2011), has the U.S. FDA announced the voluntary suspension of the sale of Roxarsone due to the presence of inorganic arsenic residues in chicken meat from chickens fed on Roxarsone supplemented feeds.

In medicine, arsenic oxide (white arsenic: As_2O_3), arsenic sulphide (red realgar: As_4S_4), and arsenic trisulphide (yellow orpiment: As_2S_3) have variously been used as antispasmodics, sedatives, hematinics, for treating skin disorders, as eye and cancer treatments, in the treatment of trichomoniasis, malaria, ulcers, and syphilis as well as a wide range of other ailments (Liu et al., 2008). Arsenic compounds were so widely used in the 18th century that it became known as the "Therapeutic Mule" (Przygoda et al., 2001). Fowler's solution was a very well-known inorganic arsenical medicine (1% arsenic trioxide in potassium carbonate with tincture of lavender) which was still being used even after World War II as a tonic and treatment for malaria, syphilis and chorea (Przygoda et al., 2001).

In the early part of the 20th century the organic arsenic compound Salvarsan, ('the arsenic that saves') was probably the best-known arsenic compound used in medicine. Salvarsan (compound 606, arsphenamine) and subsequently Neosalvarsan® (compound 914, neoarsphenamine) were developed by Ehrlich and co-workers primarily to effectively treat syphilis. Later, it was realized that once administered by injection, arsphenamine oxidized to oxophenarsine (later given the trade name Mapharsen®) that was subsequently used as the drug of choice in syphilis treatment until the introduction of penicillin (Bosch and Rosich, 2008). However, programs for the treatment of syphilis with organic arsenic compounds could last for 18 months, had serious side-effects and often also required alternating with bismuth or mercury treatments. Silver arsphenamine and silver neoarsphenamine and bismuth arsphenamine sulphonate also found therapeutic use (Gibaud and jaouen, 2010). Tryparsamide was the first arsenical that was clinically effective in treating African sleeping sickness (Trypanosomiasis), but resistance in *Trypanosoma brucei* was reported in the early 1930's, a decade after introduction of the drug. The arsenical compounds melarsoprol (Arsobal®) and melarsonyl are still used to treat sleeping sickness, and have been used to treat

other diseases including amoebic dysentery, despite serious side effects including blindness (Joliffe 2003; Jones 2007, Gibaud and Jaouen 2010), while others such as arsenilic acid (Atoxyl, 4-Aminophenylarsonic Acid), have been largely discontinued as treatments due to their toxicity (Gibaud and Jaouen, 2010). Carbarsone was introduced as an antiprotozoal organoarsenical in the early 1930's, followed by diphetarsone and arsthinol in the 1950's. They were withdrawn from market in the 1990's because of the association of arsenic exposure to a variety of abnormal growths/tumours (Gibaud and Jaouen 2010).

In higher organisms arsenic is carcinogenic, with a range of potential mechanisms involved including genotoxicity, DNA methylation and cell proliferation alterations, oxidative stress, co-carcinogenesis and tumour promotion (Hughes, 2002). Despite the reduction in use of arsenic as an antimicrobial there has been renewed interest in arsenic as an anticancer drug. In the mid-1990s arsenic trioxide was investigated as a treatment for acute promyelocytic leukemia (APL) and received U.S. FDA approval in 2000 as a sterile injectable arsenic trioxide solution TRISENOX® (Slejkovec et al 2011).

Antimony

Antimony may have been used as long as arsenic has been in medicine. In agriculture, tartar emetic (antimony potassium tartrate; $C_4H_4KO_7Sb \cdot 1/2\ H_2O$), has been used in the treatment of leishmaniasis, schistomiasis, trypanomiasis, bilharziasis and ascariasis in domestic and farm animals in the 19th and early 20th centuries, and tartar emetic was also used as a pesticide spray on crops.

Antimony was used as a cosmetic or in ointments in skin treatments in biblical times, and became popular in medicine during the 18th Century with uses of it including treatments for smallpox, syphilis, dropsy and agues (McCallum 1977). The toxic properties of antimony metal were clearly established in the 16th century and the powerful emetic effect of antimony was known in Roman times. This property of antimony was exploited in the 17th and 18th centuries to induce therapeutic vomiting, sweating and purging through ingestion of antimony either from drinking

wine which had stood for 17-24 hours in an antimony cup, or through swallowing a "perpetual pill" made from antimony, which soon reemerged from the patient. Tartar emetic was also used to induce vomiting in patients, and in tropical medicine, tartar emetic has been used as a treatment for schistomiasis. Other antimony compounds are still used as first-line treatment of visceral leishmanniasis and as treatments for schistomiasis in humans (Sadler and Guo 1999; Ashutosh et al., 2007; Ge and Sun 2007; Sundar and Chakravarty, 2010; Perry et al., 2011), though resistance to antimony drugs in *Leishmania donovani*, and *Leishmania infantum* in the Bihar region of the Indian subcontinent is now very high. Resistance in *L. donovani* to sodium stibogluconate (Pentostam) and meglumine antimonite (Glucantime) has been shown experimentally to be as a consequence of exposure of *L. donovani* in a mouse model to levels of arsenic equivalent to those that humans are exposed to in arsenic contaminated drinking water from Bihar (Perry et al., 2013).

Metal ion toxicity

Despite the documented historical use of antimicrobial metals, understanding of the detailed toxic effects of different metal ions and metalloids on bacteria are arguably incomplete. However, it is clear that the chemistry of the metals drives the biology, in terms of metal bioavailability, the biological effects that a metal will have on cells, and the resistance mechanisms that bacteria can use to detoxify or remove the metals.

Mechanisms of metal toxicity are generally agreed to be as a consequence of the metal ions' affinity for cellular components and biomolecules, or the stability of metal-biomolecule complexes formed, although the consequences can be varied. Metals and metalloids can exert toxic effects in a number of different ways: by binding to or blocking functional groups in biological molecules, by displacing essential metals in enzymes, binding to the cellular thiol pool, or participating in chemical reactions in the cell that are harmful. Ultimately the deleterious effects reported include damage to proteins, DNA and biological membranes, interference

in enzyme function and cellular processes, and oxidative stress (Nies, 1999; Hobman et al., 2007).

There have been different attempts to group metals based on their ligand affinity or toxicity, leading to rather vague classifications like "heavy metals" or "toxic metals" (Duffus 2002). The two classifications that are the most widely accepted descriptors of the potential for interactions of metal ions with biological ligands are the Irving-Williams series of divalent metal ion ligand affinities, and the classification of metals into Lewis acids. The Irving-Williams series of ligand affinity for essential divalent metal ions clearly demonstrates the affinity of biological molecules for first row transition metals: $Ca^{2+} < Mg^{2+} < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}$ and shows that divalent copper has a strong affinity for biological molecules, suggesting that it can displace other metals from the first row of transition metals from them (Waldron and Robinson, 2009). Another way of measuring the toxicity of metal ions is to consider their strength as Lewis acids. Hard Lewis acids (small, non-polarizable electron sheath) prefer ionic coordination to oxygen containing ligands. Soft Lewis acids (with a large, polarisable electron sheath) prefer covalent coordination to soft Lewis bases; primarily S and N ligands: cysteine sulphydryls and nitrogen imidazoles. Intermediate Lewis acids will relatively stably coordinate to hard and soft donor ligands (Table 1). The metals and metalloids that are known to be toxic are largely but not exclusively soft Lewis acids which are likely to be able to displace intermediate and hard Lewis acids from cysteine sulphydryls because of their higher affinity for them.

In addition to effects caused by the higher affinity of soft Lewis acids for ligands, oxidative stress is one other proposed mechanism of toxicity for some metals. Redox-active metals such as Cu, Cr, Fe and V, as well as redox-inactive metals and metalloids such as As, Cd, Hg, Ni, Pb and Sb can be involved in cellular oxidative stress damage. Although arsenate and mercuric ions can be reduced intracellularly they do not catalyse one electron transfer reactions and consequent free radical generation, such as copper, iron, chromate and vanadate do. For redox-active metals, generation of hydroxyl radicals *via* Fenton-like reactions is believed to be the probable mechanism by which oxidative stress occurs. For redox-inactive

metals and metalloids the potential mechanism of oxidative stress generation is that they bind to and inactivate cellular thiols, which normally quench reactive oxygen species that are generated during normal cellular metabolism, or can be redox metal catalysed, or metal-catalyzed oxidation of reduced glutathione can also generate hydrogen peroxide. Recent evidence suggests that iron-sulphur clusters in enzymes are key targets for toxic metals (Hobman et al., 2007; Macomber and Imlay 2009; Xu and Imlay 2012)

The broad mechanisms of toxicity for each of the commonly used antimicrobial metals are given below:

Mercury

Mercury is the most toxic metal to *Escherichia coli* (Nies 1999). Mercury toxicity has been attributed to the inactivation of enzymes and interference with other protein functions by the tight binding of mercuric ions to thiol and imino nitrogen groups in them, or displacement of other metal cofactors from enzymes. Mercuric ions also bind to nucleotides and lipids, interfering with DNA function and contributing to lipid peroxidation. Mercuric ions and organomercurials have the ability to rapidly pass through biological membranes, and organomercurials are highly lipid soluble (Clarkson and Magos, 2006).

Copper

Copper carries out an essential role as an electron donor/acceptor in many enzymes, but copper can also take part in Fenton-like reactions leading to the generation of hydroxyl radicals, hydrogen peroxide and superoxide, which can cause cellular damage (reviewed in Grass et al., 2011). This has been generally accepted as the major mechanism for Cu toxicity. However, recent experimental evidence from experiments in liquid culture has shown that copper mediated ROS generation occurred largely in the periplasm of *E. coli*, so the importance of ROS generation by copper as a cellular toxicity mechanism has been under debate

(Macomber et al., 2007). Gram-positve bacteria lack a periplasm, and although many are tolerant to hydrogen peroxide (Solioz et al., 2010), recent evidence from *S. aureus* shows oxidative stress resistance and protein misfolding repair transcriptional responses, and hydrogen peroxide scavenging defence (Baker et al., 2010). According to the Irving-Williams series copper has a higher affinity than other first row transition metals for ligands, and displacement of Fe from Fe-S clusters by copper in liquid culture experiments has been reported to be an important mechanism of copper toxicity (Macomber and Imlay 2009). There is also a role for copper and ROS in phagosome killing of bacteria (Reviewed in German et al. 2013)

The rapid killing of bacteria on solid copper surfaces is thought to be due to cellular damage caused by very high local concentrations of copper dissolving from the surface, which causes membrane rupture, coupled with ROS generation causing further cellular destruction including degradation of plasmid and chromosomal DNA (Grass et al., 2011).

Silver

Silver (as well as gold) is the second most toxic metal to *E. coli* (Nies 1999). Silver ions cause the inhibition of respiration, membrane damage, and destruction of the proton motive force. The interaction of Ag^+ with thiol groups in membrane proteins/enzymes is thought to be a major mechanism of toxicity, with data suggesting that the key toxicity event is interactions between Ag^+ and respiratory chain enzymes (Holt and Bard 2005). Proteomic studies have shown that ionic and nanoparticle silver causes destabilization of the outer membrane, collapse of the cytoplasmic membrane potential and depletion of intracellular ATP levels in *E. coli*, consistent with interference with the respiratory chain (Lok et al. 2006; Du et al. 2012). Other evidence suggests that although still toxic to bacteria under anaerobic conditions, under aerobic conditions intracellular Ag^+ ions also cause reactive oxygen species (ROS) generation and interference with DNA replication (Park et al., 2009), increased membrane permeability and increased sensitivity to

antibiotics (Morones-Ramirez et al., 2013). There is some disagreement on which ROS are important in this mechanism of Ag^+ mediated damage. Park and co-workers suggest Ag^+ ion mediated superoxide radical generation in *E. coli* and *S. aureus* (Park et al., 2009), whilst in *S. epidermidis* Gordon and co-workers suggest generation of hydroxyl radical ions through release of iron from proteins by Ag^+ ions binding sulphydryl groups, leading indirectly to hydroxyl radical formation (Gordon et al., 2010). Other work in *Vibrio cholera* showed that low levels of Ag^+ causes collapse of the proton motive force, proton leakage, and the cytoplasmic membrane is the major target for low levels of silver ions (Dibrov et al., 2002) and in *Staphylococcus aureus* silver cations also cause rapid and extensive loss of membrane integrity (Randall et al., 2012).

Arsenic and antimony

Arsenic toxicity depends on the nature of the arsenic compound. Inorganic arsenic toxicity is through allosteric inhibition of essential metabolic enzymes, with arsenite being more toxic than arsenate (Cooksey 2012). Arsenate is an analogue of phosphate and can enter cells via phosphate uptake systems and inhibits oxidative phosphorylation. Arsenite can enter cells via aquaglyceroporins, and binds to sulphydryl groups in proteins, and has been reported to bind to the vicinal thiols in pyruvate dehydrogenase and 2-oxo-glutarate dehydrogenase, affecting cellular respiration (Oreland and Stolz 2003). There is evidence that the presence of arsenic in cells leads to the generation of reactive oxygen and nitrogen species. One known mechanism for this is that Arsine (ASH_3) and methylated derivatives can generate methylarsinyl peroxyl radicals, which damage DNA (Cooksey 2012), but inorganic arsenic has also been implicated in reactive oxygen species generation, and disruption of signal transduction pathways (Kumagai and Sumi, 2007). Arsenic and antimony share some chemical and toxicological properties, and therefore may share modes of toxicity.

Bacterial metal ion homeostasis and resistance to toxic metals

The natural exposure of bacteria to bioavailable metals (both essential and toxic) has occurred over billions of years since the expansion of oxic environments that accompanied the great oxidation event (Barkay et al., 2010) and this exposure has likely been the driver for the evolution of the ability of microorganisms to control cellular levels of these bioavailable oxidised metal ions. Sometimes, these metals are found in high concentrations due to volcanic activity or other natural geological events. Bacteria have also been exposed to lethal concentrations of these metals through anthropogenic releases of toxic metals into the environment through mining, smelting, manufacture, fossil fuel burning and numerous other industrial applications, often at high localized concentrations; as well as the deliberate use of metals as antimicrobials and pesticides. Thus bacteria have evolved mechanisms to acquire essential metals, control the intracellular levels of these metals, and eliminate metals that in excess are deleterious. Similarly systems for removing from the cell, or modifying, purely toxic metals have also evolved and have been selected.

Antimicrobial metals have multiple and different cellular targets, and there are limited options available for bacteria to mitigate or nullify the effects of metal toxicity. Therefore, the potential resistance strategies that they can employ are limited to extracellular or intracellular sequestration of the metal, reduction in permeability, alteration of target sites, enzymatic detoxification, or efflux of the metal ions (Hobman and Brown 1997). These resistance mechanisms are conceptually similar to the possible mechanisms of antibiotic resistance (Courvalin 2008). Most of the mechanisms of resistance to metals that have been well characterized at the genetic level in bacteria are enzymatic detoxification, or efflux of the metals from the cell. This is because unlike organic antimicrobial compounds which can be broken down or inactivated by enzymatic cleavage, metals are immutable, and bacterial metal import systems or porins are not sufficiently discriminatory to allow in to the cell only metal ions that are required, and metal ion chaperones may also be subverted to bind to the "wrong" metal.

Mechanisms of antimicrobial metal resistance.

Although mechanisms such as methylation or demethylation of metals, (which are often by-products of normal cellular metabolism) or generalized antimicrobial efflux through multidrug efflux systems, and stress response mechanisms, may contribute to fortuitous metal ion tolerance /resistance or damage repair, specific metal ion resistance mechanisms are usually characterized by a metal ion specific response regulator, which controls the expression of structural resistance genes. The products of these genes produce a metal ion specific efflux protein or protein complex, and/or enzyme(s) which alter the metal ion into a less toxic form to the bacterial cell. There may be other proteins encoded by the resistance mechanism, their functions ranging from metal ion chaperone to metal ion transporter or metal ion reductase. The simplest general mechanism of resistance is therefore a metal specific regulator, which controls expression of a metal ion efflux system.

The specific resistance mechanisms for Hg, Cu, Ag, As/Sb will be discussed in detail below.

Mercuric ion resistance.

Resistance to mercuric ions is believed to be an ancient resistance mechanism, evolving after the biosphere became widely oxygenated, and has been found widely in bacteria and Archaea (Barkay et al., 2010). The mechanism of mercuric ion resistance to inorganic mercuric ions (narrow-spectrum resistance) is unusual for a metal ion resistance mechanism and counter-intuitive (Figure 1). Rather than direct efflux of the metal, the simplest inorganic mercuric ion resistance operon in Gram-negative bacteria, from Tn*501*, encodes proteins that chaperone divalent mercuric ions (Hg^{2+}) in the periplasm using MerP. Hg^{2+} ions are imported across the cytoplasmic membrane via MerT into the cytoplasm, where they are reduced to essentially non-toxic metallic mercury (Hg^0) by mercuric reductase (MerA). Metallic mercury is volatile at room temperature and pressure and leaves the bacterial cell by passive diffusion. Mercuric ion resistance is a very good example of how a

resistance mechanism is determined by the chemistry of the metal, as MerA requires reducing equivalents to reduce Hg^{2+} to Hg^0 and has to import Hg^{2+} to the cytoplasm in order to do this. MerP and MerT appear to prevent Hg^{2+} from damaging the cell during this process (Morby et al., 1995). In Gram-negative bacteria, regulation of the *mer* mercury resistance operon is through the activator MerR, with secondary regulation of the operon via MerD (reviewed in Brown et al., 2003). Resistance to organomercurials (or broad spectrum mercuric ion resistance) is conferred *via* organomercurial lyase (MerB). MerB cleaves the C-Hg bond in organomercurial compounds, working with a narrow spectrum *mer* operon, and is regulated by an organomercurial responsive MerR. MerE is an additional inorganic and organic mercury importer (Kiyono et al., 2009). Other Gram-negative mercuric ion resistance operons encode additional mercuric ion import proteins (such as MerC in the Tn*21 mer* operon (Sahlman et al., 1997) and MerF in pMER327/419 (Wilson et al., 2000; Hobman et al., 1994)). Most of the work on understanding mercury resistance has come from studies on the classic mercury resistances from Tn*501* and Tn*21* in Gram-negative bacteria.

The mechanism of mercuric ion resistance in Gram-positive bacteria is broadly the same as that in Gram-negative bacteria, but details of the regulation and mercuric ion import systems differ slightly. The *mer* operons in Gram-positive bacteria have been best characterized in the plasmid pI258 *mer* from *S. aureus* and in different *Bacillus* strains. The *S. aureus mer* resistance contains *merR*, *merA*, *merB* and *merT* homologues, and some additional open reading frames, as do the *Bacillus mer* resistance operons, which confer broad spectrum mercury resistance (Chu et al., 1992; Gupta et al., 1999). There is now evidence that mercury resistance in some *S. aureus* strains is carried on the $SCC_{mercury}$ element (*Staphylococcus* Cassette Chromosome reviewed in Malachowa and DeLeo, 2010). There are excellent and comprehensive reviews of mercuric ion resistance in bacteria (Summers et al., 2003; Barkay and Wagner-Döbler, 2005).

Copper homeostasis and resistance.

Copper homeostasis and copper resistance mechanisms have evolved because copper is an essential metal that can be toxic at higher intracellular concentrations, and copper is involved in host defence against pathogens.

Bacterial cells have systems that control "normal" levels of copper and others that confer resistance to very high levels of copper. In *E. coli*, there are two chromosomally encoded copper homeostasis mechanisms, the *cue* and *cus* systems, both of which have components that modify the charge in ionic copper, and efflux it (the model for the mechanism is shown in Figure 2). In the *cue* system, a MerR family copper responsive transcriptional activator, CueR, regulates expression of a copper efflux P1-type ATPase, CopA; and of CueO, a multicopper oxidase (Outten et al., 2000; Peterson and Moller, 2000; Stoyanov et al., 2001). In the *cus* system, a two component regulator CusRS activates expression of *cusCFBA*, CusCBA is a tripartite RND (resistance-nodulation-cell division) family silver/copper effluxer and CusF a periplasmic metallochaperone (Munson et al., 2000). Whilst the *cue* system is induced under very low external copper concentrations, the *cus* system has been reported to be induced under higher external levels of copper, and may be important under anaerobic conditions (Munson et al., 2000). The AcrD and MdtABC multidrug efflux pumps in *Escherichia coli* have also been reported to efflux Cu and other antimicrobials when NlpE, an outer membrane lipoprotein, which functions during envelope stress responses, is overexpressed (Nishino et al., 2010), and in *Salmonella*, enterobactin and TolC are involved in copper detoxification (Pontel et al., 2014).

In addition to the *cue* and *cus* systems some *E. coli* strains isolated from pigs fed on copper supplemented feed carry a plasmid-borne copper resistance system, *pco*, which confers additional copper resistance (Tetaz and Luke, 1983; Brown et al., 1995). The *pco* copper resistance from *E. coli* plasmid pRJ1004 contains seven open reading frames, designated *pcoABCDRSE* (Rouch and Brown 1997). The current model for the mechanism of resistance to copper salts conferred by *pco* is also shown in Figure 3. Gene expression from the *pco* operon is regulated by PcoRS, a two component regulator system homologous to the CopRS, CusRS and the SilRS regulators (from the pMG101 silver resistance plasmid) (Munson et al., 2000).

PcoR regulates expression of the *pcoABCD* genes from one promoter and *pcoE* from a separate promoter (Rouch and Brown 1997). PcoA, C and E are periplasmic proteins, PcoB an outer membrane protein and PcoD an inner membrane protein. PcoA is a multicopper oxidase, and may have a similar function to CueO, oxidizing Cu(I) to less toxic Cu(II). PcoC is a copper chaperone (Djoko et al., 2008) and PcoE may act as a periplasmic first line of defence copper 'sponge' protein, binding copper whilst the remainder of the Pco proteins are expressed (Zimmermann et al., 2012). PcoB is a predicted outer membrane protein that may interact with PcoA, and which could either oxidize Cu(I) to the less toxic Cu(II), or act to sequestrate oxidized catechol siderophores, which themselves could reduce cupric ions to the more toxic cuprous ions. (CueO may also act to prevent this by directly oxidizing catechols (Grass et al., 2004). PcoC and PcoD are required for full copper resistance (reviewed in Rensing and Grass 2003). Homologues of *pco* (albeit lacking some of the genes seen in the pRJ1004 *pco* resistance) and called *cop* have been identified in plant saprophytic and pathogenic bacteria from crops treated with copper fungicides (Bender and Cooksey, 1987; Cooksey et al., 1990).

Gram positive bacteria have a different copper homeostasis mechanism, which is probably best understood in *Enterococcus hirae*- (reviewed in Solioz et al., 2010). This mechanism involves import of copper into the cytoplasm via (a different) CopA, an ATPase; binding of excess cytoplasmic copper by a copper chaperone (CopZ), which donates it to either a copper export ATPase (CopB) or to CopY, which is a copper responsive repressor of gene expression for the *E. hirae cop* operon. The mechanism of copper homeostasis in *Lactococcus lactis* appears to be different as both CopA and CopB act as efflux ATPases. Recently a plasmid encoded a copper efflux ATPase and a multicopper oxidase have been found in *Listeria monocytogenes* (Kuenne et al., 2010). A CPx-type ATPase copper resistance efflux pump encoded by the *tcrB* gene has also been found on a conjugative plasmid carried by *Enterococcus faecium* from pigs and is related to the *copYZAB* operon in *E. hirae*. This has also found in farmed chickens and calves and is linked to macrolide and glycopeptide resistance (Hasman and Aarestrup 2002). CsoR, a copper sensing repressor regulates expression of the *copZA* promoter in response

671 to intracellular copper in *Bacillus subtilis* and *Staphylococcus* (Baker *et a*l., 2011;
672 Liu et al., 2007; Smaldone and Helmann 2007).

673 There are several excellent and comprehensive review articles on copper resistance,
674 which describe the genetics and biochemistry of resistance and the role of copper
675 resistance in pathogenicity, in great detail (e.g. Chaturvedi and Henderson, 2014;
676 Dupont et al., 2011; German et al., 2013; Osman & Cavet 2008; Rensing and
677 Grass 2003; Solioz et al., 2010).

678

679 **Silver tolerance and resistance**

680 Although bacterial silver resistance has been reported sporadically since the 1960's
681 (for reviews see Clement and Jarrett, 1994; Silver et al., 2006; Chopra 2007,
682 Mijnendonkx et al., 2013) the pMG101 *sil* system remains the only one
683 characterized in any detail at the genetic level. The 182 kb transferrable IncHI-2
684 group plasmid pMG101 from *Salmonella enterica* serovar Typhimurium was isolated
685 in 1973 from fatal infections in a burns unit in Massachusetts General Hospital,
686 Boston, USA. Plasmid pMG101 confers resistance to Cm, Ap, Tc, Sm, Su, Hg, Te
687 and Ag (McHugh et al., 1975; Gupta et al., 1999). The proposed silver resistance
688 mechanism has been predicted *via* DNA sequencing and comparison to the *E. coli*
689 *cop* and *cus* copper resistances. Several small subclones of the *sil* operon confer
690 partial silver resistance (Gupta et al., 1999). The *cus* system is known to confer
691 resistance to low levels of silver (and was called *agr* by Franke et al., 2001; 2003,
692 and *cus* by Munson et al., 2000)) and some of the *sil* genes from pMG101 are
693 closely related to the *cus* genes. There is 71% identity between SilC and CusC,
694 67% identity between SilB and CusB and 87% identity between SilA and CusA,
695 which form the efflux protein complexes SilCBA and CusCBA, respectively (Gupta et
696 al., 1999). The proposed mechanism of silver resistance is shown in Figure 4, in
697 which a two component silver responsive transcriptional regulation system SilRS
698 (homologous to CusRS and PcoRS) controls expression of a silver efflux ATPase,
699 SilP , the tripartite SilCBA silver effluxer and SilF, which is believed to be a
700 periplasmic silver chaperone. Several other genes or open reading frames are

present in the *sil* system. SilE has a role in periplasmic silver binding (Silver, personal communication) and there is a small open reading frame between *silA* and *silP* named *orf*105 which could encode a hypothetical protein of 105 amino acids, but which is of unknown function. There is another silver resistance system in the environmental bacterium *Cupriavidus metallidurans* CH34, which is composed of *silCBA* and located on one of two large plasmids, pMOL28 (Mergeay et al., 2003, Monchy et al, 2008)

Homologues of the *sil* system have been detected using *sil* specific primers in IncH1-2 group plasmids from Gram-negative bacteria (Gupta et al., 2001), in oral bacteria (Davis et al. 2005), in nosocomial isolates of *Enterobacter cloacae* (Kremer and Hoffmann, 2012), a silver resistant *E. cloacae* leg ulcer isolate (Sütterlin et al., 2012), some Gram-negative bacteria isolated from wounds (Woods et al., 2009) and surprisingly in *S. aureus* (Loh et al., 2009). There are also some reports of silver resistant pathogens which carry the *sil* genes that were isolated from burns units and even from the silver containing burns creams (Pirnay et al., 2003).

Arsenic and antimony resistance.

Arsenic resistance is very widespread amongst both Gram-negative and Gram-positive bacteria, probably reflecting the wide distribution of arsenic in the environment and its use as an antimicrobial (Silver and Phung 2005). Arsenic resistance was first identified in Gram-positive bacteria by Novick and Roth (1968), and in Gram-negative bacteria shortly afterwards.

Arsenic resistance operons in bacteria confer resistance to arsenite (AsIII), arsenate (AsV) and antimonite (SbIII). The minimum arsenic resistance operon consists of *arsR*, *arsB* and *arsC*, which encode respectively, an arsenite responsive trans-acting transcriptional repressor protein, an arsenite antiporter and an arsenate reductase. Some Gram-negative arsenic resistance operons (such as the *E. coli* plasmid-borne arsenic resistance carried on R773) also carry two additional genes: *arsD* and *arsA*. ArsA has an ATPase function, which binds as a dimer to ArsB forming an ATP energized effluxer, which is more efficient at arsenite efflux than

ArsB alone. ArsD has a minor role in transcription, but has recently been found to act as a metallochaperone for arsenite efflux via ArsAB (Lin et al., 2006). Previous work has shown that in the absence of ArsA, ArsB confers lower levels of arsenite resistance by translocating these ions into the periplasm using energy derived either from the proton pumping respiratory chain or from F_0F_1 ATPase (Dey and Rosen, 1995). The Gram positive arsenic resistance found on *S. aureus* plasmid pI258 is comprised of the simpler *arsRBC* system. The current model for arsenic and antimony resistance conferred by *ars* operons is shown in Figure 5.

There is an additional chromosomal arsenic resistance mechanism that has recently been found in some bacteria (e.g. *Alcaligenes faecalis*, *Thiomonas sp*.). This mechanism involves the use of arsenate as a terminal electron acceptor in the absence of oxygen, with a respiratory arsenite oxidase from the periplasm and a respiratory arsenate reductase converting respectively, arsenite to the less toxic arsenate (as part of a chemolithoautotrophic lifestyle), and acting as a terminal electron acceptor during anaerobic heterotrophic growth. Some of these arsenate-respiring bacteria also carry the classic arsenate resistance genes, and can tolerate very high levels of arsenate (Silver and Phung, 2005).

Antibiotic and antimicrobial metal ion resistances are often carried on mobile genetic elements in bacteria from the 'antibiotic era'.

Bacterial resistance to antimicrobial metals in clinically important bacteria was first reported in the early 1960's in *S. aureus* isolated from surgical wounds. This was attributed by Moore (1960) to the use of mercuric ions used to disinfect catgut used in sutures, and other workers to the use of mercury containing diuretics (Hall 1970) or disinfectants (Porter et al., 1982). Mercuric ion resistance (Hg^R) was then found to be genetically linked to *S. aureus* penicillinase plasmids (Richmond and John, 1964), and arsenic resistance was first identified in Gram-positive bacteria by Novick and Roth (1968) who found that *S. aureus* penicillinase plasmids carried resistance to arsenate, arsenite/antimony, lead, cadmium/zinc, mercury, and bismuth. Meynell and Datta (1966) isolated R (resistance) plasmids from clinical

761 *Escherichia coli* strains such as R46, which conferred tetracycline, ampicillin,
762 streptomycin, sulphonamide and arsenic resistance, whilst Smith (1967) also found
763 resistance to mercuric ions, nickel and cobalt in clinical isolates of *Escherichia coli*
764 and *Salmonella* sp. These resistances were later found to be located on plasmids or
765 mobile genetic elements such as transposons. Elek and Higney (1970) also
766 identified arsenic, mercury and copper resistance in *Escherichia coli* causing urinary
767 tract infections using resistogram typing. One of these strains contained the classic
768 R plasmid R773 which also conferred resistance to tetracycline and streptomycin.
769 The Hammersmith Hospital Collection of resistance plasmids collected from the
770 early 1960's onwards- contained 25% Hg^R plasmids (Schottel et al., 1974)- whilst
771 other studies showed up to 60% of hospital isolate strains at that time were Hg^R.
772 Since then, metal ion resistance genes have been regularly detected in bacteria
773 isolated from the clinic, environment, agricultural, domestic and wild animal, and
774 human sources.

775 The first descriptions of the mechanisms of antimicrobial metal resistance started in
776 the late 1960's and the detailed mechanisms of resistance and the genes encoding
777 the resistance mechanisms have been studied since then.

778 More recently clinical interest in antimicrobial metal ion resistances has decreased,
779 but there is increasing evidence that antibiotic and metal ion resistances are linked,
780 as they are carried on the same mobile genetic elements – such as transposons and
781 plasmids (Frost et al., 2005; Baker-Austin et al., 2006; Summers, 2006; Mindlin
782 and Petrova, 2013).

783

784 Antimicrobial metal ion resistances were carried on mobile genetic
785 elements in bacteria from the 'pre-antibiotic era'.

786 Our first understanding of bacterial antimicrobial metal ion resistance came from
787 clinical bacteria from the 'antibiotic era', which were originally isolated because they
788 were resistant to antibiotics. However, subsequent investigations showed that as
789 well as resistance to antimicrobial metals in contemporaneous strains of clinically

important bacteria, antimicrobial metal resistance was also present in 'pre-antibiotic era' clinical isolates stored by E.D.G. Murray in Hammersmith Hospital between 1917-1954, (although very low numbers of these strains were antibiotic resistant). Within the collection there were significant numbers of strains carrying plasmid-borne resistance to K_2TeO_4 (11/433), $CuSO_4$ (68/433), $NaAsO_2$ (61/433), but less to $HgCl_2$ (3/433). Resistance to silver was not tested (Hughes and Datta, 1983). The incompatibility groups of these plasmids were the same as are found today (Datta and Hughes, 1983).

Tn21 subgroup transposons- drug (resistance) mules

One of the best known examples of how metal ion resistance and antibiotic resistance genes are genetically linked is the understanding that Tn*21* family mercuric ion resistance transposons carry class 1 integrons. These integrons are not mobile themselves, but are responsible for the acquisition and expression of antibiotic resistance cassettes (Liebert et al., 1999).

Sulphonamides were introduced into Japan during World War II, and streptomycin, chloramphenicol and tetracycline were introduced in 1950 to tackle serious Shigellosis problems. *S. dysenteriae* strains were isolated in 1952 that were resistant to sulphonamides, and isolation of strains resistant to sulphonamides, streptomycin, chloramphenicol and tetracycline first occurred in 1955 (reviewed in Watanabe 1963). Experimental findings that these antibiotic resistances could be transferred from *Shigella* sp. to *Escherichia coli* K-12 led to the realisation that these resistances were associated with "Resistance Transfer Factors" or plasmids. Plasmid R100 (also independently isolated as R222, or NR1) is a classic example of a multiresistance plasmid that was first isolated in Japan sometime during the early to mid-1950's (Nakaya et al., 1960; Davies, 1995). R100 carries resistances to tetracycline, chloramphenicol, sulphonamides and aminoglycosides (Liebert et al., 1999). The mercury resistance transposon Tn*21* carried on R100 (NR1) can justly be regarded as the paradigm for a particular class of mercuric ion resistance found in Gram-negative bacteria, and for how a metal ion resistance transposable element

performs another role, acting as a drug (resistance) mule carrying integron elements that acquire, reassort and express antimicrobial resistance genes. In the case of Tn*21*, In*2*, the integron carried by it contains the *sulI*, (sulfonamide resistance), *qacE*Δ1, (partially deleted quaternary ammonium compound resistance) and *aadA1* (aminoglycoside adenylyltransferase) resistance genes (Liebert et al., 1999). So although Hg compounds are now rarely (if at all) used as antimicrobials in agriculture and medicine, class I integrons are being carried by mercuric ion resistance transposons in Gram-negative pathogens that are of current concern.

Examination of the Hg^R plasmids from the Murray collection showed that the Hg^R determinant carried on one of them was very similar to Tn*21*, (but was flanked at each end by copies of IS*5075*, lacked the integron, and had a small deletion at the site where In*2* has inserted into the transposon) (Essa et al., 2003). In a separate study a 10,000 year old Siberian permafrost bacterial isolate was found to contain a transposon that was virtually identical to Tn*21*, but lacked the integron (Kholodii et al., 2003). These preantibiotic era Tn*21*-like mercury resistances lacking In*2* are consistent with a model for the stepwise evolution of Tn*21* ancestor mercury resistance transposons into multiresistance transposons.

Tn*21* subgroup transposons conferring multiple antibiotic resistance and containing Class 1 integrons have subsequently been found widely in enterobacteria from commensal, clinical and environmental Gram negative bacteria (Zühlsdorf and Wiedemann 1992, Liebert et al., 1999, Wireman et al., 1997; Mazel et al., 2000, Levings et al., 2007; Partridge, 2011; and reviewed in Mindlin & Petrova 2013) (Table 2). Integron acquisition of antibiotic resistances including ESBLs (extended spectrum beta lactamases) (Novais et al., 2010) and *A. Baumannii- aba*R5 (Post et al., 2010, Post and Hall 2009) are of major concern, but perhaps of no surprise. Recently, evidence has emerged of highly efficient horizontal transfer of Tn*21*-related transposable elements by natural transformation followed by chromosomal integration between unrelated bacterial species (Domingues et al., 2012). There are at least seven independent examples (including Tn*21*) of an integron insertion into a simple mercury resistance transposon into or close to *res* sites of the transposon Mindlin and Petrova, 2013). An important example of an evolutionarily distinct

multiresistance *mer* transposon is Tn*1696* (Partridge et al., 2001) where In*4* inserted into a Tn*5036*-like transposon. Similar independent integron insertion events into *mer* transposons point to the major role of the *mer* transposon as the carrier of integron associated antibiotic resistances (a drug resistance "mule") and may be one explanation for the frequency of *mer* transposon appearance in pathogens. Correlation between high levels of antibiotic resistance and carriage of the *merA* gene has been noted in *E. coli* from human populations, with higher exposure of the human population to mercury correlating with higher levels of mercury and antibiotic resistance (Skurnik et al., 2010).

Whole genome and whole plasmid sequencing of medically important bacteria is now showing the presence of Tn*21*-related multiresistance transposons in multiple strains- this will be discussed further later in this article (see Table 2).

Microbial Genomes- snapshots of the evolution of pathogen resistance repertoires?

The dramatic increase in the number of draft or complete microbial genome sequences being produced over the last ten years or so has provided us with information on the content of bacterial genomes and particularly with high throughput sequencing technologies, on the evolution of bacterial pathogens. These genome sequences also allow us to examine the prevalence of antimicrobial metal resistance in recent isolates of medically or agriculturally important bacteria, including the genomes of existing, "new", emerging, and re-emerging pathogens, opportunistic pathogens and "Pathogenic commensals" (Alekshun and Levy, 2006). Many of these pathogens are niche pathogens, or opportunistic healthcare-associated infections in critically ill or immune compromised patients. They also represent acute clinical problems because they are Multiply Drug Resistant (MDR) presenting challenges to treatment.

What is the evidence from these genome sequences that antimicrobial metal resistances are contributing to the broader MDR problem? We have examined

evidence for carriage of mercury, copper/silver and arsenic/antimony resistance in microbial genome sequences, and will discuss them next.

Mercury Resistance: still here, but why?

Mercury resistance transposons related to Tn*21* and the similar Tn*1696* can be found on pathogen plasmids or chromosomes, associated with antibiotic resistance cassettes carried on integrons. Table 2 shows examples of these resistances from recently sequenced pathogens, some of which were originally isolated when mercury was still used as an antimicrobial, others which were isolated more recently. Examples of more recently isolated *mer* transposons include those carrying the TEM-24 ESBL resistance in the integron (Novais et al., 2010), examples of multidrug resistance *Acinetobacter baumanii*, *Yersinia pestis*, *Salmonella* Typhimurium, and the recent *E. coli* O104:H4 mass food-poisoning outbreak isolate from 2010 (see Figure 6). The widespread persistence of mercury resistance transposons in pathogens is at first sight surprising given that mercury compounds are apparently rarely used as antimicrobials.

Copper and silver resistance: a previously under-remarked genetic linkage?

The pMG101 plasmid-borne silver resistance (Gupta et al., 1999) and the independently isolated *pco* plasmid copper resistance (Tetaz and Luke 1984) are the most well characterized silver and copper resistances. During the annotation of the genome sequence of the enterohaemmorhagic *E. coli* (EHEC) H10407 (Crossman et al., 2010) we noted a chromosomal genetic arrangement where the *pco* and *sil* operons were adjacent to each other. Subsequent searches of other plasmid and genome sequences (see Table 3) have identified this arrangement (or similar) in a range of different Gram-negative bacteria, both on plasmids and on chromosomes (see Figure 6), including in the German *E. coli* O104:H4 isolate from the 2010 mass outbreak, avian pathogenic *E. coli* (Johnson et al., 2006), and

livestock isolates (our work, unpublished). This raises a number of unresolved questions regarding the contribution of *sil* and *pco* to silver and copper resistance, whether these contribute to *in vivo* survival of pathogens in macrophages, cross regulation and co-selection of these genes, as well as their mobility, and the consequences of this on MDR resistance- particularly in agricultural environments where high levels of copper are used in feed and as antimicrobials, but also in environments where silver is being used as an antimicrobial.

Arsenic/antimony resistance: not gone away, nor likely to?

Bacterial arsenic and antimony resistance is at present of marginal interest to human medicine, but resistance is still found widely in bacteria of medical importance (Table 4). Environmental exposure to arsenic or antimony, the continued use of antimonite in treating Leishmaniasis, exposure of human populations to arsenic contaminated drinking water, (Perry et al., 2011, 2013), the use of arsenic compounds as rodenticides and the current and historic use of arsenic compounds in animal husbandry could all have provided direct selection for carriage of As/Sb resistance in the commensal and pathogenic microbial flora, and may still be doing so (Eppinger et al., 2012). Co-selection of arsenic resistance alongside other antimicrobial resistances in IncH1-2 plasmids has also been proposed as an explanation for the continued retention of arsenic resistance (Ryan and Colleran 2002), but equally environmental arsenical selection may be contributing to MDR selection.

The state we are in and how we got here.

Amidst the current worldwide concerns about antibiotic resistance, it could be argued that antimicrobial metal ion resistance is of marginal importance to medical microbiology, because antimicrobial metals are currently of limited clinical significance, though their use is growing again. Despite limited or discontinued use of these metals, mercury, copper, silver, arsenic and antimony resistances are still

here. These resistance genes are often found associated with antibiotic resistance gene cassettes on the same mobile genetic elements, or these antimicrobial metal resistances are carried on MDR elements, where presumably the fitness loss of carrying them is either unimportant, or outweighed by the advantages there are to carrying them, because resistance is still needed. It is interesting that the transposons carrying mercury resistance genes found in clinically important pathogens are often carrying a far heavier "payload" of antibiotic resistance genes.

The original "R" (Resistance) plasmids isolated in the 1960s and 1970s conferred multiple antibiotic and metal ion resistances on their hosts, and high levels of Hg^R and As^R bacteria were found in healthcare environments. It was reasonably assumed that this was at least in part due to mercury (Porter et al., 1982) and arsenic compounds being widely used in medicine. There is current, and clear evidence, of the linkage of metal-ion and antibiotic resistance gene carriage in bacteria in sewage treatment plants (see Davies and Davies 2010 and references therein), as well as in terrestrial and aquatic environments (Berg et al., 2005; Stepanaskas et al., 2006; Wright et al., 2006; Wright et al., 2008; Skurnik et al., 2010). Moreover, there is a considerable literature on the problem of antibiotic resistance/metal resistance co-selection (Stepanauskas et al., 2006 ; Baker-Austin et al., 2006 ; Singer et al., 2006 ; Aminov and Mackie 2007, Allen et al., 2010). So whilst the use of mercury and arsenic in medicine has declined, and copper and silver have limited uses, antimicrobial metal resistance genes to these (and other) metals are persisting, and are co-selected with other antimicrobial resistance genes.

And herein lies the problem. Summers (2004, 2006) has already elegantly argued that although antimicrobial resistance has traditionally been viewed as a treatment (failure) problem, the propagation of resistance to antimicrobials is actually an ecological problem, and that both human and agricultural uses of antimicrobials have contributed to this situation. Summers (2006) has also argued that understanding the role of the agricultural and commensal microbiota and the mobile genetic elements involved in resistance gene movement is also very important in understanding these multidrug resistance and transmission

phenomena. We can find nothing to disagree with there: the role of commensal bacteria as a reservoir for antimicrobial resistance genes is now gaining more recognition (for example Fricke et al., 2008; Marshall et al., 2009) and it has been long recognized that antibiotic resistance in agricultural bacteria is a significant problem (reviewed in: Khatchatourians 1998, Wise et al., 1998; Levy and Marshall, 2004; Silbergeld et al., 2008). The phenomenon of antimicrobial metal resistance gene emergence and spread is in our opinion conceptually identical to the problem of the evolution and dissemination of antibiotic resistance which was outlined by Courvalin (Courvalin, 2005, 2006). One strategy proposed to reduce antibiotic resistance is to attempt to delay the emergence and dissemination of resistance to new antibiotics (Courvalin 2006). Unfortunately we cannot delay the emergence of antimicrobial metal resistance. It has already happened, and those resistances are still apparently highly successful, widespread and mobile in Gram-negative bacteria, and may also be important in Gram positive bacteria such as *S. aureus*. Is it now time to have a serious debate about the non-medical uses of antimicrobial metals in relation to the dissemination of MDR? Or are we too late? Will new formulations and uses of antimicrobial metals overcome existing resistance mechanisms? Or will lethal selection drive evolution of resistance?

Conclusions

Bacterial antimicrobial metal ion resistances, which have been found in pathogens and non-pathogens, were present long before microbiologists realized that these resistances existed. Even now, the genetic elements encoding metal ion resistance appear to be playing a powerful role in facilitating MDR resistance and horizontal gene transfer, through co-carriage and/or co-selection of antibiotic resistance with the metal resistances. The presence of antimicrobial metal resistance genes in bacteria not only reflects the anthropocentric view of microbiology (Aziz, 2009), which is the history of human antimicrobial use in infectious disease (Toleman and Walsh 2011) but also microbial exposure to these metals from industry and agriculture; and predating all human uses: the exposure of microorganisms over millennia to localized high levels of bioavailable toxic metals from natural

environmental releases, and interactions with organisms that predate humans. The continuing widespread presence of antimicrobial metal resistance genes often intimately associated with other antimicrobial resistance genes suggests that it is unlikely that they are going to go away soon, and we must take resistance gene co-carriage and co-selection into account when we think about strategies to combat antimicrobial and antibiotic resistance. Persistence of these metal resistance genes points to what the future for antibiotic resistance gene persistence could be.

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Figure Legends

Figure 1: Model of the Gram negative bacterial mercuric ion resistance mechanism from Tn*21*. Modified from Hobman and Brown 1997; Barkay et al.,

2003. Divalent mercuric ions (Hg^{2+}) enter the periplasm via porins in the outer membrane, where they bind to cysteine residues in MerP. They are then passed on to the inner membrane located MerT and/or alternate importers MerC or MerF. Mercuric ions are transferred via cysteine pairs in MerT and emerge in the cytoplasm, where they are reduced by mercuric reductase (MerA) to Hg^0. This is volatile at room temperature and pressure, and leaves the cell as mercury vapour. Expression of the mercury resistance structural genes are regulated by MerR. MerD acts as a co-regulator of expression. In "broad spectrum" mercury resistances, which confer resistance to inorganic and organic mercury compounds, "broad spectrum" carry an organomercurial responsive MerR and encode an enzyme, organomercurial lyase (MerB), which cleaves the organic moiety from mercury. MerB is often found located between *merA* and *merD*. An additional importer MerE is reported to import organomercurial ions (Sone et al., 2013)

Figure 2: Model of the *Escherichia coli* chromosomal *cue* and *cus* copper homeostasis/efflux mechanisms. Modified from Stoyanov et al.,2001; Outten et al., 2000; Munson et al., 2000; Rensing and Grass 2003). Copper enters the cytoplasm and induces expression of *copA* which encodes a copper efflux P1-type ATPase and of CueO, a multicopper oxidase. Both genes are regulated by CueR, a copper responsive MerR family regulator. In the *cus* system, a two component regulator CusRS activates expression of *cusCFBA* via phosphorylated CusR. CusCBA is a tripartite RND (resistance-nodulation-cell division) family silver/copper effluxer and CusF a periplasmic metallochaperone. CusCBA effluxes Cu^+ directly to the outside the cell, and copper can directly enter the CusCBA complex from the cytoplasm, the periplasm or from CusF.

Figure 3: Model for plasmid-borne (*pco*) copper resistance from *E. coli* plasmid pRJ1004. Modified from Rensing and Grass 2003; Djoko et al., 2008; Zimmermann et al., 2012. The *pco* system operates in addition to the chromosomal *cue* and *cus* systems. The *pco* copper resistance from *E. coli* plasmid pRJ1004

contains seven open reading frames: *pcoABCDRSE* (Rouch and Brown 1997). Copper enters the periplasm, possibly through porins, and gene expression from the *pco* operon is regulated by the two component sensor-kinase regulator system PcoRS which responds to the presence of copper. Phosphorylated PcoR regulates expression of the *pcoABCDRS* genes from one promoter and *pcoE* from a separate promoter (Rouch and Brown 1997). There is a likely role for pigments / catechol siderophores in *pco* copper resistance, though this is not well understood. PcoE acts as a first line of defence, copper 'sponge' protein, binding copper in the periplasm, whilst the other Pco proteins are expressed. PcoA is a multicopper oxidase, which oxidizes Cu(I) to less toxic Cu(II) bound to PcoC in the periplasm (Djoko et al., 2008). PcoB is a predicted outer membrane protein that may interact with PcoA/C to export copper (Djoko ey al., 2008), or may act to sequestrate oxidized catechol siderophores (Rensing and Grass 2003). PcoD is an inner membrane spanning protein which may import copper into the cytoplasm or PcoC may provide copper to PcoD, for loading on to PcoA, which is exported to the periplasm via the TAT pathway (Rensing and Grass 2003; Djoko et al., 2008).

Figure 4: Hypothetical model for silver resistance encoded on *Salmonella* Typhimurium plasmid pMG101. (Modified from Silver, 2006). Silver ions (Ag^+) enter the cell, and are detected in the periplasm by SilS, the sensor component of a two component silver responsive transcriptional regulation system SilRS. The SilRS sensor/regulator regulates its own expression via phosphorylated SilR, and that of *silE*, which is believed to have a role in periplasmic silver binding. SilRS also regulates the expression of *silCBA* , *silF, silP* and a small open reading frame *orf*105 the product of which is of unknown function. SilCBA is a tripartite RND (resistance-nodulation-cell division) family silver effluxer, SilF is believed to encode a small periplasmic silver chaperone, and SilP is an efflux ATPase. The model predicts SilCBA effluxes Ag^+ directly to the outside the cell, and Ag^+ (by homology to the *cus* system) can directly enter the SilCBA complex from the cytoplasm, the periplasm or from SilF. SilE may work like its homologue PcoE, as a periplasmic metal binding "sponge" giving initial protection against Ag^+ damage.

Figure 5: Arsenic and antimony resistance operon from *E. coli* plasmid R773. (Modified from Silver 1998, Nies 1999, Kruger et al., 2013). Arsenate (ASO_4^{3-}) As^{5+} and arsenite (AsO_2^-) As^{3+} or antimonite (SbO_2^-) can enter the *E. coli* cell via specific or non-specific transport systems (arsenate can enter cells via the phosphate import system). ArsR is an arsenite responsive trans-acting transcriptional repressor protein which senses As^{3+} in the cytoplasm and regulates expression of the structural arsenic resistance genes. ArsC, is an arsenate reductase, which is required to reduce arsenate to arsenite so that it can be effluxed by ArsB, an arsenite antiporter. ArsA has an ATPase function, and binds as a dimer to ArsB, forming an ATP energized effluxer, which is more efficient at arsenite efflux than ArsB alone. ArsD has a minor role in transcription, but has recently been found to act as a metallochaperone for arsenite efflux via ArsAB (Lin et al., 2006).

Figure 6: Tn*21* family mercury resistance transposon from *E. coli* O104:H4

Tn*21* family transposon from the sequenced enteroaggregative, enterohaemorrhagic *E. coli* German outbreak strain. The integron contains: trimethoprim, sulphonamide (*sul*I and *sul*II), and aminoglycoside (aminoglycoside phosphotransferase and aminoglycoside kinase) antibiotic resistance genes and encodes a multidrug effluxer protein related to QacE and EmrE. Upstream of the mercury resistance transposon is a tetracycline resistance gene cluster.

Figure 7: *pco/sil* resistance gene clusters found in different bacteria.

H10407- Enterotoxigenic *E. coli* strain H10407; pLVPK- *K. pneumoniae* CG43 pLVPK plasmid; Ent- *Enterobacter* sp. AgI isolated from the gut of *Anopheles gambiae* mosquito; O104:H4- E. coli O104:H4 enteroaggregative/enterohaemmorhagic strain from the 2011 German foodborne outbreak., Cit' - *Citrobacter* sp.30_2 from the human microbiome project-a human intestinal biopsy specimen.

1834

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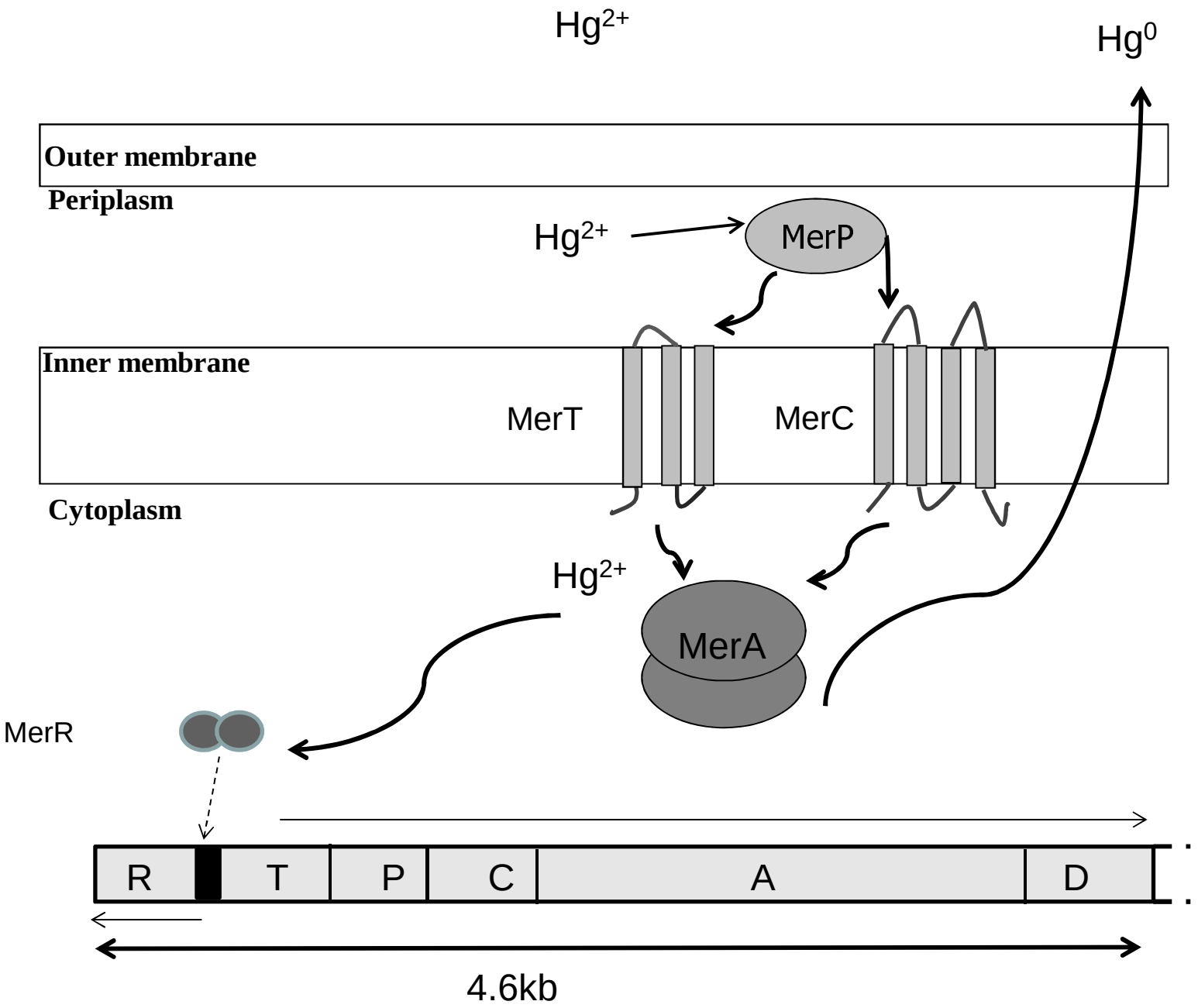
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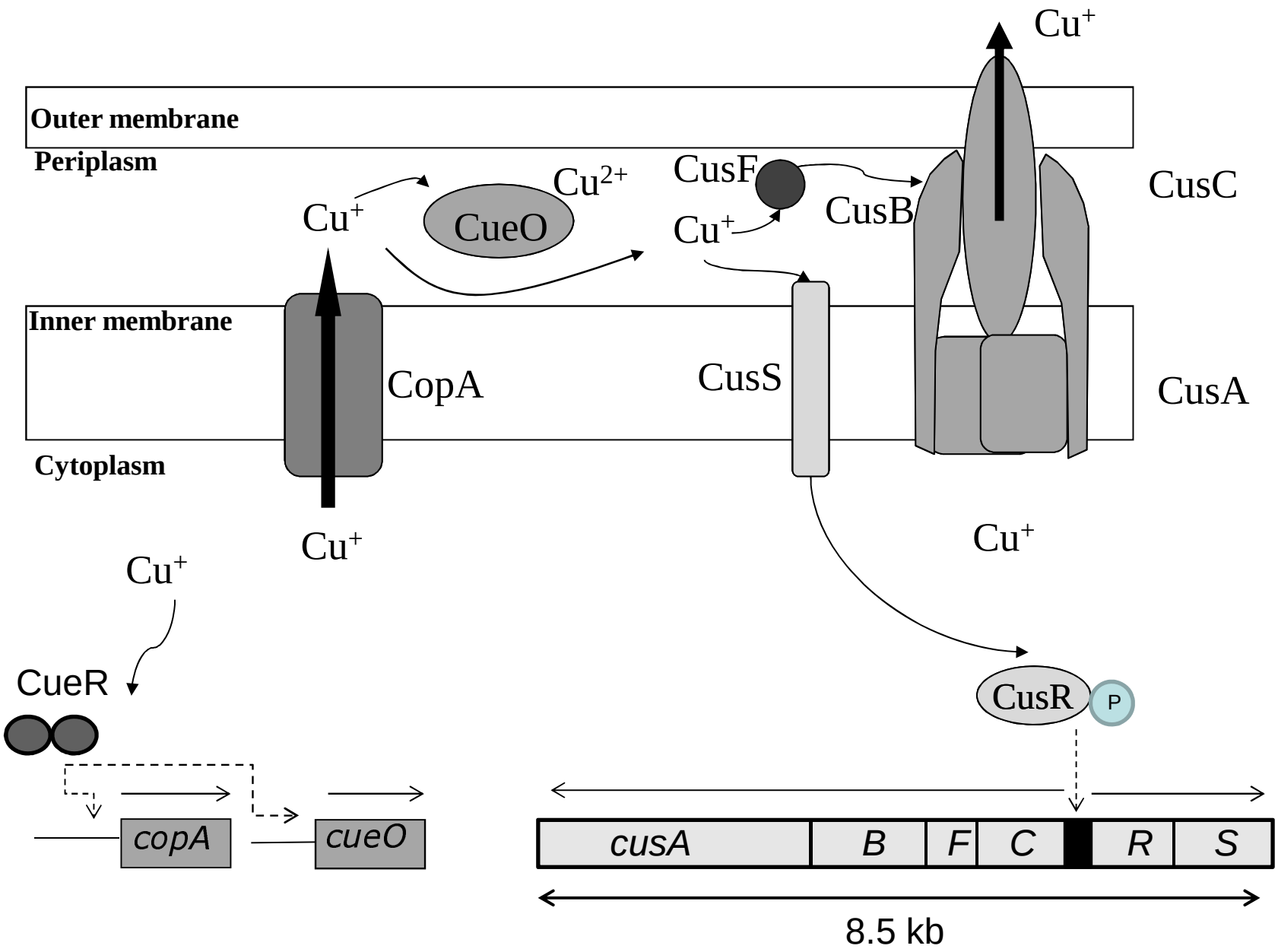
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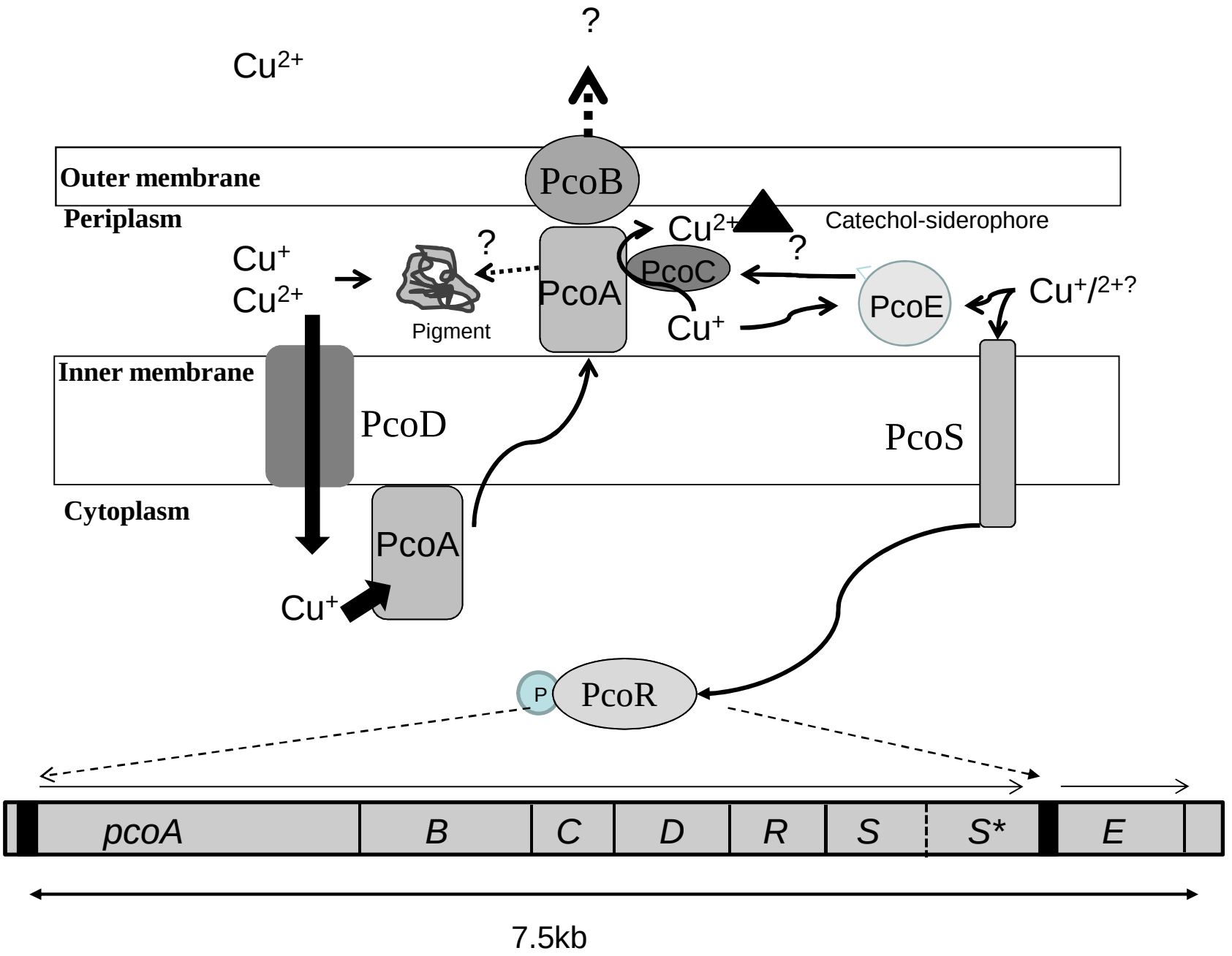
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Cu+/2+?
Pigment
Cu+
Inner membrane
PcoD
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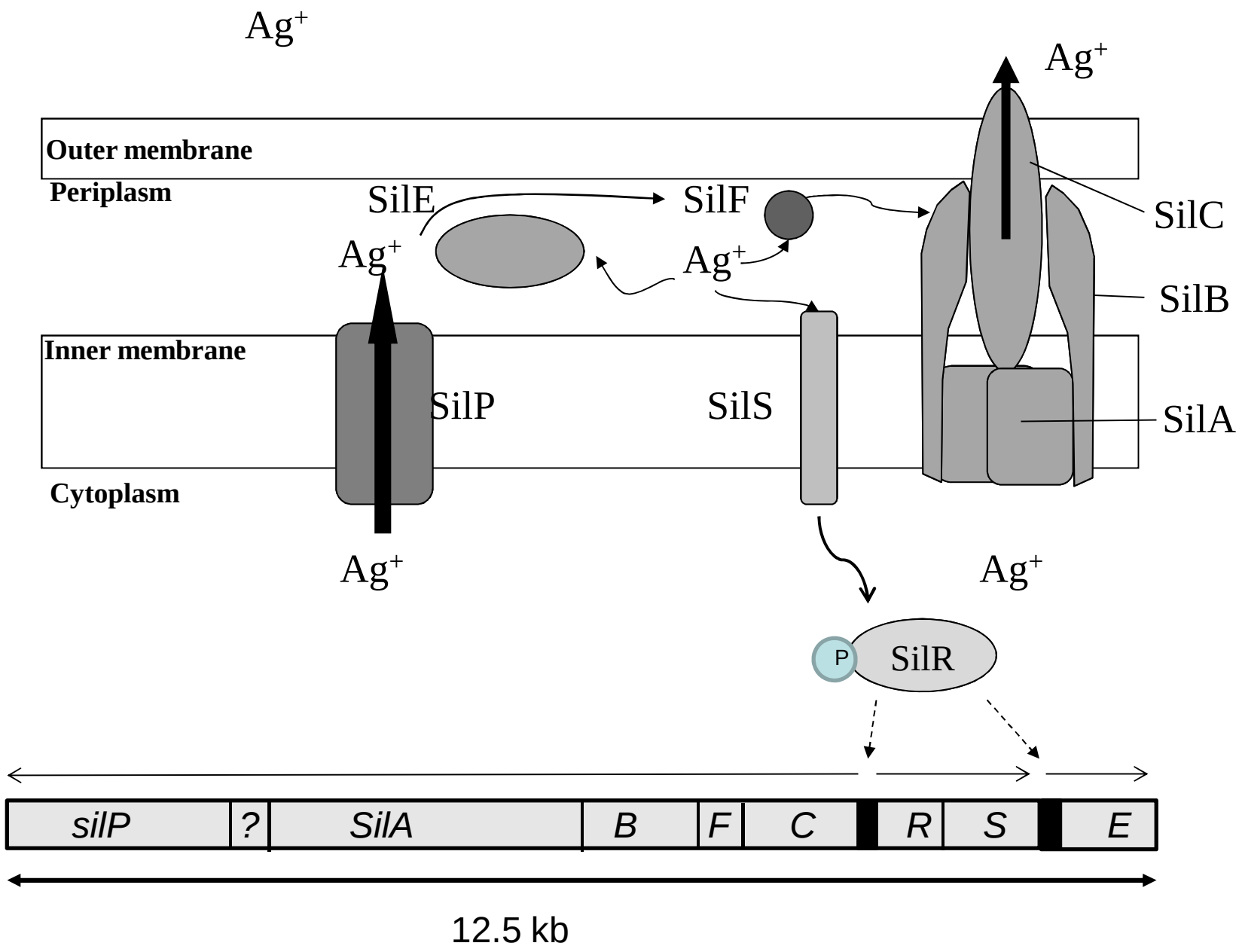
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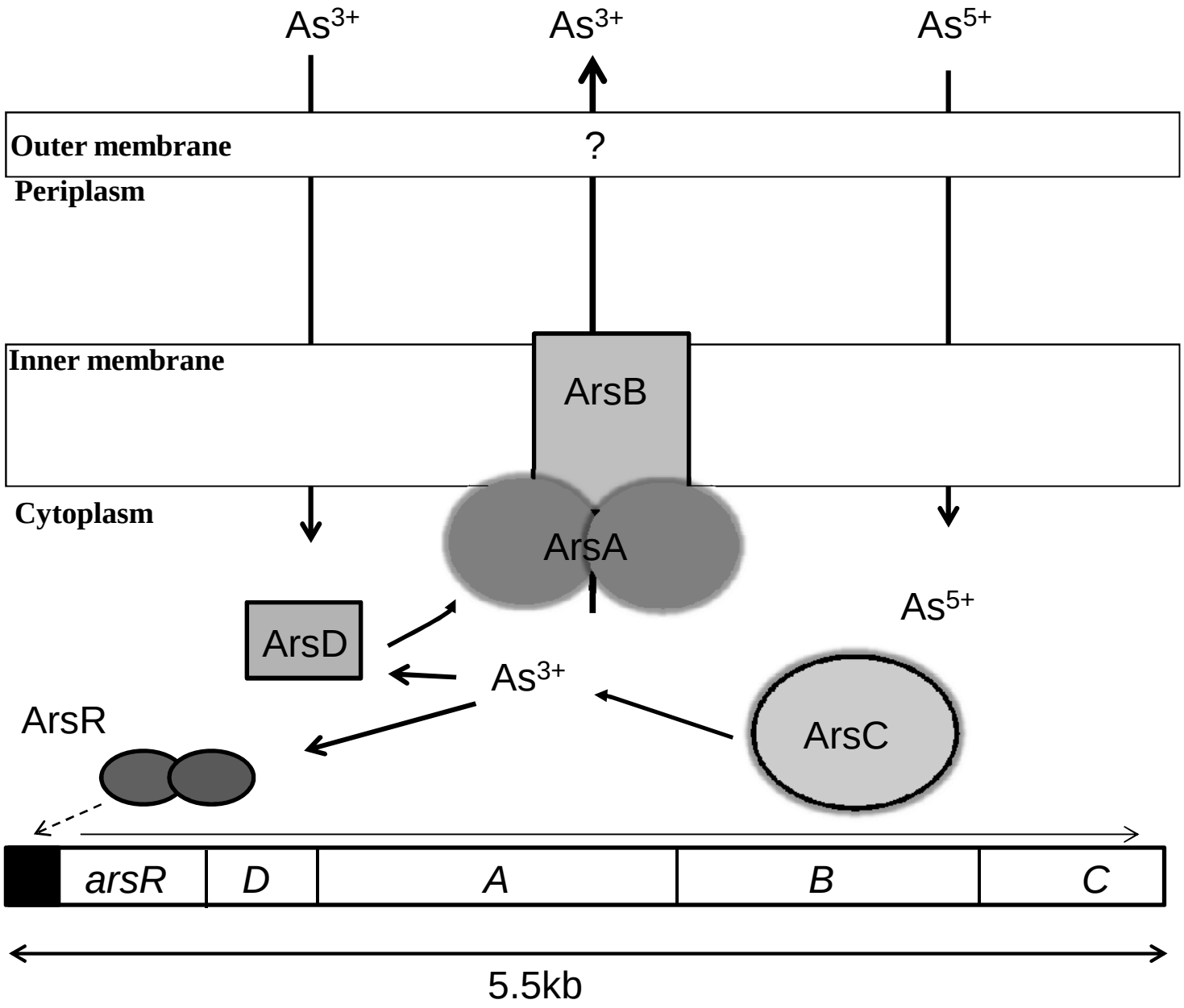
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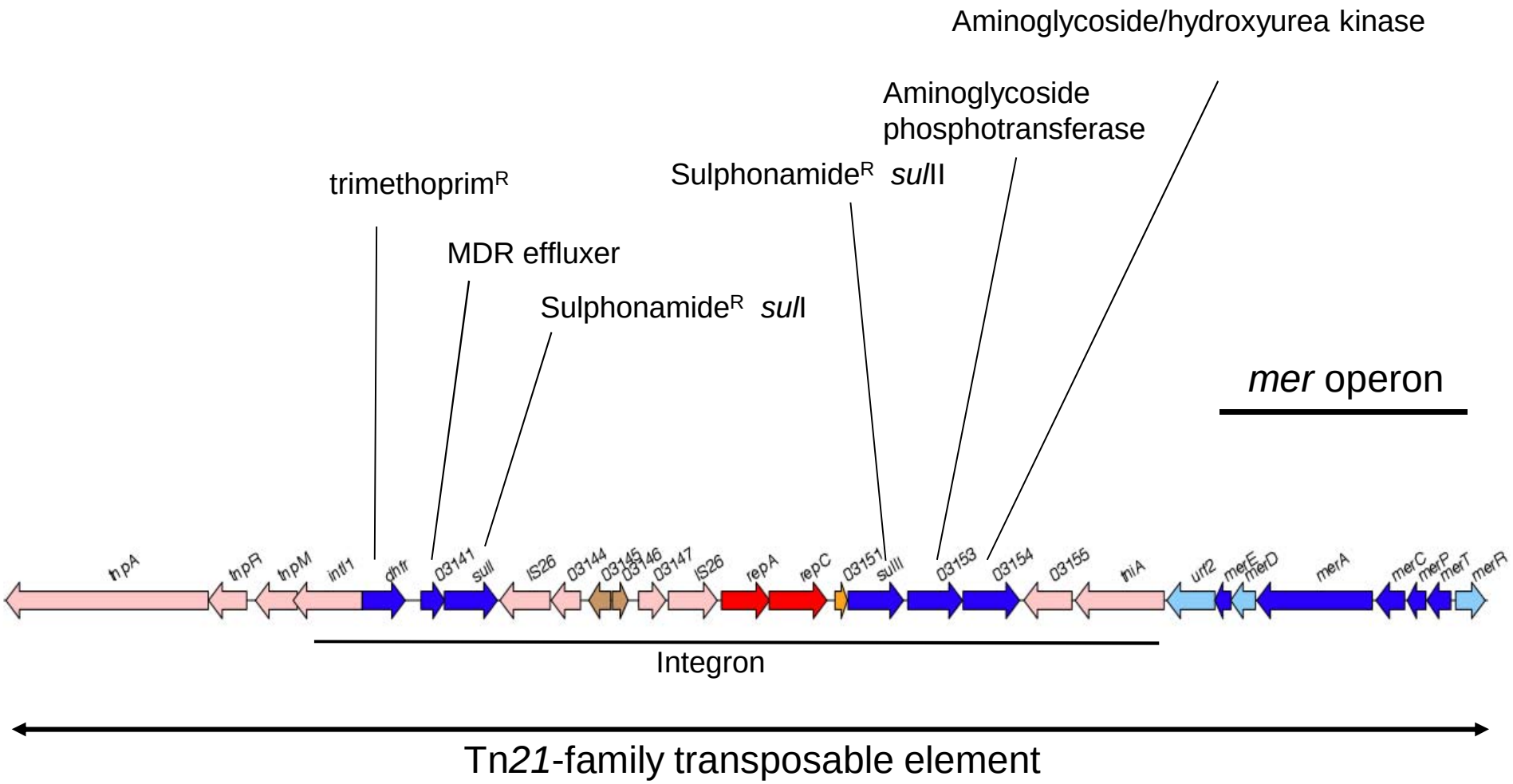
Aminoglycoside/hydroxyurea kinase
Aminoglycoside phosphotransferase
trimethoprim^{R}
Sulphonamide^{R} *sul*II
MDR effluxer
Sulphonamide^{R} *sul*I
mer operon
tnpA
tnpR
tnpM
intI1
dhfr
03141
sulI
IS26
03144
03145
03146
03147
IS26
repA
repC
03151
sulII
03153
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urf2
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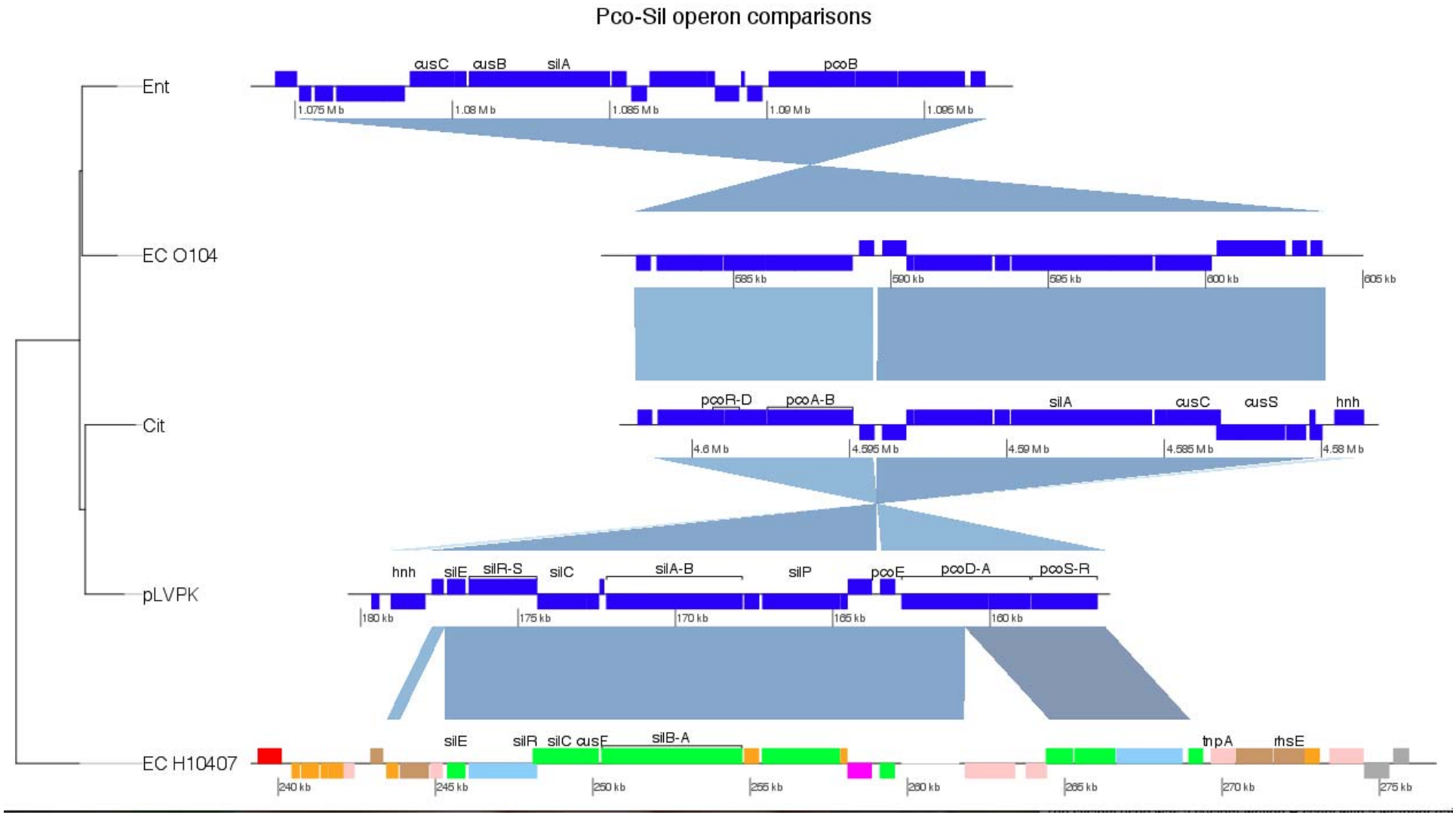
merA
merC
merP
merT
merR
Integron
Tn*21*-family transposable element


Pco-Sil operon comparisons
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EC O104
Cit
pLVPK
EC H10407
cusC
cusB
silA
pcoB
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pcoA-B
silA
cusC
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Class A and Class B metal ions
Class A (hard) metals Lewis acids (electron acceptors) of small size and low polarizability (deformability of the electron sheath or hardness) Li, Be, Na, Mg, Al, K, Ca, Sc, Ti, Fe(III), Rb, Sr ,Y, Zr, Cs, Ba, La, Hf, Fr, Ra, Ac, Th. ***Borderline (intermediate) metals*** V, Cr, Mn, Fe(II), Co, Ni, Cu(II), Zn, Ga, As, Rh, Pb(IV), Sn, Sb ***Class B (soft) metals*** Lewis acids (electron acceptors) of large size and high polarizability (softness) Cu(I), Pd, Ag, Cd, Ir, Pt, Au, Hg, Ti, Pb(II).

Table 1: Classification of metals in terms of polarizability (Modified from Niebor and Richardson 1980, Duffus et al., 2002). Class A metal ions form ionic bonds with oxygen containing ligands that are mobile and easily displaced, Class B metals form covalent bonds and prefer coordination to nitrogen and sulphur centres in biological molecules.

Table 2

Tn21 related mercury resistances			
Strain	**Genetic element**	**Additional information**	**Reference**
Aeromonas salmonicida subsp. salmonicida A449	Tn*21* subfamily composite transposon containing an In*2* integron encoding resistance to streptomycin/ spectinomycin, quaternary ammonia compounds, sulphonamides and chloramphenicol.	Causative agent of furuniculosis in fish	Reith et al., 2008
Acinetobacter Baumannii AYE	Tn*21*-like element contained within an 86 kb chromosomal "resistance island" (AbaR1) containing 45 antimicrobial resistance genes.	Epidemic strain in French hospitals. Multiply antibiotic resistant.	Fournier et al., 2006
Acinetobacter Baumannii 3208	Chromosomal resistance island AbaR5, carrying residual sequences of Tn*1696*	Australia 1997 blood infection isolate	Post and Hall 2009

	(Tn*21*-related transposable element carrying In*4*)		
Acinetobacter baumannii AB0057	Tn*21*-like element contained within an 86 kb chromosomal "resistance island" AbaR3	USA 2005 MDR bloodstream isolate	Adams et al., 2008
Escherichia coli O42	Chromosomal Tn*21*-subfamily related Tn*2411* carrying mercuric chloride resistance and resistances to sulphonamide, streptomycin and ethidium bromide on an In2-like integron. Tn*2411* is flanked by tetracycline and chloramphenicol resistances.	Enteroaggregative *E. coli* type strain isolated in Bangladesh in 1970	Chaudhuri et al., 2010
Escherichia coli EHEC O26:H$^-$ strain O6877	Plasmid pO26-CRL contains a Tn*21* subfamily composite transposon containing an In*2* integron encoding resistance to mercuric chloride, trimethoprim, β-lactams, sulfathiozole, streptomycin, and kanamycin.	Australian isolate of Enterohaemmorhagic *E. coli* isolated in 1990s	Venturini et al., 2009

Escherichia coli EAHEC O104:H4 TY2482	Genomic island 3 (GI-3)	German epidemic enteroaggregative haemorrhagic *E. coli* outbreak 201. Multidrug resistant. Related to *E. coli* 55989	Grad et al., 2013
Klebsiella pneumoniae	Plasmid pFOX-7a carrying a Tn*1696* derivative Tn*6234*	Outbreak in a neonatal intensive care unit. IncL/M plasmid carrying a Tn3-like transposon carrying $bla_{\text{TEM-1a}}$ and Tn6234 carrying $bla_{\text{FOX-7}}$ (an AmpC-type ESBL beta lactamase) and other resistance determinants.	Di Pilato et al., 2014
Pseudomonas aeruginosa PA7	Tn*21*- like element contained within a chromosomal "transposon dump"	A non-respiratory clinical isolate from Argentina. Unusually antibiotic resistant and contains Tn*21*, Tn*1721* and Tn*5393*	Roy et al., 2010
Pseudomonas aeruginosa	Plasmid R10003	Tn*1696* carrying In*4* Originally isolated in 1970's. Resistance to gentamicin,	Partridge et al., 2001

		streptomycin, spectinomycin and chloramphenicol. Independent evolution by insertion of In*4* into a Tn*5036*-like transposon	
Salmonella enterica serovar Typhi CT18	Plasmid pHCM1	Is*5075* flanked *mer* resistance related to Tn*21*	Parkhill et al., 2001
Salmonella enterica serovar Paratyphi A	plasmid pAKU_1 incHI1 group, similar backbone to pHCM1 and pRK27 24kb composite multidrug resistant transposon	Isolated in 2002 in Karachi, Pakistan.	Holt et al., 2007
Salmonella enterica serovar Typhimurium T000240 strain (DT12)	This strain contains a unique 82-kb genomic island, designated as GI-DT12, which contains a Tn*2670*-like composite transposon containing an integron, multiple antibiotic resistance genes and a Tn*21*-like mercury resistance.	Isolated from a human gastroenteritis sufferer in 2000. Fluoroquinolone resistant	Izumiya et al., 2010
Shigella flexneri	Plasmid R100 containing	The index isolate of Tn*21*.	Reviewed in Liebert et al.,

	Tn21, Tn10 (Tetracycline) and a Tn9 homolog (Chloramphenicol)	Isolated in Japan n the mid 1950's. Tn*21* contains In*2*	1999
Other mercury resistances			
Staphylococcus aureus sequence type 239 *(TW)*	Plasmid-pTW20_1 borne SCC*mec* (beta-lactamase) with SCC*mercury* and *cadA* ATPase in a region flanked by IS*431*.	The chromosomal SCC*mercury* region contains streptomycin and erythromycin resistance. SCC*mec* carries ψTn*554* carrying cadmium resistance.	Holden et al., 2010
Staphylococcus aureus	Tn*6009- a combination of Tn916* and a *mer* operon	Found in both Gram-positive and Gram-negative bacteria from oral and urine samples	Soge et al., 2008
Mycobacterium abscessus CIP 104536T (ATCC 19977)	Hg^R carried on a 23Kb plasmid similar to pMM23 from *M. Marinum*	Strain originally described in 1953 from a human knee infection, but *M. abscessus* strains recognized to cause pseudotuberculous lung disease, particularly in cystic fibrosis patients	Ripoll et al 2009

Table 2: Tn*21*-like mercury resistance transposons and associated integrons, and other mercury resistances, in sequenced plasmids or bacterial chromosomes from pathogens.

Strain	Genetic element	Additional information	Reference
Citrobacter sp. 30_2	*pco/sil*	Reference genome for the human Microbiome project sequenced by the Broad Institute	GenBank assembly GCF_00158355.2
Cronobacter sakazakii BAA-894	Chromosomal location of *pco/sil*	Type strain for bacterial meningitis associated with infant formula milk	Kucerova et al., 2010
Enterobacter cloacae subsp. *cloacae* ATCC 13047	The strain is reported to encode 37 multidrug efflux proteins, 7 antimicrobial peptide resistance proteins, 11 β-lactamases. Multiple metal ion resistances: chromosome: 2 x *sil*, 3 x *ars*, 1 x *mer* and 1 x *cop* operon. Plasmid pECL_A: 1 x *sil*, 1 x *ars*, 2 x *mer*, 1 x *cop* and 1 x *ter*	Type strain Isolated in 1890 from human cerebrospinal fluid by Edwin Oakes Jordan	Ren et al., 2010
Enterobacter hormaechei	Plasmid pQC carried in *E. hormaechi* hospital outbreak strain. Resistance to aminoglycosides and third generation cephalosporins. Reduced sensitivity to fluoroquinolones	Nationwide nosocomial outbreak in the Netherlands. Plasmid related to R478.	Paauw et al., 2009
Enterobacter sp. Ag1	*pco/sil* detected in the draft genome sequence of this strain	Isolated from the gut of *Anopheles gambiae* mosquito	Jiang et al., 2012
Escherichia coli C ATCC 8739	Chromosomal location of *pco/sil*	Test strain for testing antimicrobial handwashes and assaying antimicrobial	GenBank accession number CP000946

		preservatives	
Escherichia coli H10407	Chromosomal location of *pco/sil*	Enterotoxigenic *E. coli* (ETEC) type strain Bangladesh	Crossman et al., 2010
Escherichia coli APEC O1	Inc HI2 plasmid pAPEC-O1-R carrying *pco/sil* and resistance to tellurite, streptomycin, gentamycin, tetracycline, quaternary ammonium compounds and sulphonamides	Plasmid found in Avian Pathogenic *Escherichia coli* isolates in USA.	Johnson et al., 2006
Escherichia coli EAHEC O104:H4 TY2482	*Pco/Sil* carried on chromosome	German epidemic enteroaggregative haemorrhagic *E. coli* outbreak 2011	Ren et al., 2013 and This article
Klebsiella pneumoniae CG43	Plasmid pLVPK carrying *sil*, *pco* and *Pb* resistance	IncHI-2 plasmid Taiwan, hospital isolate	Chen et al., 2004
Klebsiella pneumoniae	Multiresistance plasmid pUUH239.2 carrying *sil*, *pco* and *ars* resistance with multiple antibiotic resistances. Resembles *E. coli* ST131 plasmids	2005 nosocomial outbreak isolate from Sweden. CTX-M-15 Extended spectrum beta-lactamase	Sandegren et al., 2012
Serratia marcescens	Plasmid R478 *pco/sil*. Also confers resistance to tetracycline, chloramphenicol, kanamycin, mercury, arsenic, and tellurite.	IncHI-2 group plasmid isolated in USA in 1969.	Gilmour et al., 2004
Salmonella Typhimurium	Plasmid pMG101contains *sil*. Also confers resistance to tetracycline, chloramphenicol, kanamycin, ampicillin, streptomycin, sulphonamide,	Isolated from a Burns unit at Boston General Hospital, Boston, USA 1973. IncHI-2 group plasmid containing prototypical *sil* operon.	McHugh et al., 1975

	mercury, arsenic, and tellurite.	Plasmid is partially sequenced.	
Sil only			
APEC *Escherichia coli*	pAPEC-O2-R IncF plasmid, an avian pathogenic *Escherichia coli* transmissible R plasmid carrying *sil* and resistances to quaternary ammonium compounds, tetracycline, sulphonamides, aminoglycosides, trimethoprim and beta-lactams	An avian pathogenic *Escherichia coli* isolated from a chicken with colibacillosis.	Johnson T.J., et al (2005)

TABLE 3. Examples of *pco/sil* gene clusters in sequenced bacterial type strains and pathogens.

Strain	Genetic element	Additional information	Reference
Acinetobacter Baumannii AYE	Arsenic resistance contained within an 86 kb chromosomal resistance island (AbaR1) which is a composite transposon containing 45 antimicrobial resistance genes.	Epidemic strain in French hospitals. Multiply antibiotic and antimicrobial resistant.	Fournier et al., 2006
Acinetobacter baumannii AB0057 and related strains	Multiple antimicrobial resistance: arsenate, mercury and multiple antibiotic resistance carried on AbaR3, which shares resistance island homology with AYE strain.	Isolated in 2004 from a patient at Walter Reed Army Medical Center, USA.	Adams et al 2008
Acinetobacter baumannii 3208	Arsenic and multiple antimicrobial resistance carried on the AbaR5, similar to AbaR3.	Isolated in 1997 in a hospital in Sydney, Australia, from a blood sample.	Post and Hall 2009
Burkholderia cenocepacia J2315	Resistant to aminoglycosides, macrolides, β-lactams imipenem and piperacillin,	Epidemic pathogen of cystic fibrosis patients. Isolated from the sputum of a CF	Holden et al 2009

	cotrimoxazole (trimethoprim-sulfamethoxazole) and also exhibits intermediate resistance to fluoroquinolones. Carries arsenic resistance.	patient in 1989 in Edinburgh. UK index case of the ET12 lineage.	
Campylobacter jejuni RM1221	Carries a four gene arsenic resistance cluster. Resistant to cephalosporins, β-lactams and sulphonamides	Originally isolated from a chicken carcass.	Wang et al., 2009; Fouts et al., 2005
Klebsiella pneumoniae DA15000	Plasmid pUUH239.2: A CTX-M-15-encoding multiresistance plasmid. It confers resistance to: beta-lactams, aminoglycosides, tetracyclines, trimethoprim, sulphonamides, quaternary ammonium ions, macrolides, silver, copper, and arsenic.	*Klebsiella pneumoniae* strain involved in a large nosocomial outbreak in Uppsala University Hospital between 2005-2011.	Sandegren et al., 2012
Listeria monocytogenes serotype 4b	Serotype 4b strains contain a 35kb genomic island containing arsenic and	Isolated in 1983 from a foodborne listeriosis outbreak. The Scott A strain	Briers et al., 2011; Lee et al., 2013, 2014

	cadmium resistance.	is widely used as a reference strain	
Salmonella enterica serovar Typhimurium	Plasmid R64. Resistance to streptomycin, tetracycline and arsenic.	Inc I group plasmid, isolated in the 1960s.	Sampei et al., 2010
Serratia marcescens	Plasmid R478. Also confers resistance to tetracycline, chloramphenicol, kanamycin, mercury, copper, and tellurite.	IncHI-2 group plasmid isolated in USA in 1969.	Gilmour et al., 2004
Staphylococcus aureus clonal complex 130	30kb SCC*mec* element carrying *mecA*, *blaZ* , and arsenic resistance	Isolated in 2010 in Ireland	Shore et al., 2011
Staphylococcus capitis NRCSA strain CR01	Novel 60.9kb composite staphylococcal cassette chromosome *mec* (SCC*mec*) methicillin resistance and an SCC*cad/ars/cop* carrying arsenic copper and cadmium resistance	Isolated in 2007 in France from an infant with late onset sepsis in a neonatal Intensive Care Unit.	Martins-Simoes et al., 2013
Staphylococcus haemolyticus SH32	28kb SCC*mec* (SH32) carrying the methicillin	Isolated in 2003 in China from the blood of inpatient in	Yu et al., 2014

	resistance *mec* gene complex, arsenic, and copper resistance	a hospital	
Staphylococcus pseudointermedius CC45	Carries a novel $SCCmec_{57395}$ element. Resistance to oxacillin and penicillin, chloramphenicol, tetracycline, kanamycin, gentamicin, streptomycin, erythromycin, clindamycin, ciprofloxacin and arsenic, cadmium and copper resistance	Methicillin resistant *Staphylococcus pseudointermedius* are an emerging problem in animal healthcare and can cause severe infections in humans	Perreten et al., 2013
Stenotrophomonas maltophila SM777	Carries resistance to cadmium, lead, cobalt, zinc, mercury, silver, selenite, tellurite, uranium.	Increasingly important as a nosocomial pathogen of cystic fibrosis patients and the immunocompromised.	Pages et al., 2008 ; Crossman et al., 2008
Yersinia enterocolitica	Arsenic resistance carried on the 70kb pYV virulence plasmid.	Tn*2502* confers resistance to arsenite and arsenate.	Neyt et al., 1997
Yersinia Pestis JAVA 9	Carries 4 plasmids each of which carries Tn*2503* encoding arsenic resistance	Isolated in 1957 in Java from a dead rat. The strain is fully virulent in non-human	Eppinger et al., 2012

	related to Tn*2502* carried *on* *Yersinia enterocolitica* pYV virulence plasmid.	primate and rodent models, but lacks the *Y. pestis*-specific plasmid pMT.	

Table 4: Arsenic resistance in recently sequenced strains.