COPPER RESISTANT BACTERIA BETTER TOLERATE COMMERCIALLY AVAILABLE ANTIMICROBIAL TREATMENTS BASED IN SILVER AND SILVER-COPPER IONS

By

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Oscar Hernando Torres Urquidy

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Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College. I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

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Signed_____Oscar Hernando Torres Urquidy_____

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DEDICATION

To my family, friends, and all the persons who contributed maximally or minimally to complete this dissertation work.

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ABSTRACT

The Civilizations have used copper and silver with disinfecting purposes for at least 4000 years. Copper is not toxic unless it is concentrated excessively and is especially harmful against bacteria. On the other hand, silver presents very high antimicrobial activity given its optimal concentration and exposure time. Both copper and silver are used to treat water systems, are given to animals as health supplements, are applied on catheters, textiles and other surfaces for antibacterial purposes. Due to exposure to sublethal doses of copper during the antibacterial treatments, bacteria are believed to develop resistance against the metal. The reportedly copper resistant bacteria used in this study were *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas putida* and *Salmonella enterica* strains S9, S19 and S20. Both in the Gram positive and Gram negative bacteria, the molecular mechanisms in charge of providing homeostasis of copper and/or silver, and perhaps causing the metal resistance, are the hyperactivity of membrane efflux pumps and metal oxidases and reductases, the occlusion and/or lack of cellular entrance channel proteins, and the presence of metallo-chaperone proteins. Thus, it is suggested that the mechanism believed to be responsible for the homeostasis (resistance) of 1 metal may participate in the homeostasis (resistance) of the other.

INTRODUCTION

I. PROBLEM DEFINITION

Bacterial resistance to copper (Cu) disinfection has been observed since as early as 1983 (Andersen *et al*., 1991; Marco and Stall, 1983). The bacterial resistance to copper disinfection is not well understood, but the previous exposure to sublethal copper concentrations on crops (Andersen *et al*., 1991; Marco and Stall, 1983), in pigs' intestines that are given copper supplements for growth (Hasman, 2005; Aarestrup *et al*., 2002), and on copper alloy coins (Espírito Santo *et al*., 2010); the long term bacterial disinfection with copper ionization systems (Lin *et al*., 2002) and mutations resulting in a degree of copper impermeability in cells (Lutkenhaus, 1977) have all separately been implicated as precursors in the development of copper resistant strains. Since copper alloys were registered as official antimicrobial materials to prevent microbial colonization and dissemination (U.S. Environmental Protection Agency, 2008), it is expected to have an increase in the applications of copper coatings on general use surfaces (fomites) but even more on fomites that contribute to hospital acquired infections (Copper Development Association, 2009). The environmental surfaces play an important role for spreading disease caused by human pathogens, for cross-contamination can easily be completed by depositing the pathogens directly from an infected patient to a surface, from the surface to the healthcare workers' hand, clothing or equipment and finally, from the former to the non infected patients or additional surfaces (Kramer *et al*., 2006).

The spread of copper applications on fomites has lead to the assumption that copper resistant bacterial strains will proliferate in analogy to the their multiplication in other environments (Espírito Santo *et al*., 2010; Hasman, 2005; Aarestrup *et al*., 2002; Lin *et al*., 2002; Andersen *et al*., 1991; Marco and Stall, 1983; Lutkenhaus, 1977); thus, the new challenge of developing an effective antimicrobial agent that combines 2 metals and kills such resistant strains has arisen to prevent these strains' infective dissemination.

Enterocci (Vincent *et al*., 2009; Bonten *et al*., 1996), *Pseudomonas spp*. and *Escherichia coli* are hospital relevant pathogens and are priorities for on-surface spread prevention, for in North America and worldwide, they are the causative agents of several infections identified in the Intensive Care Units (ICU) in hospitals (Vincent *et al*., 2009). On the other hand, *Salmonella* is a pathogen that contaminates food while the food is being processed on various surfaces. *Salmonella* effects on humans result in great economic loss in the U.S. (Butzby and Roberts, 1996), and in Canada, it has been known to cause severe dairy stock outbreaks where non antimicrobial fomites have served as infection intermediaries (Radke *et al*., 2002).

This work describes the antibacterial effects in physiological saline solution of the AgION® zeolite containing copper, silver, and their combination against reported copper-resistant and clinically relevant *Enterococcus faecium*, *Escherichia coli*, *Pseudomonas putida*, and *Salmonella* strains S9, S19 and S20. The AgION® zeolites are currently applied and used on surfaces on human contact to prevent their microbial colonization. The importance of researching antimicrobial properties of Ag and Cu in conjunct disinfection against copper resistant strains is that, if common molecular mechanisms

make bacteria resistant against the two metals (Franke *et al*., 2001; Stoyanov *et al*., 2001; Munson *et al*., 2000; Outten *et al*., 2000; Solioz and Odermatt, 1995; Odermatt *et al*., 1994), a disinfective treatment, such as the AgION® zeolite combining both copper and silver, may not be effective against the reportedly copper resistant strains. Consequently, the usage of such disinfective agent on surfaces may not have an efficacious action versus such resistant strains in the environment.

II. LITERATURE REVIEW

The following is a review about the interaction of microbes and copper and silver when these metals are used as antimicrobial agents.

III. COPPER AND SILVER AS ANTIMICROBIALS

III.A. **Historical Perspective of Copper and Silver Use**

In 2000 B.C. in ancient Egypt, copper was used to disinfect water and wounds. The ancient Phoenicians also fixed copper strips to ships' hulls to prevent fouling and thus increase ship speed and maneuverability (Borkow and Gabbay, 2009). In ancient Greece (400 B.C.), Hippocrates recommended copper for use on leg ulcers that were the product of varicose veins (Borkow and Gabbay, 2009; Michels and Anderson, 2008). In addition, knowledgeable persons prescribed copper for treating lung infections and for treating drinking water (Borkow and Gabbay, 2009). During the times of the Roman Empire, copper cooking utensils were used to prevent the spread of disease (Borkow and Gabbay, 2009) and Pliny (23 to 79 A.D.) treated intestinal worms with a combination of copper oxide and honey (Michels and Anderson, 2008). In North America, the Aztecs used copper oxide and malachite for treating skin conditions (Borkow and Gabbay, 2009) and practiced gargling with mixtures containing copper to alleviate inflamed throats (Michels and Anderson, 2008). Early American explorers, traveling west across the American continent, deposited copper coins in large wooden water containers to provide safe drinking water for their long trip. By the 18th century, copper had been adopted for

various medical uses in the Western world in the treatment of mental and pulmonary disorders. During World War II, Japanese soldiers placed pieces of copper in their water bottles to help prevent dysentery. Even today, persons both in Africa and Asia practice a custom from the past in which copper sulfate is used to cure sores and skin diseases. Due to the healing properties ascribed to the compound in these regions, it is highly prized (Borkow and Gabbay, 2009).

The antimicrobial properties of silver (Ag) have also long been recognized (Silvestry-Rodríguez *et al.*, 2007; Yoon *et al*., 2007). For more than 2,500 years, silver has been used for the purification of water and to control diseases with no reports of toxic reactions in the hundreds of millions of persons exposed to the metal. Reports describe the use of silver as a link to humankind's earliest attempts to improve his environment. Since ancient times in Europe, silver vessels have been used (Shashikala *et al*., 2007) to preserve foods (Silverstry *et al*., 2007), vinegar, water, and wine (Shashikala *et al*., 2007). In ancient times in Mexico, water and milk were kept in silver containers (Davis and Etris, 1997).

The antimicrobial activity of silver ions was first scientifically identified in the 19th century (Demling and Desanti, 2001). In 1881, silver nitrate drops were found to reduce (by approximately 99%) the number of cases of ophthalmia neonatorum in newborns in a hospital. These infections of the eye in newborns are contracted during passage through the birth canal and are thus venereal in origin (Thygeson, 1936). During the following years, silver nitrate prophylaxis was established and required by law or by health departments in many countries around the world (Hoyme, 1993). In the 1920s, the

United States Food and Drug Administration (FDA) accepted colloidal silver as an effective treatment for wound management (Demling and Desanti, 2001).

III.B. **Antimicrobial Applications for Copper and Silver**

At prescribed levels, copper is deemed to be safe for human consumption since it is an essential trace element used in several human physiological and metabolic pathways (Uauy *et al*., 1998) and allergic reactions with human skin are rare (Borkow *et al*., 2008). Copper is also necessary for wound repair (Borkow *et al*., 2008). Many microorganisms have been demonstrated to be highly susceptible to copper injury (Borkow and Gabbay, 2005). Due to its antimicrobial properties, copper and copper-based compounds have been used in numerous applications. Copper was recently accepted as an antibacterial material by the U.S. Environmental Protection Agency (EPA) (U.S. Environmental Protection Agency, 2008) which regulates antimicrobial claims that are placed on commercial products. This may serve to fuel more studies regarding copper's bactericidal capabilities along with an increased use in antibacterial applications (Copper Development Association, 2009).

With the appropriate time exposure, silver shows a high level of antimicrobial activity (Silvestry-Rodríguez *et al*., 2007). Both the Environmental Protection Agency (EPA) and the World Health Organization (WHO) regard silver as safe for human consumption. On the basis of epidemiological and pharmacokinetic data, a lifetime limit of 10 grams of silver can be considered a No Observable Adverse Effect Level (NOAEL)

for humans (World Health Organization, 1996). Silver is often effective at concentrations in the range of parts per billion (i.e., µg/L). As such, silver has also been used in many areas as an antimicrobial.

III.B.1. Water treatment

The use of as much as 0.1 mg/L silver for water disinfection could easily be tolerated without risk to human health since the total absorbed dose would only be half of the NOAEL over 70 years of life (World Health Organization, 1996). In Europe, silver has been used as antimicrobial for drinking water (Clarke and Berman in Block, 1983). Silver has been used as an integral part of the EPA and National Sanitation Foundation (NSF) approved point-of-use (POU) water filters to prevent bacterial growth. Home water purification units (e.g., faucet-mounted devices and water pitchers) in the U.S. contain silverized activated carbon filters along with ion exchange resins (Gupta *et al*. 1998; Silver, 2003). Fifty million consumers obtain drinking water from POU devices that utilize silver (Water Quality Association, 2001). These products leach silver at low levels (up to 0.05 mg/L) with no known adverse health effects. In South America, silver has been added to ceramic filters, resulting in a very compact and portable water filtration system effective for the elimination of both bacteria and protozoa (Lantagne, 2001).

Copper compounds may be added to water reservoirs used for fishing and swimming areas, watering livestock, and for horticulture in areas where greens and ornamental plants receive fairway and turf irrigations to prevent the growth of algae and

other parasites (SePro Company, 2008). The usage of copper pipes for water installations in buildings allows for copper ion release into the water, accounting for its partial elimination of bacteria (ACE hardware corporation, 2011).

Copper-silver ionization by electrolysis is an effective alternative for disinfecting water systems (Stout and Yu, 2003; Liu *et al*., 1994) that has been used for more than two decades. Hot water is passed through a flow cell where the addition of copper and silver ions is done by applying an electric current between two electrodes composed of an alloy made of 90 parts copper and 10 parts silver. Addition of the ions only into the hot water recirculating lines permits strictly limited contact with the operators (Lin *et al*., 1996).

III.B.2. Foods and health supplements

Silver has been used to treat vinegar, fruit juices, and effervescent drinks and wine (Foegeding and Busta, 1991). It is also available in Mexico as colloidal silver in gelatin for use as a consumer produce wash and in the U.S. as an alternative health supplement or in silver citrate complexes as food additives (Silver 2003; Silvestry-Rodríguez *et al*., 2007).

III.B.3. Medical applications

Contemporarily, silver has numerous uses in medicine. For instance, silver has been added to bandages for treating wounds of various origins (Michaels *et al*., 2009),

particularly for burn patients. Silver impregnated fiber materials are placed directly on burn wounds to prevent infection. Silver is released from the fibers onto the tissues and liquids surrounding the burnt area (Ip *et al*., 2006b). Marketed as Silvazine in the U.S., a topical cream composed of 1% silver sulfadiazine and 0.2% chlorhexidine digluconate in a water immiscible cream is used for reducing infections of large open wounds in humans and animals (Silver, 2003).

Pure silver coatings can be applied to the surfaces of fracture fixation devices, vascular grafts, sutures, (Darouiche, 1999) and heart valve sewing rings (Auer *et al*. 2001; Darouiche, 1999), but these coatings have not consistently provided antibacterial efficacy *in vivo* despite promising results demonstrated *in vitro* (Darouiche, 1999). Silver coated plastic indwelling catheters are designed to impede the growth of microbial biofilms while infection by nosocomial bacteria is prevented (Silver, 2003).

In 1994, the FDA approved a 10-year claim of efficacy and safety for a Cu releasing TCu380A intrauterine device based on a low incidence of pelvic inflammatory diseases *in utero* when this copper device was used for long periods (Bilian, 2002). Moreover, due to the copper's antimicrobial properties, other medical related devices have been made of copper which include nebulizers (European Medical Device Technology, 2011) and hospital gas pipes (PlumbingHelp CA, 2011).

A mixture of copalite and copper powder has shown to prevent caries (cavities) when it is applied on the root surfaces of teeth and an anti-cariogenic layer can be left semi-permanently (Thneibat *et al*., 2008). Also, cuprous thiocyanate has been included in toothpastes that stay on teeth as a semi-permanent coating with antibacterial effects

(Gladstone, 1973). Additionally, the antibacterial properties of dental amalgams have been proven with amalgams that contain copper and silver. Such amalgams were able to reduce the bacterial populations significantly when tested in nutrient rich media (Morrier *et al*., 1998).

III.B.4. Textiles

Pure cotton (Renaud *et al*., 2006), pure polyester (Chen *et al*., 2010), and cotton-polyester (Takai *et al*., 2002) textiles impregnated with metal mixtures of silver, zinc and copper (Renaud *et al*., 2006; Takai *et al*., 2002) can be worn by surgeons and medical staff to control the survival and spread of bacteria on garments (Takai *et al*., 2002). For civilians, a demand for hygienic clothing and active wear made of nylon, wool (Gao and Kranston, 2008), and polyester (Chen *et al*., 2010) has created an important market for antimicrobial textile products (Gao and Kranston, 2008). These products are clothing pieces with silver that can prevent unpleasant smells by inhibiting odor producing bacteria (Chen *et al*., 2010). Additionally, fabrics used in the filters of respirators (SpectraShield 9900) are impregnated with the copper-silver zeolite to kill the microbes that come into contact with the filter. The passage of air through the fabric provides the user with sterile air (Fong, 2007).

III.B.5. Antimicrobial or coated surfaces

Silver has also been added to polymers (Brady *et al*., 2003) inorganic ceramics (Cowan *et al*. 2003; Galeano *et al*. 2003; Kim *et al*. 2004; Kim *et al*. 1998;, and plastics (Silver 2003) to confer antimicrobial activity. The result is consumer products such as washing machines, refrigerators, ice machines, public telephones, calculators, and toilets (in Japan), plastic toys, and food contact surfaces that have incorporated silver (Silver 2003; AgION 2009). Hundreds of formulations containing silver have been registered with the EPA in the United States. In Japan, silver-substituted zeolite has been developed as the most common antimicrobial agent incorporated into plastics. The zeolite is laminated as a thin layer (3–6 μm) on food contact surfaces and release silver ions upon contact with aqueous solutions from foods (Ishitani, 1995 in Quintavalla and Vicini, 2002).

Copper has also been used in many antimicrobial surfaces. For instance, a uniform coating of multi-layered copper granules can be applied on roofs of homes to prevent attachment and growth of algae and moss (3M Industrial Mineral Products Division, 2004). Cuprous oxide and cuprous thyocyanate have been used in antifouling paints for the bottom of ships to prevent the growth of microbial biofilms (Vetete *et al*., 1997). Self-sterilizing metallic copper (Noyce *et al*., 2006; Faúndez *et al*., 2004) or copper containing materials (Borkow *et al*., 2004) have been used for surfaces for cutting, mixing, wrapping, packaging, storing or transporting meats and vegetables (Borkow and Gabbay, 2009; Noyce *et al*., 2006; Borkow *et al*., 2004; Faúndez *et al*.,

2004). Copper antimicrobial coatings and fixtures can also be installed in commercial, residential and health care facilities to reduce the microbial load on surfaces and thus lower the transfer of pathogens to hands or instruments (Casey *et al*., 2010; Weaver *et al*., 2008; U.S. Environmetnal Protection Agency, 2008; Noyce *et al*., 2006).

III.C. **Copper and Silver Toxicity**

III.C.1. Copper

The potential symptoms of overexposure to copper dusts and mists are irritation of the eyes, nose, and pharynx, nasal perforation, a metallic taste, and dermatitis. The potential symptoms of overexposure to copper fumes are irritation of the eyes and upper respiratory system. Metal fume fever can also occur which includes symptoms such as muscle aches, chills, nausea, fever, cough, weakness, lassitude, and dry throat; milder effects can include a sweet or metallic taste and a discoloration of hair and skin (O'Neil *et al*., 2001).

The number of reported cases of copper poisoning in the general population is markedly low (Bremner, 1998). The sporadic reports of acute copper toxicosis in cases such as contamination of drinks from copper-made containers or dispensers usually include symptoms such as anuria (lack of urine flow), oliguria (low urine flow) hematuria (red blood cells in the urine), hemoglobinuria (hemoglobin in the urine), jaundice,

diarrhea, metallic taste, nausea, and vomiting (Chuttani *et al*., 1965). In reality, low chronic copper toxicosis may be more health-detrimental than the acute intoxication, for the chronic toxicosis is characterized by a gradual copper accrual in the liver and can affect the liver eventually. Thus, the chronic consumption of 2.2-3.5 mg Cu/L from tap water for more than 9 months resulted in the death of a child, but the sudden ingestion of 100 g Cu in an attempted suicide by an adult resulted in the patient's total recovery ([Jantsch *et al*., 1985; Müller-Höcker *et al*., 1988] in Committee on Copper in Drinking Water, National Research Council, 2000). However, copper poisoning's effects can vary depending on various characteristics based on conditions such as age, diet, genetics, and physiological condition (Bremner, 1979 in Nriagu, 1979). Adult animals seem to be more tolerant to high copper concentrations than nurturing and neonatal animals, for young animals have very high level of copper absorption and possess immature biliary excretory mechanisms. This may explain why young children prevail in reports of copper-induced cirrhosis (Tanner, 1997). Particular individuals may also be more prone to suffer copper toxicosis within any given species (Bremner, 1998).

In all the organisms, many of the clinical adverse effects of excessive copper ingestion are cogent with oxidative damage to macromolecules and/or membranes. Copper is active in oxidation-reduction processes, and, through the Haber-Weiss reaction, it is able to catalyze the formation of hydroxyl radicals (Kadiiska *et al*., 1992). The generation of hydroxyl radicals also induces DNA damage (Bremner, 1998).

III.C.2. Silver

Potential symptoms of silver dust overexposure are argyria, ulceration and irritation of skin (O'Neil *et al*., 2001; Nordberg and Gerhardsson, 1988); however, ingestion is the primary route of exposure in humans. For instance, it is released from dental amalgams (Silver, 2003; Danscher, 1981), leaches from water filters (Lee and Jeong, 2004), is taken orally as colloidal silver (Silver, 2003), and is used in water treatment (e.g., silver/copper ionization in hospitals) (Lin *et al*., 1996). If long-term inhalation or ingestion of soluble silver compounds or colloidal silver reaches a total dose ranging from 0.91-30 grams of metallic silver (Hill and Pillsbury, 1939), argyria (Gulbranson *et al*., 2000) and/or argyrosis may develop (Lansdown, 2006; Nordberg and Gerhardsson, 1988; Hill and Pillsbury, 1939). Argyria consists of a characteristic, irreversible bluish-gray or ash gray pigmentation of the skin and nails, but it is most prominent on sun exposed body parts (Drake and Hazelwood, 2005; Gulbranson *et al*., 2000, Agency for Toxic Substances and Disease Registry, 1990; Greene and Su, 1987; Shelley *et al*., 1987). This condition is accompanied by deposition of silver in organs and tissues (Drake and Hazelwood, 2005; Agency for Toxic Substances and Disease Registry, 1990). Argyria and argyrosis may be classified as either localized or generalized (Greene and Su, 1987). The localized form is caused by direct external contact with silver (Drake and Hazelwood, 2005; Agency for Toxic Substances and Disease Registry, 1990; Greene and Su, 1987). It is believed that when the body absorbs silver compounds, they form complexes primarily with proteins, but also with DNA and RNA by binding to amino,

carboxyl, imidazole, phosphate and sulfhydryl groups (Lansdown, 2006; Danscher, 1981). Nevertheless, despite the various clinical effects that silver can induce when it is absorbed in the body, when tested in rats, silver did not prove carcinogenic effects (Furst and Schlauder, 1978).

IV. BACTERIAL RESISTANCE / TOLERANCE TO COPPER AND SILVER

IV.A. **Evidence for copper resistance / tolerance**

The development of resistance or tolerance to either copper or silver may come from the exposure of bacteria to continuous doses that do not completely kill them (Lin *et al*., 2002). The following are reports of bacterial strains isolated from environments where exposure to unusual metal levels may have promoted the development of copper resistance/tolerance [demonstrated by laboratory minimum inhibitory concentration (MIC) tests].

Resistance against copper injury in *Enterococcus faecium* has been studied in Europe since 1998 (Hasman and Aarestrup, 2002) where bacterial strains are believed to develop this resistance due to exposure to copper sulfate, a food additive that is commonly used in the production of food animals (Danish Agricultural Advisory Center in Hasman and Aarestrup, 2002). In several studies, *E. faecium* strains from farms with animals destined for human consumption have been isolated and these isolates have been tested for their capacity to resist high copper concentrations (Hasman *et al*., 2006; Hasman and Aarestrup, 2005; Aarestrup and Hasman, 2004; Aarestrup *et al*., 2002; Hasman and Aarestrup, 2002). In general, *E. faecium* has a very high tolerance to copper; therefore, a copper susceptible strain is assumed to have a copper MIC of 4 mM. The *E. faecium* isolates from these farms were exposed to a copper concentration range from 35

to 175 ppm in the feed and showed Cu MICs of 16 mM and 28 mM (Hasman and Aarestrup, 2002).

Lately, in addition to the increase of resistance of *Escherichia coli* against antibiotics in the U.S. (Gupta *et al*., 2001; Gupta *et al*., 1999) there have reports of an increase of resistance against the toxic action of metals, particularly copper. Copper resistance in *E. coli* has been reported as early as 1983 (Tetaz and Luke, 1983) and commonly over the last several decades (Aarestrup and Hasman, 2004; Williams *et al*., 1993). Cu^r (copper resistant) *E. coli* strains have been isolated from cattle directly through their bodily excretions (Aarestrup and Hasman, 2004; Williams *et al*., 1993) or from their surroundings (Tetaz and Luke, 1983). The reason for the proliferation of copper resistant *E. coli* strains in cattle or pigs is the inclusion of copper sulfate in their feed as a growth promoter. This may lead to the selection of copper resistant strains either by eliminating non-resistant strains (Tetaz and Luke, 1983), or by sub-lethal exposure which activates resistance mechanisms (Lin *et al*., 2002). Generally, a copper sensitive *E. coli* K-12 strain has a MIC of 4 mM Cu (Tetaz *et al*., 1983). Tetaz and Luke (1983) reported four *E. coli* isolates with a Cu MIC ranging from 12 mM to 20 mM. Williams *et al*. (1993) found four strains with a Cu MIC of 18 mM, and Aarestrup and Hasman (2004) described 202 strains whose MICs varied from 8 mM to 24 mM Cu.

A *Pseudomonas putida* strain (08891) was originally isolated from a tomato seed lot by Cooksey *et al*. (1990). Its copper MIC was established at 2.4 mM, yet the measurements were taken in an organic rich medium which may have led to an overestimation of the value. This strain is more resistant to copper than *P. putida* R5-3 (a

moderately resistant strain with an MIC of 1.8 mM) (Cooksey *et al*., 1990) and than a copper sensitive wild type strain PNL-MK25 (with an MIC of 0.4 mM (Adaikkalam and Swarup, 2005). However, *P. putida* CZ1 isolated from a heavy metal contaminated soil exhibited a copper MIC of 5 mM (Chen *et al*., 2006).

In a large study, 156 *Salmonella* strains isolated from farms and their animals fed with copper supplements had a very high copper tolerance with MIC's ranging from 20 mM to 28 mM (Aarestrup and Hasman, 2004). A copper resistant *Salmonella* strain recovered from the wastewater from a tannery had a Cu MIC of 6.0 mM and two others had a MIC of 6.30 mM (Shakoori and Muneer, 2002). In comparison, a copper sensitive strain, *S.* Typhimurium (ATCC 14028s) had an MIC of 5.5 mM (Espariz *et al*., 2007).

It is important to mention that the majority of such copper MIC tests are carried out in media rich in organics. In such media, copper may be bound and thus prohibited from interacting directly with the bacteria (Schmidt *et al*., 2007; Chen *et al*., 2006; Lin *et al*., 2002; Calomiris *et al*., 1984). This may lead to an overestimation of copper MIC's for tested strains.

IV.B. **Molecular mechanisms of Copper homeostasis, tolerance, and resistance**

The molecular mechanisms for copper homeostasis provide the means necessary for the bacterial cells to survive certain concentrations of copper. Given the protective

nature of the mechanisms, it is likely that their over expression (e.g., redundant mechanisms being expressed at the same time or the expression being up-regulated) or a loss of repression (e.g., physical alteration in the repressor or mutation in the promoter) could lead to resistance. The mechanisms for bacterial copper homeostasis and/or resistance are discussed below.

IV.B.1. Copper oxidase protection in the periplasm

The increased anaerobic toxicity of copper for *E. coli* is theorized to result from a shift in total copper from the Cu^{2+} to the Cu^{1+} oxidation state as oxygen is depleted (Beswick *et al*., 1976). This shift could be due to the reduction of Cu^{2+} to Cu^{1+} by biological mechanisms such as cell-surface reductases, small molecule reductants, or other components of the electron transport machinery (Outten *et al*., 2001).

The CueO enzyme constitutes an oxidative protection mechanism for achieving copper resistance in *E. coli*. CueO is a periplasmic multi-copper oxidase enzyme which couples the one electron oxidation of copper to full reduction of molecular oxygen to water by utilizing a functional unit formed by three classes of copper-binding locations with different spectroscopic and functional properties (Solomon *et al*., 1996). The observation that Cu^{1+} is more toxic than Cu^{2+} suggests that the role of CueO during aerobic growth may be to convert periplasmic Cu^{1+} to a less toxic form, namely Cu^{2+}. Thus, one factor that may contribute to Cu^{1+} toxicity and its accumulation is the loss of protein CueO function in the absence of oxygen. Experimentally, a CueO-minus *E. coli*

mutant showed only a mild aerobic copper sensitivity in comparison to wild type strain. Disruption of CueO function may lead to an increase in the Cu^{1+} concentration gradient on the periplasmic side of the inner membrane (Outten *et al*., 2001). The Cu^{1+} accumulation could then occur via passive or active uptake of Cu^{1+} through yet unidentified inner membrane factors, possibly involving nonspecific uptake systems for other metals such as sodium or potassium. However, protein CueO has not been proven to use Cu^{1+} as an electron donating substrate *in vivo*, and it has been proposed that it may have another biochemical function, such as that of a terminal oxidase that helps energize a copper transport pathway (Outten *et al*., 2001).

IV.B.2.Copper efflux proteins

In the Gram-negative bacterium *E. coli*, CopA is a Cu^{1+} translocating P-type ATPase, regulated by Cu^{1+} and Ag^{1+}, that is activated by the CueR protein (Stoyanov *et al*., 2001; Outten *et al*., 2000). CopA transports Cu^{1+} across the cytoplasmic membrane by extruding the cytoplasmic cation into the periplasm; it is the main element for copper homeostasis in *E. coli*, and it is required for copper resistance under both aerobic and anaerobic environments (Outten *et al*., 2000). Cells disrupted in the *copA* gene, which do not express the CopA efflux pump, have increased sensitivity to copper over wild type cells (Outten *et al.*, 2001). In order to clear the cytoplasm of excessive copper, CueR, as a cytoplasmic Cu^{1+} sensor, is ideally placed to up-regulate the primary copper efflux system represented by *cueO* and *copA* genes (Outten *et al*., 2000). In the cytoplasm, as

copper stress increases, the *cue* system is activated to a maximal induction of ~12-fold from its basal level. In this sense, *cueR, copA* and *cueO* genes and their proteins confer copper tolerance under both moderate and high copper concentrations (Outten *et al*., 2000).

In Gram-negative bacteria, the CusCFBA system is a family of 4 transport proteins that are usually encoded by a single operon (Franke *et al*., 2003). The central pump protein, CusB, belongs to the resistance nodulation cell division family (RND). RND proteins are secondary transporters believed to be energized by proton-substrate antiport (Transporter Classification Database, 2010). The three other CusCFBA components are CusA, a membrane fusion protein (MFP) (Dinh *et al*., 1994) that is anchored to the cytoplasmic membrane and CusC, an outer membrane factor (OMF) (Johnson and Church, 1999) that is anchored to the outer membrane, and CusF, which may act as a metallochaperone and may be involved in copper tolerance through the selection of metal substrates (Franke *et al*., 2003). Only the metal efflux CusCBA protein complex extends from the cytoplasm through the periplasm to the cellular exterior (Munson *et al*., 2000), while the CusF protein is expected to reside in the periplasm (Loftin *et al*., 2007). The *cusCFBA* operon is regulated by the *cusR* gene and its product CusR. Deletion of *cusR* hinders *cusCFBA* from being expressed when copper challenges the *E. coli* cells, leaving *E. coli* without the active CusCFBA copper resistance mechanism. Under aerobic conditions, the copper sensitivity of a *cusR*-minus strain was not different from wild type *E. coli*, implying that CusCFBA does not confer significant copper resistance under aerobic conditions. Surprisingly, the opposite result was observed

when the same *cusR* mutant was challenged with copper under anaerobic conditions, with increased copper sensitivity measured (Franke *et al*., 2003; Outten *et al*., 2000). Furthermore, it was found that copper accumulation increased markedly in the *cusR* deleted strain compared with the wild-type strain, suggesting that anaerobic copper export is disrupted in the absence of a functioning CusCFBA system (Franke *et al*., 2003; Outten *et al*., 2000). Overall, the CusCFBA system plays an important role in copper tolerance under anaerobic growth and under extreme copper stress when complementing the CopA/CueO system during aerobic growth (Franke *et al*., 2003; Outten *et al*., 2000).

In Gram-positive bacteria, the best studied mechanism of copper homeostasis has been that of *Enterococcus hirae*. Genetically, the copper homeostatic system consists of four genes arranged in the *cop* operon. The gene products of *copA* and *copB* are copper transporting ATPases, *copY* encodes a copper-responsive repressor, and *copZ* encodes a chaperone which can catalyze intracellular copper routing (Solioz and Stoyanov, 2003). The *cop* operon is constitutively expressed (Solioz and Odermatt, 2003) and is regulated by the copper-responsive repressor CopY (Odermatt and Solioz, 1995), which detaches from the *cop* operon once the CopZ chaperone transfers Cu^{1+} directly to CopY (Solioz and Stoyanov, 2003). CopB is a Cu^{1+}/Ag^{1+} cell extruding P-type ATPase *in vivo*, facilitating copper and silver resistance (Solioz and Odermatt, 1995; Odermatt *et al*., 1994). In *E. hirae*, CopB serves as the P-type ATPase copper extrusion pump, being the equivalent of CopA in *E. coli*.

Interestingly, it was found that all the Cu^r (copper resistant) *Enterococcus faecium* strains isolated from three different European countries all contained a particular gene,

tcrB (Hasman *et al*., 2006; Hasman and Aarestrup, 2005; Aarestrup *et al*., 2002; Hasman and Aarestrup, 2002). A search in the NCBI BLAST database of the TcrB protein revealed an overall amino acid identity of 46% to the CopB protein from *E. hirae* as the closest homologous protein, and only 30% identity to any gene of *E. coli* origin (Hasman and Aarestrup, 2002).

The copper resistance found in the Gram-negative *Pseudomonas putida* strain 08891 is believed to come from a *cop* operon homolog (Cooksey *et al*., 1990). BLAST searches show the systems that are likely to be involved in the detoxification of copper in *P. putida* are chromosomal *cueA* (Adaikkalam and Swarup, 2002), *copAB* and *cus*. Moreover, the *cop* system appears to be duplicated in *P. putida* but not in its close relative, *P. aeruginosa* (Cánovas *et al*., 2006). Such duplication may allow a Cu efflux pump to substitute another when one is not functional, or may function in redundancy which may be translated in the copper resistance equivalent to the sum of the individual copper effluxes' actions.

IV.B.3.Copper protein chaperones

In studies in yeast, copper in the form of Cu^{1+} is transported across the plasma membrane by proteins which further transfer the metal to small, soluble cytoplasmic Cu transporters known as Cu chaperones. Thus, Cu chaperones are proteins which transport Cu directly to other specific proteins located in different cellular compartments (Valentine and Gralla, 1997). Under physiological conditions, there has been no detection

of intracellular copper ions in yeasts. This suggests that all intracellular copper is bound to chelators, metallochaperones, or other proteins (Rae *et al*., 1999). In the Gram-positive bacterium *Bacillus subtilis*, the Cu^{1+} mediated chaperone-transporter interaction consists of a direct cation transfer from the chaperone to the transporter. This gated flow of Cu^{1+} seems to be the essence of how cells avoid toxic Cu^{1+} in solution that may result in cell death (Singleton and Le Brun, 2007).

In the *E. coli* periplasm, CusF, a protein encoded by the copper tolerance *cusCFBA* operon, may act as a metallochaperone and be involved in copper tolerance through the selection of metal substrates or it may regulate the efflux complex CusCFBA through protein–protein interactions. The arrangement of CusF ligands effectively sequesters Cu^{1+} from its periplasmic environment and thus may play a role in protecting the cell from the toxic ion; however, its role as a chaperone (which would have to pass the metal to another acceptor protein) has been questioned due to the chemical structure of the CusF copper binding site since the Cu^{1+} transfer site between CusF and the acceptor protein is physically blocked by an amino acid. Therefore, a gating mechanism involving a CusF physical rearrangement seems to be necessary for metal transfer, and this physical rearrangement should be induced by the protein-protein–donor-acceptor interaction (Loftin et al., 2007).

In Gram-positive bacteria, specifically *E. hirae*, it has been suggested that the metallo- chaperone CopZ can bind uptaken copper directly from the influx protein CopA. There is first an electrostatic attraction between CopZ and CopA which facilitates the direct contact between the two proteins. Once the proteins are in proximity (Solioz and

Stoyanov, 2003; Multhaup *et al*., 2001), copper up-modulates the two protein interaction through the copper binding motif, or the cystein-X-X-cystein pattern in the N-terminus that is present in the CopA protein (Multhaup *et al*., 2001). After the chaperone CopZ is loaded with Cu^{1+}, copper delivery to CopY occurs, and ultimately CopY de-represses the *cop* operon (Solioz and Stoyanov, 2003).

IV.B.4. Porins and copper impermeability

Outer membranes, like other biological membranes, are fundamentally built as a bilayer of lipids. As such, they have little permeability for hydrophilic solutes, including most nutrients. Therefore, they contain channel-forming proteins which allow for the influx of nutrients and perhaps for the extrusion of waste products. Porins were defined as proteins that form such nonspecific channels and in 1976, they were found in every species of Gram-negative bacteria investigated and even in a group of Gram-positive (and in some cases acid-fast) bacteria, the *Corynebacterium–Nocardia–Mycobacterium* complex. X-ray crystallographic analysis determined that porins exist as transmembrane β-barrels (Nikaido, 2003). Porins are believed to be the copper entrance mechanism in Gram-negative bacteria. Shortly after the discovery of porins, porin-devoid *E. coli* K-12 mutants were identified and tested for their tolerance against high copper concentrations. Higher copper tolerance was shown by the mutants, so it was hypothesized that such porins allow for the entrance of copper into the cell where it can then reach copper sensitive sites perhaps within the cytoplasm or the periplasm. It was suggested that even

though the mutant was more resistant, copper was entering the *E. coli* cells through other porins, for copper still entered the cells but at a slower rate. Additionally, the mutant died when really high copper concentrations were reached (Lutkenhaus, 1977).

IV.B.5.Copper influx pumps in Gram-positive bacteria

Of the Gram-positive bacteria, *E. hirae* is perhaps the best studied microorganism in copper homeostasis. By indirect evidence (Solioz and Stoyanov, 2003), the entry of copper is believed to take place by an active influx pumping when copper is available in limited conditions (Wunderli-Ye and Solioz, 1999). The influx pump responsible for this uptake is named CopA, a copper P-type ATPase that is very similar to the efflux pump with the same name found in *E. coli*. *E. hirae* cells disrupted in the influx pump *copA* gene grew poorly in a medium scarce in copper, but grew abundantly when the copper concentrations were plentiful (Wunderli-Ye and Solioz, 1999).

IV.B.6.Membranes and teichoic acids protect against copper

Ferroplasma acidarmanus Fer1, an archaeon isolated from a highly acidic environment, is able to grow and respire at concentrations of 20 g/L of copper in mineral salts medium. A notable characteristic of extremely acidophilic archaea is the impervious nature of their cell membranes (van de Vossenberg *et al*., 1998). This feature is likely to

be an important feature of highly metal-resistant microorganisms (Baker-Austin *et al*., 2005).

In bacteria, cell membranes differ in chemical structure between Gram-positive and Gram-negative species. Such structural differences include the presence of teichoic acids in most Gram-positive cell walls. Teichoic acids are bacterial polysaccharides of glycerol phosphate or ribitol phosphate linked via phosphodiester bonds. Teichoic acids differ in their chemical compositions among bacterial phylogenetic groups, but in general, these acids have been recognized for two essential functions: 1) maintaining cation homeostasis and assisting in the assimilation of magnesium (Hughes *et al*., 1973; Heptinstall *et al*., 1970) and other metal cations, and 2) for defining the electromechanical properties of the cell wall (Neuhaus and Baddiley, 2003). They also have additional roles in adhesion, biofilm formation, acid tolerance, intrageneric co-aggregation, protein folding, antibiotic resistance, Ultra Violet sensitivity, and virulence. Most of the time, the general electrostatic charge in teichoic acids, cellular membranes and walls, is negative. D-alanine, an amino acid known to form part of the teichoic acids, shows an overall positive charge. If it is present in enough quantities along the teichoic acid chains, it alters the overall electrostatic charge along the acids, on the cellular membrane and wall, and may also change the overall cellular surface charge from negative to positive. This charge alteration is believed to play a primordial role in fomenting resistance against the action of positively charged antimicrobial peptides (Kristian *et al*., 2005), and against positively charged germicidal metals, whose action is greatly determined by electrostatic attraction to the microbe's negative charge (Kristian *et*

al., 2005). Likely, the presence of teichoic acids in Gram-positive bacteria, depending upon the species, may provide resistance against positively charged metals.

IV.C. **Evidence for silver resistance / tolerance**

Reports of bacterial silver resistance have been acknowledged since the 1970's in hospital units where silver is used as an antibacterial for treating burns (McHugh *et al*., 1975). Silver resistant bacteria have also been isolated from silver mines (Haefeli *et al*., 1984).

The *Salmonella enterica* serovar Typhimurium MGH strain harboring the silver determinant *sil* genes in the pMG101 plasmid first became famous for its colonization of three patients that suffered from bacteremia and died in a hospital burn ward in 1973. The transferable plasmid pMG101 conferred multiple antibiotic and silver resistances. When tested for $AgNO_3$ resistance with the TYE agar-plate dilution technique, the silver resistant (Ag^r) *S.* Typhimurium MGH showed a Ag MIC of 10 mM, an MIC much higher than the one displayed by 10 other *S.* Typhimurium strains which despite their being multiply antibiotic resistant, were silver sensitive (Ag^s) with a silver MIC of 0.6 mM.

The *Enterobacter cloacae* strains Ag703 and Ag1157 isolated from teeth carrying metal containing restorations were defined as Ag^r because their $AgNO_3$ MIC was 1 mM Ag, the same $AgNO_3$ MIC shown by Ag^r *Pseudomonas stutzeri* AG259. Contrarily, these *E. cloacae* isolates did not exhibit resistance against silver sulfadiazine (AgSd) with a MIC of 0.05 mM Ag. In the study, Ag^s strains of *Staphylococcus aureus* (NCTC 6571;

Oxford strain), *P. aeruginosa* (NCTC 10662), and *E. cloacae* (strain 05) served as negative controls, with an MIC of 0.25 mM for $AgNO_3$ and 0.1 mM for AgSd. The two Ag^r *E. cloacae* isolates were four times more $AgNO_3$ resistant, but two times more sensitive to the AgSd than their Ag^s *E. cloacae* counterpart. The MIC determinations were performed using the Mueller-Hinton agar dilution technique (Davis *et al*., 2005); in other words, in a nutrient rich medium.

Since additional evidence of silver resistance is provided by the genetic analyses, it is worth mentioning that from the genomic DNA sequence of the *E. cloacae* Ag703 strain, a PCR product (Accession number AY679159) was obtained that shared the greatest homology with the Ag^r *silE* gene of *E. coli* strain pTJ100 (99% homology/300 bp) (Accession number AY214164) and shared slightly lower homology (96% homology/300 bp) with the *silE* from the *Salmonella* pMG101 plasmid (Accession number AF067954) (Davis *et al*., 2005). The SilE protein is a small periplasmic metal-binding protein coded in the silver determinant plasmid pMG101 of *S.* Typhimurium (Silver, 2003; Gupta *et al*., 1999) and is known to enhance cellular tolerance to Ag^{1+} (Gupta *et al*., 1999) by binding five toxic periplasmic Ag^{1+} cations per peptide (Silver, 2003).

In a recent study, 172 bacterial strains were obtained from chronic wounds in humans and horses and were examined for the presence of the silver resistance gene cassette (*silCBA, silE, silF, silRS,* and *silP*). Six strains (2 from humans, 4 from horses) containing the *sil* resistant genes were found. These were all discovered to be *E. cloacae* and the genes were demonstrated to be extra-chromosomal. This suggests Ag^r (silver

resistant) *E. cloacae* affects both human and veterinary medicine and implies the possibility that the presence of *sil* genes in *E. cloacae* is inherent. Two types of Ag MIC determinations were conducted: 1) where $AgNO_3$ was diluted in Mueller-Hinton broth and 2) where Ag-containing dressings created an inhibitory area on bacterial lawns grown on Tryptic Soy Agar. The six *E. cloacae* strains containing the silver resistance genes showed a $AgNO_3$ MIC $\geq$ 46.4 μM Ag in contrast to strains (both *E. cloacae* and *E. coli*) lacking these genes that had MIC's ranging from 9.3 μM to 23.2 μM Ag. Thus, the inhibitory concentrations of $AgNO_3$ were only slightly higher in *sil* containing strains compared to *sil*-negative strains. Since other Ag^r *E. cloacae* strains have shown Ag MIC values of up to 50.4 mM (Ip *et al*., 2006a), the isolates from this study appeared only to have low level phenotypic resistance (Woods *et al*., 2009). The experiments to determine the efficacy of Ag wound dressing on the *sil*-positive and -negative strains showed that the silver containing dressing gauze eliminated all strains after an initial 30 minute contact time with areas of inhibition clearly apparent around the gauze. However, results showed a significant difference (P = 0.0003) between the zones of inhibition for the *sil*-containing strains and the larger zones for the *sil*-devoid strains (Woods *et al*., 2009). Woods *et al*. (2009) proposed that the condition of silver resistance may be similar to that associated with *Staphylococcus aureus* methicillin resistance in that silver resistance is restricted to a particular species of bacterium and is not easily transferable.

Other environments have also harbored putative Ag^r strains. The *Pseudomonas stutzeri* AG259 and AG257 strains were isolated from the soil of a silver mine in Utah and both strains possessed silver resistance supposedly provided by a plasmid named

pKK1. In this study, the MIC was determined from the ability of bacteria to form single colonies on LB agar containing $AgNO_3$. Strains AG257 and AG259 were found to form colonies on plates with a silver concentration of at least 25 mM $AgNO_3$. It was proposed that the examined plasmid-determined silver resistance might involve the production of a compound which binds with Ag^{1+} to form an inert complex or that might change the availability of halides that bind Ag^{1+} so that Ag^{1+} is unable to affect cellular components (Haefeli *et al*., 1984).

IV.D. **Molecular mechanisms of silver homeostasis, tolerance, and resistance**

Bacterial silver resistance is usually encoded by genes located on plasmids (Gupta *et al*., 2001), but also may be found on the chromosome (Franke *et al*., 2001; Gupta *et al*., 2001; Li *et al*., 1997) as are other metal resistant determinants (Silver and Phung, 1996). The determinant studied in most detail was originally found on the Gram-negative *Salmonella* plasmid pMG101 (McHugh *et al*., 1975) and encodes resistance to Ag^{1+} (Gupta *et al*., 1999; McHugh *et al*., 1975). No silver resistant determinant has yet been identified in Gram-positive bacteria (Percival *et al*., 2005). The silver resistance determinant from *Salmonella* plasmid pMG101 contains nine genes (Silver *et al*., 2006; Silver, 2003; Gupta *et al*., 1999) but the functions for only eight named genes and their respective proteins have been designated, mainly based on homologies with other known bacterial proteins involved in metal resistance systems. These eight genes are *silF* (Silver

et al., 2006), *silP*, *silA*, *silB*, *silC*, *silR*, *silS*, and *silE* (Gupta *et al*., 1999). The silver resistance determinant is controlled and regulated by a two-component gene pair, named *silRS*, whose encoded proteins are a membrane sensor kinase and a transcriptional regulatory responder. These two genes are homologous to other members of the two-component family, including PcoRS found on plasmids (Brown *et al*., 1995) and CusRS found on the chromosome that belong to copper resistance systems (Munson *et al*., 2000). The other silver determinant genes, according to the functions of their proteins, are discussed in the following sections.

IV.D.1. Silver oxidation-reduction protective mechanism in the periplasm

Since Ag^{1+} cannot be oxidized to Ag^{2+} easily, Ag^{1+} should not be able to damage periplasmic components by oxidation (Nies, 2003). Also, since the reduction of of Ag^{1+} to Ag^{0} does not take place during but rather after cellular growth is complete (Silver, 2003), and the reduction occurs in both resistant and sensitive bacterial strains (Silver and Phung, 1996), neither post-growth respiratory chain reduction of Ag^{1+}, nor its oxidation, seems to be a silver resistance mechanism (Silver, 2003; Silver and Phung, 1996). What in reality may cause the cellular damage is Ag^{1+} binding to the cellular components (Nies, 2003). Due to the fact that silver is not a metal required for any known physiological function, no protection based on its oxidation or reduction has been described, and likely is non-existent (Nies, 2003, Silver, 2003; Silver and Phung, 1996).

IV.D.2. Silver efflux proteins

In experiments dealing with clinical isolates, Ag^{1+}-resistant *E. coli* mutants were selected (Li *et al*., 1997) and their resistances were shown to be due to active Ag^{1+} efflux derived from a chromosomally encoded system, perhaps the CusCFBA efflux protein system (Gupta *et al*., 2001), which had not yet been identified at that time (Silver *et al*., 2006). Encoded in the Gram-negative *Salmonella* pMG101 plasmid, the SilCBA Ag^{1+} efflux system constitutes a three-polypeptide complex that belongs to the resistance, nodulation, and cell division (RND) superfamily of cation efflux pumps that is driven by a membrane potential based on cation/proton exchange among the cytoplasm, periplasm and cellular exterior (Nies, 2003; Silver, 2003). The SilA component is a large (over 1,000 amino acids in length) cytoplasmic membrane cation pump protein; SilB is a periplasmic "membrane fusion protein" that contacts both the SilA cytoplasmic membrane protein and the SilC outer membrane protein. SilA has a membrane embedded domain and a domain in the periplasm that forms a cavity/pore/funnel pathway for Ag^{1+} to flow from the cytoplasm directly to the outer membrane protein, SilC. This three-protein system ensures Ag^{1+} or other substrate movement across the periplasmic space of Gram-negative bacteria directly to the outside of the cell (Nies, 2003) without their release into the periplasmic space (Silver *et al*., 2006).

Another efflux protein pump, SilP, is the product of the last gene of the silver resistance determinant in the pMG101 plasmid, and is predicted to be a P-type ATPase, a member of another large family of homologous heavy metal cation resistance efflux

ATPases (Silver and Phung, 2005). There are several salient features in the SilP sequence in regard to its Ag^{+1} pumping ability, starting from the N-terminus proximal poly-histidine, mono-aspartate (H5DH2) putative Ag^{1+}-binding domain in the cytoplasm that is considered equivalent to the Cu^{2+}-binding cysteine-X2-cysteine motif present in related ATPases (Silver *et al.*, 2006) of bacterial to human origin (Silver and Phung, 1996), including the closely related *E. coli* copper efflux CopA (Silver *et al.*, 2006). Interestingly, the SilP and CopA sequences are unrelated for the N-terminal cation recognition domains of about 275 amino acids and then closely similar for the remaining regions (Silver *et al.*, 2006). Thus, SilP, as a cytoplasmic membrane P-type ATPase, probably pumps Ag^{1+} from the cell cytoplasm to the periplasmic space (Gupta *et al.*, 1999). How periplasmic Ag^{1+} is removed is not understood, but probable explanations are Ag^{1+} ions exit through a non-specific outer membrane protein and/or by the Ag^{1+} sequestration by SilE, sequestration that may or may not be followed by transport across the outer membrane via the SilCBA complex (Silver *et al.*, 2006, Silver, 2003).

In Gram-positive bacteria, as a derivative of studying the copper homeostesis molecular system (*cop* operon) of *E. hirae*, probable silver efflux pump features have been recognized (Solioz and Odermatt, 1995; Odermatt *et al.*, 1994; Odermatt *et al.*, 1993). From the *E. hirae cop* operon, the CopB is a Cu^{1+}/ Ag^{1+} cell extruding P-type ATPase (Solioz and Odermatt, 1995; Odermatt *et al.*, 1994), but this ATPase does not seem to provide real silver resistance, for CopB deletion mutants were as sensitive to silver as were the wild-type counterparts (Odermatt *et al.*, 1993) and it has been shown that in wild-type cells, CopB Ag^{1+} extrusion cannot exceed CopA Ag^{1+} incorporation to

avoid cellular death (Wunderli-Ye and Solioz, 1999). It will be interesting to see if the experiments conducted by Odermatt *et al*. (1993) could be conducted with *E. hirae* or other Gram-positive bacteria believed to be silver resistant so that an evaluation of the role of CopB as an efflux pump is assessed in resistant rather than in wild-type strains.

IV.D.3. Silver protein chaperones

Encoded in the *Salmonella* plasmid pMG101, the SilE protein is a small periplasmic metal-binding protein that is homologous to the PcoE protein of the *E. coli* copper resistance determinant (Silver, 2003; Gupta *et al*., 1999). The gene's RNA synthesis is more abundant than any other silver determinant's expression when *E. coli* cells are grown under Ag^{1+} presence (Gupta, 1999). Although how SilE interacts with other periplasmic components is not known, its lack of functionality is recognized to decrease the overall Ag^{1+} tolerance in the bacterial cell (Gupta *et al*., 1999). SilE is thought to frame itself in large alpha-helical secondary structures interspersed with beta sheets and collectively, the secondary structures with their ten histidine imidazole N atoms coordinating the cation binding, fix five Ag^{1+} cations per peptide (Silver, 2003).

In the pMG101 plasmid, between the *silC* and *silB* genes, a small gene was initially not assigned a function as it lacked homologs (Gupta *et al*., 1999), but its protein product is now called SilF as it is about 50% identical in sequence to the chromosomal gene product CusF (Silver *et al*., 2006). The SilF periplasmic chaperone protein is thought to carry Ag^{1+} from the periplasmic site of release by SilP to the periplasmic

uptake site of SilA, part of the SilCBA complex. Since SilF is believed to be very similar to CusF, the CusF Ag^{1+} binding site description is offered in replacement of that of SilF. The CusF cation binding site is constituted by two beta strand regions with the cation bound by a single histidine (His36) N atom and two methionine (Met47 and Met49) S atoms (Loftin *et al*., 2005). With SilE and SilF periplasmic Ag^{1+}-binding chaperones being ascribed different functions, it is easily believed that the proteins may be somehow similar and/or related; however, SilE and SilF are basically unrelated (Silver *et al*., 2006).

In spite of what metal ions are incorporated by the copper and silver resistant determinant proteins, the chaperones SilE, PcoE, SilF, and CusF bind both monovalent cations Ag^{1+} and Cu^{1+}, but no divalent cations including Cu^{2+}. Careful experimental measurements of cation preference and the identification of sequences for binding are needed in the future (Silver *et al.*, 2006).

IV.D.4. Porins and silver impermeability

The Gram-negative *E. coli* K-12 and B mutants missing two putative outer-membrane porins (namely 1a and 1b) showed a higher resistance to silver ions than the parent strains carrying functional porins 1a and 1b. The absence of proteins 1a and 1b from the outer membrane is caused by a mutation in the *ompB* locus which results in cellular impermeability (Pugsley *et al*., 1978). Nevertheless, it was proposed that the absence of major porins alone cannot explain the silver resistance because various well-characterized OmpF- and/or OmpC-deficient mutants showed almost identical

susceptibilities to Ag^{1+} as their non-mutant counterparts. Thus, active efflux pumping may be the main mechanism by which resistant and even susceptible *E. coli* cells tolerate higher Ag^{1+} concentrations and adiminished outer membrane permeability aids the efflux mechanism synergistically to raise the level of resistance (Li *et al*., 1997). Single silver determinant gene knockouts must be used in silver resistance tests to elucidate the amount of Ag^{1+} resistance that each single gene contributes within the bacterial strains of interest.

IV.D.5. Silver influx pumps in Gram-positive bacteria

Similar to copper entrance into *E. hirae* (see section II.B.5), Ag^{1+} ions are also believed to access cells through the CopA Ag^{1+} influx protein because CopA-minus mutants could grow in a 5 μM $AgNO_3$ concentration that inhibited any growth from the Ag^{1+} efflux CopB-minus or wild-type *E. hirae* (Odermatt *et al*., 1994; Odermatt *et al*., 1993). Thus, a lack of function of the *E. hirae* CopA may provide certain imperviousness to Ag^{1+} that may allow *E. hirae* survival in higher Ag^{1+} concentrations.

IV.D.6. The membrane and teichoic acids protect against silver

A non-specific protection against silver is also provided by bacterial membranes and by the teichoic acids found in the cell wall of Gram-positive species (see section II.B.6).

IV.E. **Resistant strains included in the present study**

The bacterial strains *E. faecium* 75-30733-5, *E. coli* 77-30013-2 and *P. putida* 08991 were obtained from Dr. Christopher Rensing and the strains *S. enterica* S9, S19 and S20 were obtained from Dr. Sadhana Ravishankar, both at the University of Arizona in Tucson, AZ. All the strains above are reported to be copper resistant (Elguindi *et al*., 2011; Ravishankar *et al*., 2010; Cooksey *et al*., 1990). The copper sensitive strains employed in this research were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and included *E. faecium* 19579, *E. coli* 25922, *P. putida* 31483, and *S. enterica* 23564.

E. faecium is a Gram-positive, non-motile, non-spore forming coccus which occurs in pairs or chains of variable length (Gleeson and Gray, 1997; Schleifer and Kilpper-Bälz, 1984), yields a positive result when tested with Lancefeld group-D antisera (Gleeson and Gray, 1997), and contains a peptidoglycan type Lys-D-Asp (Schleifer and Kilpper-Bälz, 1984). It exhibits notable tolerance to unfavorable growth conditions,such as 6.5% sodium chloride, pH 9.6, and,temperatures of 10 and 50°C. This bacterium is more frequently found in the human intestinal tract than it is in that of animals ([Farrow *et al*., 1983; Kilpper-Bälz *et al*., 1982; Collins and Jones, 1979; Deibel and Seely, 1974; Schleifer and Kandler, 1972] in Schleifer and Kilpper-Bälz, 1984).

E. faecium is the causative agent of approximately 20% of nosocomial Enterococcal bloodstream infections and is much more frequently associated with vancomycin, teicoplanin, penicillin, and high-level gentamicin resistance. In fact, greater

than 85% of *E. faecium* hospital isolates are resistant to penicillin and >50% are resistant to high-level gentamicin (Jones, 2001).

E. coli is a motile, Gram-negative, lactose fermenting, non-spore-forming rod with peritrichous flagella that belongs to the family Enterobacteriaceae (Myrvik *et al*., 1974). This bacterium serves as the prototype of the group of bacterial coliforms and is used as an indicator of fecal contamination because of its abundance in feces, its durability in external environments, and its ease of detection. Despite the fact that *E. coli* is not highly pathogenic, it poses great medical importance because of the frequency and potential of the infections (Myrvik *et al*., 1974). Normally, non-pathogenic strains live in the human intestine without causing harm, but may instigate disease in other areas of the body, especially the urinary tract and the peritoneum (Myrvik *et al*., 1974). Nonetheless, several *E. coli* strains are frank pathogens, producing gastroenteritis, and some of these strains are identified as enterotoxigenic (ETEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enterohemorragic (EHEC) (Nataro *et al*., 1998; Neill *et al*., 1994 in Hui *et al*., 1994) and enteroinvasive (EIEC) (Neill *et al*., 1994 in Hui *et al*., 1994).. *E. coli* infections are usually foodborne (Neill et al., 1994 in Hui et al., 1994). Epidemiologically and economically, some *E. coli* are pathogenic microorganisms of utmost importance, for the high cost of illness due to EHEC infections suggests that additional efforts to control this pathogen might be enforced (Frenzen *et al*., 2005).

P. putida belongs to the proteobacteria subclass Gamma (Kersters *et al*., 1996) and is a member of rRNA group I of the genus *Pseudomonas* (Ramos-Díaz and Ramos, 1998; Kersters *et al*., 1996). Morphologically speaking, *P. putida*, as a Pseudomonad, is

similar to the Enterobacteriaceae members, but differs from them in that *P. putida* has polar flagella, is strongly oxidase-positive, and is a strict aerobe. Pseudomonads have a substantially greater G+C content than Enterobacteriaceae in their DNA and most metabolize sugars via the 2-keto-3-deoxygluconate (Entner-Dourdoroff) pathway rather than through glycolysis. *P. putida* produces a yellow fluorescent pteridine pigment (Davis *et al*., 1980).

P. putida, although is an uncommon pathogen, can become a serious infective agent to treat (Treviño *et al*., 2010; Lombardi *et al*., 2002), one that can survive in the hospital setting and occasionally cause difficult-to-treat nosocomial infections in severely ill patients (Treviño *et al*., 2010). Most patients contracting an infection from *P. putida* are neutropenic, cancer patients (Anaisse *et al*., 1987), or newborns (Perz *et al*., 2005). Since the mid-1990's, the gene bla_{IMP} and its coded enzyme, both responsible for resistance against broad spectrum antibiotics, have been identified in a multi-antibiotic resistant *P. putida* isolate. The spread of this gene is proposed among all the Gram-negative rods, at least in Japan (Senda *et al*., 1996).

The *P. putida* strain used in the current study was originally isolated from a tomato seed lot and was designated *P. putida* strain 08891 by Cooksey *et al*. (1990). Its copper resistance was mainly believed to come from a *cop* operon homolog held in the strain's plasmid and chromosome (Cooksey *et al*., 1990). Its copper MIC was established at 2.4 mM, yet the measurements were taken in an organic rich medium which may have led to an overestimation of the value. This strain is more resistant to copper than *P. putida* R5-3 (a moderately resistant strain with an MIC of 1.8 mM) (Cooksey *et al*., 1990)

and than a copper sensitive wild type strain (PNL-MK25 with an MIC of 0.4 mM) (Adaikkalam and Swarup, 2005). However, *P. putida* CZ1 isolated from a heavy metal contaminated soil exhibited a copper MIC of 5 mM (Chen *et al*., 2006). The systems that are likely to be involved in the detoxification of copper in *P. putida* are chromosomal *cueA* (Adaikkalam and Swarup, 2002), *copAB* and *cus*. Moreover, the *cop* system appears to be duplicated in *P. putida* but not in its close relative, *P. aeruginosa* (Cánovas *et al*., 2006). Such duplication may allow a Cu efflux pump to substitute another when one is not functional, or may function in redundancy which may be translated in the copper resistance equivalent to the sum of the individual copper effluxes' actions.

Salmonella are gram-negative bacteria comprising 2 species and 6 subspecies (Coburn *et al*., 2007); the most important of which is *Salmonella enterica* ssp. *Enterica (*Callaway *et al*., 2008), with hundreds of different serotypes, all of which are known for their pathogenicity (Myrvik *et al*., 1974). Salmonellosis can produce a range of symptoms from mild gastroenteritis to severe illness or death. In the United States, the FoodNet survey collected 2010 outbreak data and lab results from 10 states, and 4,200 hospitalizations and 68 deaths from nine food-borne diseases were identified. *Salmonella*, a group of roughly 2,500 strains of intestinal bacteria, was responsible for most of those cases, including 23 of the deaths (The FoodNet, 2010 in Vergano, 2011). It is recognized that *Salmonellosis* rates started to drop in the 1990s, but lately, they have actually increased by 10% in the past few years so that they now equal the 1996 rate of 17.6 cases for every 100 000 persons (Hagen, 2011 in Vergano, 2011). The disease is mainly

transmitted through food because the bacterium can grow on beef and poultry, which can be contaminated by feces (Callaway *et al*., 2008).

Salmonella spp are of great epidemiological relevance. The average annual number of culture confirmed salmonellosis cases reported to the Centers for Disease Control and Prevention (CDC) was 35,621. Based on the number of estimated cases in the U.S. by FoodNet, 97% go unreported. The CDC has calculated that 95% of *Salmonella* infections are foodborne. Economically speaking in the U.S., the estimated annual cost (in 1998 dollars) of medical care and lost productivity due to foodborne *Salmonella* infections was either $0.5 billion or $2.3 billion (Frenzen *et al*., 1999).

V. EVIDENCE FOR IMPROVED EFFICACY OF METAL COMBINATIONS WHEN USED AS ANTIBACTERIAL TREATMENTS.

The settings where metal combinations with antimicrobial purposes have been used at least in an experimental stage include water systems in hospitals, cooling towers for buildings, surfaces of constant contact in hospitals, medical devices, metal dental restorations, nanoparticles impregnated on paper with Zn whiskers, textiles and mortars. For a detailed review about the metal combinations used as antibacterial treatment, see Appendix A.

VI. ANTIMICROBIAL ZEOLITE TECHNOLOGY

AgION™ zeolite powder consists of a multi-faceted ceramic crystal carrier or ion exchanger that provides a three dimensional release mechanism for incorporated metal ions. With ambient moisture in contact with the zeolite coating, sodium ions are exchanged for the antimicrobial metal ions held in the zeolite crystals; thus, the liberated biocidal metal ions control the microbial populations on a coated surface. AgION™ comes in various formulations containing different percentages of incorporated metal ions (w/w) including the following: 1). 20% Ag, 2). 2.5% Ag, 3). 10% Cu, and 4). 3.5% Ag + 6.5% Cu (AgION™ antimicrobial, 2010).

AgION™ antimicrobials are registered with the United States Environmental Protection Agency for an ample range of uses including food and water contact surfaces, building products, appliances, cosmetics and personal products (AgION™ antimicrobial, 2010), and fibers and textiles (AgION™ antimicrobial, 2010; Victor group, 2010; Takai *et al*., 2002). Additionally, AgION™ antimicrobials are listed under the U.S. Food and Drug Administration food contact substance notification for use in all types of food contact polymers. They are listed by the U.S. Department of Agriculture for non-food compounds maintained by the U.S. National Science Foundation for food processing plants (AgION™ antimicrobial, 2010).

In a study by Bright *et al*. (2002), AgION™ powder formulations of 2.3% Ag alone, a 3.1% Ag/5.4% Cu mixture, and a 2.5% Ag/14% Zn mixture were separately added to experimental flasks and assessed against *Staphylococcus aureus* in solution. At

room temperature after four hours, all formulations reduced *S. aureus* numbers by approximately 1-$\log_{10}$. This was significant with regard to the control. After 24 hours, the formulations containing metal mixtures achieved greater population reductions than those achieved by the Ag formulation. Moreover, the Ag/Zn combination statistically (P=0.009) outperformed the Ag/Cu after 24 hours. Due to the apparent highest antimicrobial efficacy of the Ag/Zn formulation in solution, steel panels were then coated with the 2.5% Ag/14% Zn formulation and these were also tested against *S. aureus*. Such a test somewhat mimics what occurs on a daily use fomites (inanimate surface) when it is microbially contaminated. *S. aureus* populations were significantly reduced by > 2 $\log_{10}$ (>99%) and > 3 $\log_{10}$ (>99.9%) respectively after one and 24 hours of inoculation, and at the latter time, the Ag/Zn panels reduced the population numbers below the detection limit of 10 CFU (Bright *et al*., 2002).

According to the literature, tests of the zeolite's antimicrobial properties on surfaces have usually been conducted with this 2.5% Ag/14% Zn formulation which is the one that has been incorporated into numerous commercial products. In another study, three sets of experiments were conducted where *S. aureus*, *E. coli*, *P. aeruginosa* and *Listeria monocytogenes* were inoculated on stainless steel surfaces covered with the 2.5% Ag/14% Zn zeolite, and the surviving colonies were enumerated. In one experiment, *S. aureus* was reduced by > 3 $\log_{10}$ (>99.97%) after two hours and the reduction reached 5 $\log_{10}$ (99.999%) after 24 hours (Cowan *et al*., 2003). Apparently, *S. aureus* is very labile to the action of the Ag/Zn combination as suggested by these two different investigations (Cowan *et al*., 2003; Bright *et al*., 2002). *E. coli* was reduced by 5 $\log_{10}$ (99.999%) after

six hours following inoculation and was completely eliminated after 24 hours. *P. aeruginosa* numbers were diminished by > 1 $\log_{10}$ (95.885%) after four hours and completely eradicated after 24 hours. Finally, *L. monocytogenes* was reduced by > 1 $\log_{10}$ (98.726%) after four hours and by > 6 $\log_{10}$ (>99.9999%) after 24 hours (Cowan *et al*., 2003).

Furthermore, *Legionella pneumophila*, an important pathogen involved in outbreaks in hospitals, has also been tested for its capacity to survive on 2.5% Ag/14% Zn coatings on steel panels. Contrary to the studies where detection limits for bacterial counts or complete elimination are reached after 24 hours, most of the *L. pneumophila* populations reached at least a 99.9% reduction in a short lapse of two hours. After 24 hours, most *L. pneumophila* samples do not seem to be present in higher numbers than the detection limit. Thus, *L. pneumophila* seems to be more susceptible to the zeolite's release of both Ag and Zn ions than most of the other bacterial strains tested to date (Rusin *et al*., 2003).

In order to elucidate how effective AgION™ 2.5% Ag/14% Zn coated steel panels are in the disinfection of bacterial spores, Galeano *et al*. (2003) conducted survival tests of the spores in addition to the vegetative cells of *Bacillus anthracis* Sterne, *Bacillus cereus* T, and *Bacillus subtilis* 168 in a humidity chamber at 25°C, with an 80% relative humidity. Spores from all the strains mentioned above were seeded on either bare stainless steel or AgION-coated stainless steel panels for up to 24, 48, and 46 hours, respectively. No spore inactivation was apparent, and after graphing the data, there was no indication of any distinguishable trend lines. Interpretation of the data by analysis of

variance rendered no significant differences in the spore titers on bare steel versus AgION-coated steel. Conclusively, spores of the three *Bacillus* spp. appeared to be completely resistant to the antibacterial treatment. On the other hand, the vegetative cells of the three *Bacillus* spp. were not inactivated after 24 hours of contact with bare stainless steel; viable counts of *B. anthracis* Sterne and *B. subtilis* 168 were actually observed to increase slightly. In contrast, vegetative cells were inactivated by at least 3 orders of magnitude by 24 hours of contact with the Ag/Zn AgION-coated stainless steel. Interestingly, the kinetics of vegetative cell inactivation differed for each of the three species studied. Statistically significant inactivation by the AgION-coated steel relative to the control was first detected at two hours with *B. anthracis* Sterne ($P = 0.011$), at six hours with *B. subtilis* 168 ($P = 0.024$), but not until 24 hours with *B. cereus* T ($P < 0.001$). These data suggest that there may be species-specific differences in the sensitivity to the Ag and Zn ions within the AgION antimicrobial, suggesting the need for further testing of the coating for disinfection against known foodborne and clinical *Bacillus* isolates (Galeano *et al*., 2003).

Bright *et al*. (2009) assessed the antiviral characteristics produced by a zeolite containing both 6.5% copper and 3.5% silver. This formulation produced the greatest antiviral effects in comparison to formulations with silver alone or a combination of silver, zinc and zinc oxide. When tested in physiological saline with the zeolites, the feline infectious peritonitis virus was reduced by $> 3 \log_{10}$ and the human coronavirus 229E by $> 2 \log_{10}$ with the copper and silver formulation after four hours of treatment. After 24 hours, neither virus was detected. Additionally, De Muynck *et al*. (2010)

conducted research testing the antibacterial properties of a zeolite containing the 6.5% copper and 2.5% silver mix. The zeolite was mixed with mortar, so weight/weight percentages of the zeolite and mortar mixes rendered zeolite compositions of 1%, 2%, 3%, 4% and 4.65% on cement weight base. Surviving *S. aureus*, *E. coli*, *L. monocytogenes* and *Salmonella enterica* numbers were counted 24 hours at either 4°C and 20°C after the strains were separately inoculated on the mortars with a particular percentage composition of the zeolite. It was concluded that at 20°C, a 4.65% is the minimal zeolite percentile composition required to achieve a significant bacterial reduction for all strains. Nonetheless, a significant reduction in *S. enterica* populations could also be achieved by 3%, 4% and 4.65% zeolites. In general, the bacterial reductions were significant for all bacterial strains when these were tested at 4°C with either the 4% or 4.65% zeolite, but all the reductions were less effective than the equivalent ones at 20°C (De Muynck *et al*., 2010). The negative effect on disinfection caused by lower temperatures has also been described for copper surfaces when their antimicrobial activity was tested against *S. enterica* and *Campylobacter jejuni* (Faúndez *et al*., 2004).

A great drawback of all of the studies previously mentioned is that, with the exception of the study by Bright *et al*. (2002), the individual bactericidal capacity that each metal contributes to the zeolites containing mixtures of metals cannot be determined. There is a lack of zinc only and copper only zeolite tests to assess each isolated metal's antibacterial power. In order to attain a better understanding of how two-metal zeolites work, it is imperative to analyze the properties of zeolites containing individual metals in addition to those containing combinations of metals.

VI.A. **Antibacterial capabilities against copper resistant**.

The antibacterial efficacy of zeolites containing silver or copper ions or a combination of these metals has been assessed against several diverse copper resistant (Cu^R) and copper sensitive (Cu^S) strains of clinically relevant bacterial species. The species include *Pseudomonas putida*, *Escherichia coli*, *Salmonella enterica* and *Enterococcus faecium*. For futher details of this work, see Appendix B.

DISSERTATION FORMAT

The appendices of this dissertation report are 1) a literature review and compilation of experiments and uses of devices or systems using a combination of metals as means of disinfection, and 2) the findings of a conjunction of experiments undertaken by the candidate. Thus, 1) is entitled “EVIDENCE FOR IMPROVED EFFICACY OF METAL COMBINATIONS WHEN USED AS ANTIBACTERIAL TREATMENTS” and 2) is entitled “COPPER RESISTANT BACTERIA BETTER TOLERATE COMMERCIALLY AVAILABLE ANTIMICROBIAL TREATMENTS BASED IN SILVER AND SILVER-COPPER IONS.” This dissertation includes material that will be submitted to peer-reviewed scientific journals for publication (Appendices A and B).

The dissertation author was responsible for the literature review and compilation presented in the manuscrips appended as A, and was responsible for all of the research presented in the manuscripts appended as B in this dissertation. Doctors Charles P. Gerba and Kelly R. Bright supervised and were frequently consulted on the experimental design of the study in Appendix B.

PRESENT STUDY

This dissertation is composed of 2 appendices. A summary of each appendix is below and each describes the major findings along the literature review and the research work developed by the author of this dissertation.

The manuscript in Appendix A provides a highly detailed literature review of the antimicrobial properties of a combination of different metallic materials used in both experimental and daily life instances. Nonetheless, information on the settings where metal combinations with antimicrobial purposes have been used at least in an experimental stage is provided, and these settings include: water systems in hospitals, cooling towers for buildings, surfaces of constant contact in hospitals, medical devices, metal dental restorations, nanoparticles impregnated on paper with Zn whiskers, textiles and mortars. The advantages of the metal combinations in disinfection are discussed. The manuscript in Appendix B provides a description of the experimentation conducted by the author of this dissertation. The experimentation consisted of testing the disinfective capabilities of a commercially available metallic agent (AgION®) on copper resistant bacterial strains. The metallic agent formulations tested were copper alone, silver alone and copper and silver in combination. The copper resistant bacterial strains are those that can withstand copper concentrations higher than those withstood by another strain within the same species. The copper resistant strains used in the study were *Enterococcus faecium* 75-30733-5, *Escherichia coli* 77-30013-2, *Pseudomonas putida* 08991, and *Salmonella enterica* strains S9, S19 and S20. The disinfective agent was poured in a physiological saline solution contained in flasks where the bacteria were dispensed into.

The bacteria were challenged for 24 hours, and samples were taken at 0, 3, 6 and 24 hours, time points when the survivors were enumerated. *E. faecium* showed great tolerance against any treatment, especially to that of silver alone, for no treatment achieved a successful reduction not even after 24 hours. The copper resistant *E. faecium* showed a significantly higher tolerance to the treatment with copper alone when compared to the copper sensitive *E. faecium*. Surprisingly, the reportedly copper resistant *E. coli* and *P. putida* showed no higher resistance to any of the treatments than the putative copper sensitive control strains. Actually, the putative copper resistant strains proved slightly more vulnerable, at least to the metal combination treatment, than the copper sensitive strains. The former could be explained by the fact that the high metal resistance condition in the two "sensitive" control strains may have not been tested in the past and therefore was not reported. Finally, the *S. enterica* strains S9, S19 and S20 showed a higher resistance to all the treatments than the ones shown by the copper sensitive *S. enterica*, suggesting that in these strains, the copper resistance may confer resistance to silver and to the copper and silver combination. For achieving a population reduction of 3 $\log_{10} \leq$, most resistant bacterial strains had to be challenged for 24 hours at least.

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APPENDIX A:

EVIDENCE FOR IMPROVED EFFICACY OF METAL COMBINATIONS WHEN USED AS ANTIBACTERIAL TREATMENTS: A REVIEW.

To be submitted to the FEMS Microbiology Reviews

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1. ABSTRACT

Since 4000 years ago, the antibacterial properties of single metals have been used to keep water, comestibles and human daily use products free from microorganisms. The settings where metal combinations with antimicrobial purposes have been used at least in an experimental stage is provided, and these settings include: water systems in hospitals, cooling towers for buildings, surfaces of constant contact in hospitals, medical devices, metal dental restorations, nanoparticles impregnated on paper with Zn whiskers, textiles and mortars. In the U.S., the effectiveness of the silver-copper ionization systems in eradicating bacteria has been so high that over a hundred hospitals have implemented the systems in their facilities. Additionally, the ceramic agent (EEKO-BALL), that combines copper and silver, has shown promise in eradicating bacteria in cooling towers. Lately, there has been a search for materials that pose a permanent toxicity to pathogens that can spread on surfaces. Thus far, heavy metals and their combination have been the opportune candidates for coating the surfaces, so in 2008, copper alloys with application on surfaces were registered as antimicrobial materials by the EPA. For the catheters' coatings, depending on the bacterial species tested, the coating containing copper, either alone or in combination with silver reduces the bacterial populations better than the coating with silver alone. In a study, nanoparticles containing silver alone, Cu alone and Zn alone, and their combination were used to kill *P. putida*. The most effective bactericidal nanoparticle was that of silver alone and silver and copper used in combination. In the paper industry, silver nanoparticles have practical use when applied

on paper sheets to prevent their bacterial colonization. Another antimicrobial agent with application on metal surfaces, textiles and mortars are the zeolites. The zeolites are composed of metal combinations such as copper and silver, silver and zinc, and copper, silver and zinc. In general, the formulations with combinations of metals achieve better disinfective rates than the formulations with one metal in each of the materials where they are used.

2. INTRODUCTION

Heavy metals are members of a broadly-defined subset of elements that exhibit metallic properties and a wide variety of densities (the definition of heavy metal according to the field of interest may be found in Duffus, 2002). Heavy metals include the transition metals, some metalloids, lanthanides, and actinides (Singh *et al*., 2011). The antibacterial properties of metals in general have been recognized for centuries and have represented some of the most significant discoveries in medicinal history (Elsome *et al*., 1996).

This review summarizes the uses of heavy metals and combinations of heavy metals and their compounds as antimicrobials. In addition, a discussion of metals used in combinations with other antibacterials is included.

3. INDIVIDUAL METALS

3.1. Silver (Ag)

3.1.1. Silver toxicity

In humans, silver is relatively non-toxic via both inhalation and ingestion. Exposure to high doses (gram quantities) is required over long periods to result in an irreversible discoloration of the skin known as argyria or argyrosis (Hill and Pillsbury, 1939; Nordberg and Gerhardsson, 1988; Gulbranson *et al*., 2000; Lansdown, 2006). Additionally, when silver nitrate is applied in excessive doses, problems with tissue regeneration and oxidation of the skin have been found in burn patients (Atiyeh *et al*., 2007).

3.1.2. Silver uses and antimicrobial action

The antimicrobial properties of silver (Ag) have been reviewed extensively (Silver 2003; Silvestry-Rodríguez *et al*., 2007). It is believed that the effectiveness of Ag^{1+} as an antimicrobial resides in its binding to thiol (–SH) groups on proteins, causing conformational changes and resulting in denaturation of enzymes (Lansdown, 2006; Ghandour *et al*., 1988), its modifying the phospholipid membrane causing cellular proton leakage (Dibrov *et al*., 2002) and its distorting and complexing the DNA and RNA ladder

structures (Ghandour *et al*., 1988). Silver has been used as an antimicrobial for thousands of years. Since ancient times, silver vessels have been used to store foods, vinegar, water, and wine (Davis and Etris 1997; Shashikala *et al*., 2007; Silverstry-Rodríguez *et al*., 2007). Recently, silver has seen increased use as an antimicrobial in numerous applications including the prophylactic use of silver nitrate eye drops for newborns (Hoyme, 1993), the eradication of microorganisms in potable water (Silver, 2003; Lantagne, 2001; Clarke and Berman in Block, 1983), use as a preservative in beauty products (Ishitani, 1995; Quintavalla and Vicini, 2002), use in sportswear to prevent odors (Takai *et al*., 2002), use in burn wound dressings and bandages (Ip *et al*., 2006), and use in medical devices such as in vascular grafts, sutures (Darouiche, 1999), heart valve sewing rings (Darouiche, 1999; Auer *et al*. 2001), and catheters (Silver, 2003).

3.2. Copper (Cu)

3.2.1. Copper toxicity

Despite the extensive use of copper by humans for thousands of years, the number of reported cases of copper poisoning in the general population is low (Bremner, 1998). The sporadic reports of acute copper toxicosis usually include varying temporal symptoms (Chuttani *et al*., 1965). Low chronic copper toxicosis can be more detrimental than acute intoxication by eventually affecting the liver and leading to death (Bremner, 1998).

3.2.2. Copper uses and antimicrobial action

The antimicrobial uses of copper (Cu) have also been reviewed extensively (Borkow and Gabbay, 2009). Its antimicrobial properties have been recognized since the beginning of human civilization. In 2000 B.C. in ancient Egypt, copper was used to treat water and wounds, and the ancient Phoenicians affixed copper strips to the hulls of ships to prevent biofouling and thus increase speed and maneuverability (Borkow and Gabbay, 2009). Copper has been revisited in recent years as an antimicrobial for use in hospitals, appliances, medical devices, pipelines, and sound instruments such as bells and trumpets. Today, copper is used in antimicrobial birth control devices (Bilian, 2002), for the reduction of caries and prevention of plaque formation (Thneibat *et al*., 2008), to prevent foodborne disease via self-sterilizing food contact surfaces (Borkow and Gabbay, 2004; Faúndez *et al*., 2004; Noyce *et al*., 2006; Borkow and Gabbay, 2009), and to prevent the spread of diseases in public facilities via self-sterilizing surfaces (Noyce *et al*., 2006; U.S. Environmental Protection Agency, 2008; Weaver *et al*., 2008; Casey *et al*., 2010).

Copper's antimicrobial action consists of inhibiting the respiratory chain on the bacterial membrane by not allowing the transfer of electrons through the metal. This generates free oxidative radicals (Rodríguez-Montelongo *et al*., 1993) that ultimately cause lipid peroxidation of cellular membranes, direct oxidation of proteins, and the rupture of DNA and RNA (Peña *et al*., 1999).

3.3. Titanium (Ti)

3.3.1. Titanium toxicity

Titanium's toxicity is extremely low. An average human body contains approximately 700 mg of titanium. It is believed that an average person ingests 0.8 milligrams of titanium per day, most of which is not absorbed. The human body can therefore tolerate large amounts of titanium; therefore, it has been used extensively for pinning human bones together (Emsley, 2001).

3.3.2. Titanium uses and antimicrobial action

Titanium (Ti) has been used as an antimicrobial in various forms such as titanium nanoparticles (Martinez-Gutierrez *et al*., 2010; Yeung *et al*., 2009), titania-silica aerogels (Yeung *et al*., 2009), and TiO_2 films (Yeung *et al*., 2009; Foster *et al*., 2010) mixed with water-based interior paints (Hochmannova and Vytrasova, 2010). Studies have shown that TiO_2 nanoparticles alone or in combination with silver are effective against clinically relevant pathogens (Martinez-Gutierrez *et al*., 2010). The antimicrobial properties of the photo-induced TiO_2 are the result of its super hydrophilicity and the generation of reactive oxygen species (Carp *et al*., 2004). It has been suggested that a great advantage of the photo-activated Ti films is that they may not generate resistance in the

microorganisms they are used against because of the oxidative mode of action (Ireland *et al*., 1993; Wilson, 2003).

3.4. Zinc (Zn)

3.4.1. Zinc toxicity

Zinc (Zn) exhibits both acute and chronic toxicity. The acute adverse effects of high zinc intake include abdominal cramps, diarrhea, headaches, appetite loss, nausea and/or vomiting (Plum *et al*., 2010; Office of Dietary Supplements-National Institutes of Health, 2011); however, acute intoxication is rather rare (Plum *et al*., 2010). In one case of acute intoxication, severe nausea and vomiting developed within 30 minutes after the ingestion 4.0 g of zinc gluconate (570 mg elemental zinc) (Lewis and Kokan, 1998). In cases of chronic overexposure (daily doses ranging from 150 to 450 mg of zinc), chronic effects such as deficiency in immune function, deficiency in copper by its replacement with Zn, and low levels of high-density lipoproteins have been observed (Hooper *et al*., 1980).

3.4.2. Zinc uses and antimicrobial action

Zinc has shown antimicrobial potential as nanoparticles incorporated into fabrics (Rajendran *et al*., 2010). Zinc oxide (ZnO) in thin antimicrobial films has been applied on medical devices (Gittard *et al*., 2009) and in combination with zinc borate, has been

patented as an antimicrobial coating for daily use ceramics (Olsson and Swofford, 2007). Moreover, ZnO has shown superior bactericidal capabilities in comparison to those of TiO_2 when a ZnO nano-film embedded in an interior paint was photo-induced (Hochmannova and Vytrasova, 2010). The antimicrobial properties of ZnO have been demonstrated against *Staphylococcus aureus* and have had extensive application in items such as root canal filling cones in dentistry (Moorer and Genet, 1982),

ZnO nanoparticles may kill Gram-negative bacteria via the disruption of the cell membrane and through severe oxidative stress (Xie *et al*., 2011). The antimicrobial capabilities of ZnO nanoparticles in bacterial media have been extensively researched and have exhibited a potential bactericidal effect depending on the bacterial strain (Gajjar *et al*., 2009; Xie *et al*., 2011)**.** ZnO nanoparticles have shown both bactericidal (Xie *et al*., 2011) and bacteriostatic effects (Gajjar *et al*., 2009). ZnO nanoparticle concentrations ranging from 0.1 mg/ml to 0.5 mg/ml reduced *Campylobacter jejuni* titers by >8-$\log_{10}$ within one to four hours (Xie *et al*., 2011). The high sensitivity of *C. jejuni* to ZnO nanoparticles contrasted with the higher tolerance of both *Escherichia coli* O157:H7 and *Salmonella enterica*, which were reduced by approximately 1-$\log_{10}$ within eight hours of exposure with concentrations of 5 mg/ml or 10 mg/ml (Xie *et al*., 2011). In another study, Soil dwelling *Pseudomonas putida* colonies were treated with ZnO nanoparticles and these only delayed but did not eliminate its growth when compared to the control. Additionally, in the same study, ionic Zn was 10-fold more toxic than ZnO nanoparticles (Gajjar *et al*., 2009). Nonetheless, the ZnO nanoparticles, similar to Zn and Ag zeolites (Takai *et al*., 2002), have shown the potential to confer antibacterial properties to textiles

which could be used in health care settings (Rajendran *et al*., 2010). When applied to textiles, the ZnO nanoparticles exerted a greater antibacterial effect than ionic Zn (Rajendran *et al*., 2010).

3.5. Gold (Au)

3.5.1. Gold toxicity

Pure gold (Au) and gold salts are generally low in toxicity due to their weak absorption by the body. Potential side effects are diarrhea and skin rash. In 1985, the Food and Drug Administration approved the usage of Auronofin, a gold salt, as a therapeutic agent with a dose of 6 milligrams per day. This amount does not cause any adverse effects (Emsley, 2001).

3.5.2. Gold uses and antimicrobial action

The antimicrobial properties of gold compounds were extensively investigated in the 1990's (Fricker, 1996). In general, the antibacterial action of gold compounds against Gram-negative bacteria is limited in comparison to that against Gram-positive species (Elsome *et al*., 1996). *In vitro* tests involving a gold compound were conducted with methicillin resistant *Staphylococcus aureus* (MRSA), *Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa,* and the fungus *Candida albicans* (Elsome *et al*.,

1996). After 18 hours at a low dose of ≤ 4 mg/L, the compound containing gold reduced MRSA, *E. faecalis*, and *P. mirabilis* populations by 3-$\log_{10}$ (99.9%), but a dose of 64 mg/L was needed to achieve the same reductions in *P. aeruginosa* and *C. albicans*. In an *in vivo* assay, the gold compound at a dose of ≤ 4 mg/L reduced methicillin sensitive *S. aureus* (MSSA), *E. faecalis*, and *C. albicans* by 3-$\log_{10}$ (99.9%), but a dose of 32 mg/L was required to achieve the same reduction with *P. aeruginosa* and *P. mirabilis.* It was concluded that the gold compound was effective in treating bacteria on skin. A study encompassing more diverse microorganisms evaluated the antimicrobial capabilities of gold compounds against Gram–positive and Gram–negative bacteria, against the fungi *C. albicans* and *Aspergillus niger*, and against the protozoan *Trichomonas vaginalis.* The minimal inhibitory concentrations ranged from 0.061 μg/ml for *Staphylococcus epidermidis*, 125 μg/ml for *T. vaginalis*, to > 250 μg/ml with 5 gold compounds for *P. aeruginosa*, and > 250 μg/ml with at least 2 compounds for *E. coli*, *C. albicans* and *A. niger*. It was suggested that the antimicrobial activity of these compounds was not related to only the gold content, but also to the nature of both the phosphine and the aminothiol to which the metal is linked (Novelli *et al*., 1999).

More recently, experiments have been conducted with gold nanoparticles in combination with antibiotics and gold containing chitosan nanoparticles capable of delivering ampicillin. In this study, Chamundeeswari *et al*. (2010) determined the minimal inhibitory concentrations of ampicillin delivered by the gold nanoparticles. The optimum level of inhibition for *E. coli* was 27.4 μg/ml, but was 20.6 μg/ml for both *S. aureus* and *Klebsiella mobilis*. These values were two-fold less than the minimal

inhibitory concentration required with free ampicillin, suggesting that the gold nanoparticle delivery system could facilitate the use of lower antibiotic concentrations, thereby diminishing the side effects of the antibiotics (Chamundeeswari *et al*., 2010). Other antibiotic-delivery gold nanoparticles have demonstrated minimal inhibitory concentrations of 100 and 10 μg/ml for *E. coli* and *S. aureus* respectively, suggesting that a disparity in minimal inhibitory concentrations between Gram–negative and Gram–positive bacteria. (Rai *et al*., 2010). However, while several studies on the effects of gold nanoparticles in combination with vancomycin have demonstrated enhanced antimicrobial effects (Huang *et al*., 2007; Gu *et al*., 2003), gold nanoparticles combined with gentamicin have not (Burygin *et al*., 2009). Similar to silver ions, the antibacterial action of gold is likely caused by its binding to sulhydryl groups (Watkins *et al*., 1987). This may explain the higher susceptibility of Gram-positive bacteria that have proteins in their cell walls that are more available for binding (Elsome *et al*., 1996).

In other studies evaluating the combined antibacterial effects of gold nanoparticles and X-rays, the combination did not exert an enhanced antibacterial effect compared to that of X-rays alone (Simon-Deckers *et al*., 2008).

3.6. Mercury (Hg)

3.6.1. Mercury toxicity

There are two important routes of mercury exposure: food and inhalation. To calculate the amount of mercury in the human body, mercury concentration in urine is considered to be the analysis of choice. Normal levels of mercury found in the urine are less than 20 μg/L (100 nmol/L). However, urinary mercury concentrations do not correlate with ambient mercury levels due to the long half-life of mercury in the body (approximately 40–70 days). On the other hand, serum or whole blood mercury concentration is a sensitive indicator of acute mercury loading, especially with inorganic mercury, with a normal serum mercury concentration being 1 μg/dl (< 0.05 μmol/L). In cases of acute mercury toxicosis, initial serum mercury concentrations in two patients with acute occupational mercury exposure were 550 and 490 μg/dl (27 and 24 μmol/l) (Klein and Snodgrass, 2003). Workers with chronic mercury exposure that were occupationally exposed for 20–35 years with peak urinary mercury levels of 0.6 mg/L (3000 nmol/l) were found to suffer from neurological symptoms such as an abnormal Babinski reflex (up-pointing and flared toes), decreased sensation, and tremors and weakness.

3.6.2. Mercury uses and antimicrobial action

Probably the first set of antibacterial experiments using a metal compound was Koch's investigation of mercuric chloride with anthrax spores (Elsome *et al*., 1996). With the suspected antimicrobial activities of mercuric compounds, such studies have been conducted since the mid-twentieth century (Brewer, 1939; Grossowicz *et al*., 1947; Brewer, 1948; Engley, 1950). In the second half of the twentieth century, research demonstrated the higher susceptibility of *P. aeruginosa* to the mercuric compound than *S. aureus*. Mercury dichloride was found to be the best disinfectant tested (Elkhouly and Yousef, 1974). Additional research from the 1970's on mercuric compounds demonstrated their potential for use against renal infections caused by *P. mirabilis* (Kunin, 1976). The mercuric compound chlormerodrin, in concentrations of 15 to 35 and 20 to 45 μg/ml, were microstatic and microcidal, respectively, when tested against several bacterial strains and a fungus (Pandey *et al*., 1979).

In more recent years, research has focused on thimerosal (merthiolate), an ethylmercury-containing compound whose composition is 49.55% mercury (Geier *et al*., 2007). The *in vitro* antimicrobial activity of thimerosal compared to that of amphotericin B and natamycin was studied testing 244 ocular fungal isolates including *Fusarium* spp., *Aspergillus* spp., and *Alternaria alternate*. The thimerosal minimal inhibitory concentrations ranged from 0.0078 to 0.0625 μg/ml. These concentrations were 256 times, 512 times, and 128 times lower, respectively for these species, than those for

natamycin, and 64 times, 32 times, and 32 times lower, respectively, than those for amphotericin B (Xu *et al*., 2010).

During the 1980's, mercuric compounds were tested against several strains of penicillase-producing and non-penicillase-producing *Neisseria gonorrhoeae* to evaluate their ability to eliminate antibiotic resistant bacteria. The mercuric compounds, including phenilmercuric borate, mercuric chloride, and thimerosal, had greater bactericidal capabilities than those of metallic compounds based on silver, copper, or selenium. The minimal inhibitory concentrations for eliminating 90% of the strains tested with thimerosal and phenylmercuric borate were comparable (5 mg/L and 2.5 mg/L, respectively). The 90% minimal inhibitory concentration of the inorganic mercury compound (20 mg/L) was higher than those of the organic compounds, probably because the free Hg^{2+} ions found with this compound were bound to compounds of the very rich GC agar base medium employed in the study. Importantly, for all the antimicrobial metallic compounds used, no difference in susceptibility between penicillase producing and non-penicillase producing strains was detected, supporting the suggestion that metallic compounds could be useful for treating antibiotic resistant strains (e.g., silver nitrate drops for the eyes of newborns to prevent *N. gonorrhoeae* infections) (Peeters *et al*., 1986).

However, with the increased interest in studying the relationship between mercury and antibiotic resistance, more recent studies have shifted towards identifying mercury-resistant bacteria. The minimum inhibitory concentration of mercuric chloride ($HgCl_2$) for 52 streptococcal strains and the reproducibility of the minimal inhibitory

concentration values for mercury-sensitive and mercury-resistant strains were determined using 11 different media (Pike *et al*., 2002). It was found that the addition of blood increased the minimal inhibitory concentration values. Also, tryptone soy agar (with or without blood) could not discriminate between mercury-sensitive and mercury-resistant strains, suggesting the importance of the medium used for such tests. Mueller-Hinton (without blood) appears to be the most suitable medium for the isolation of mercury resistant oral streptococci (Pike *et al*., 2002).

It is believed that the interaction of mercury with sulfhydryl groups in proteins results in its antimicrobial ability, as the microstatic effects of chlormerodrin were reversible in the presence of cysteine, an amino acid containing a sulfhydryl group (Pandey *et al*., 1979).

4. METAL COMBINATIONS

4.1. Silver and copper

4.1.1. Antimicrobial usage in hospitals and cooling towers

Silver and copper ions have been demonstrated to have a synergistic antimicrobial effect when used in combination (Lin *et al*., 1996). In other words, the combined antimicrobial effect is greater than the sum of the individual antimicrobial effects of the two metals. Since the mid 1990's, this metal combination has been used commonly in water

distribution systems in hospitals (Liu *et al*., 1994). In hospitals in the U.S. (Stout and Yu, 2003) and Germany (Rohr *et al*., 1999), hot water is passed through a flow cell where the addition of silver and copper ions is achieved by applying an electric current between two electrodes composed of an alloy made of 90 parts copper and 10 parts silver. When the ionization system was tested in the laboratory, the combined metal concentrations found to be effective against *Legionella pneumophila in vitro* were 0.02 to 0.04 mg/L silver with 0.2 to 0.4 mg/L copper (Liu *et al*., 1994); nevertheless, higher concentrations appear to be necessary in larger water systems in buildings, depending on the water quality (Liu *et al*., 1994; Liu *et al*., 1998).

EEKO-BALL is a type of silver-copper oxide which covers specific ceramics and coating materials structured as a ball. The silver-copper oxides are released into water when submerged within water in cooling towers. When tested in the laboratory, the silver-copper combination of EEKO-BALL eliminated *E. coli* more effectively than did ionic silver or ionic copper alone (Kim *et al*., 2004). Moreover, when EEKO-BALL was tested in three cooling towers, the initial populations of heterotrophic plate count (HPC) bacteria ranging from 10^5 to 10^6 colony forming units (CFU)/100 ml dropped to $<10^1$ CFU/100 ml after a week following treatment, and no bacteria were detectable after 7 and 9 weeks (Kim *et al*., 2004).

Zeolites are ceramic powders (sodium aluminosilicates) with a three-dimensional crystalline structure in which metal ions can reside. Such zeolites can be used to coat hard surfaces or can be incorporated into various materials to create antimicrobial products. The zeolite acts as an ion exchanger, exchanging the incorporated metal ions for other

cations in the immediate environment. In the initial stages of testing of antimicrobial zeolites containing metal ions, tests were conducted in solutions to assess their effectiveness as a metal delivery system. Bright *et al*. (2002) evaluated formulations of 2.3% Ag alone, a 3.1% Ag/5.4% Cu mixture, and a 2.5% Ag/14% Zn mixture to compare their bactericidal efficacy against *S. aureus.* The two metal mixtures reduced the bacterial populations significantly more ($P < 0.05$) than the treatment with silver alone. These results set the precedent for several experimental trials that followed which focused on the zeolite with Ag/Zn ions (Cowan *et al*., 2003; Galeano *et al*., 2003; Rusin *et al*., 2003). Zeolites containing a mixture of copper and silver have been incorporated in mortars or cements applied to construction surfaces. To test the antibacterial properties of the mortars, the survival of *S. aureus, E. coli, Listeria monocytogenes,* and *Salmonella enterica* were measured following their inoculation on the antimicrobial mortars under a wide range of temperatures. In general, when 4.65% of the total composition of the mortar contained a 6.5% Cu / 3.5% Ag zeolite, the population of each bacterial species was reduced significantly ($P < 0.05$) after 24 hours of exposure. *S. enterica* seemed to be the most sensitive species and was reduced significantly ($P < 0.05$) on mortars containing lower zeolite ratios of 3% and 4% (De Muynck *et al*., 2010).

Antimicrobial metal coatings have been used on medical devices such as catheters that are in direct contact with tissues and are subject to contamination and therefore have the potential to cause infections. To evaluate the antibacterial effects of Ag and Cu coatings on catheters, tests were conducted to determine the survival of *S. aureus, S. epidermidis,* and *P. aeruginosa* when coatings contain Ag alone, Cu alone, or Ag/Cu

were applied on butyl rubber disks frequently used as catheter materials. Generally, the Ag/Cu combination had the greatest antibacterial action. The enhanced antimicrobial effect of the Ag/Cu film could possibly be due to a moderate electrical field and galvanic effect produced by the silver-copper electrochemical pair (Blenkinsopp *et al*., 1992; McLean *et al*., 1993).

A study was conducted to determine the effects of nanoparticles containing either AgO or CuO or their combination against *P. putida*. The antimicrobial effect of the AgO nanoparticles was approximately 50 times greater than that of the CuO nanoparticles after one hour of exposure. Additionally, the treatment with ionic copper had a higher antibacterial activity than the CuO nanoparticle form. When combining the nanoparticle types, the AgO and CuO combination showed an enhanced antibacterial effect than the individual nanoparticles and also had a greater bactericidal effect than the AgO/ZnO combination (Gajjar *et al*., 2009).

4.2. Silver and zinc

Following the studies of Bright *et al*. (2002) in which a Ag/Zn zeolite was found to be more effective than that of a Ag zeolite or a Ag/Cu zeolite, a series of experiments with stainless steel panels coated with Ag/Zn zeolites were conducted. Reductions greater than 3-$\log_{10}$ or 99.9% of the total populations were achieved in two hours for *L. pneumophila* and *S. aureus*, six hours for *E. coli*, 24 hours for *Listeria monocytogenes* and *P. aeruginosa*, and 25 hours for *Bacillus anthracis*, *Bacillus cereus* and *Bacillus subtilis*

vegetative cells (Cowan *et al*., 2003; Galeano *et al*., 2003; Rusin *et al*., 2003); however, none of the endospores of the three *Bacillus* spp. were reduced significantly with the Ag/Zn coated panels after 24 hours of exposure (Galeano *et al*., 2003).

Ag/Zn zeolites have been applied on textiles of medical use to prevent microbial colonization. Medical use fabrics containing Ag/Zn/ammonium and Ag/Zn/Cu zeolites have been studied in which it was demonstrated that combining more than two metals for an antimicrobial application may not be the best method for eliminating bacteria. When the survival of *S. aureus* strain 209P was measured on the fabric after 24 hours post-inoculation, the Ag/Zn/ammonium fabric achieved reductions > 3-$\log_{10}$ (>99.9%) while the Ag/Zn/Cu zeolite did not. With MRSA and MSSA *S. aureus* strains and *P. aeruginosa*, the time to achieve reductions > 3-$\log_{10}$ (>99.9%) was around 24 hours, but this $\log_{10}$ reduction fluctuated greatly depending on the ambient humidity (Takai *et al*., 2002).

Other types of antibacterial coatings based on nanoparticles have been applied in the paper industry. Silver nanoparticles have been adhered to ZnO “whiskers” or prickly structures that can be incorporated onto surfaces of paper sheets to prevent microbial colonization. When compared to paper sheets impregnated with elemental silver, silver nitrate, or ZnO, the paper sheets with the Ag nanoparticle-ZnO whiskers achieved the best bacterial reductions of *E. coli*, and had the longest lasting antibacterial effect with consecutive uses (Koga *et al*., 2009). The authors attributed this antibacterial effect solely to the silver nanoparticles and not to their combination with the ZnO whiskers; however,

the Ag nanoparticles in combination with the ZnO whiskers could activate the ZnO whiskers' antibacterial effects in a potentiation effect.

ZnO nanoparticles appear to exert a bacteriostatic action rather than a bactericidal one, and their toxicity may be considerably lower than that of ionic zinc. In a study by Gajjar *et al*. (2009), ZnO nanoparticle combinations with either AgO or CuO were not more effective in reducing *P. putida* populations than AgO or CuO nanoparticles tested alone, suggesting that the ZnO nanoparticles did not contribute to the antibacterial action. A combination of AgO and CuO nanoparticles had better antibacterial effects than the AgO/ZnO combination.

4.3. Silver and titanium

A number of studies have shown increased activity in antimicrobial action of Ag combined with TiO_2 to form antimicrobial layers combining the antibacterial properties of Ag with the photocatalytic advantages of TiO_2 (Sökmen et al., 2001; Keleher *et al*., 2002; Machida et al., 2005; Kim *et al*., 2006). These Ag-TiO_2 bilayers have demonstrated a broad spectrum of action by killing the Gram-negative *E. coli* and the Gram-positive *S. epidermidis* by > 6-$\log_{10}$ (99.9999% reduction) within 60 minutes and by inactivating the bacteriophage T4 by approximately 9-$\log_{10}$ (99.9999999%) in 80 minutes (Sheel *et al*., 2008). Nonetheless, monolayers composed of either Ag or TiO_2 sometimes display higher antibacterial activity than a bilayer composed of the two (Foster *et al*., 2010). From experiments with *E. coli* held in the dark, it was suggested that

a monolayer of silver alone can be more effective than monolayers composed of Cu/CuO, TiO_2, or layers with various metal combinations (e.g., Ag with TiO_2 or Cu with TiO_2). Under illuminated conditions, the best antibacterial activity was found with TiO_2 followed closely by the activity of Ag, Cu/CuO, TiO_2, and Cu-TiO_2. Under both dark and illuminated conditions, the Ag monolayer serves efficiently as a bactericide. Nonetheless, the clinically relevant MRSA and *P. aeruginosa* are much more resistant than *E. coli* to the action of the Ag-TiO_2 layer under illuminated conditions. The higher resistance in MRSA may be attributed to its Gram-positive peptidoglycan cell wall (Foster *et al*., 2010). Contrary to previous findings where a mixture of Cu and TiO_2 displayed antimicrobial synergy (Ditta *et al*., 2008), the Ag/TiO_2 nanoparticles did not achieve a greater antimicrobial effect than that of the 20-25 nm Ag nanoparticles (Martinez-Gutierrez *et al*., 2010).

4.4. Copper and zinc

The bactericidal effects of the combination of CuO and ZnO nanoparticles were evaluated against a *P. putida* soil strain. This combination did not exhibit a synergistic effect. The formulation of AgO alone or AgO/CuO in combination was more effective than CuO/ZnO in combination (Gajjar *et al*., 2009).

4.5. Copper and titanium

A number of studies have demonstrated increased activity of TiO_2 when it is combined with Cu (Sunada *et al*., 2003; Tian *et al*., 2007; Yates *et al*., 2008), including activity against viruses in which the combination of photocatalysis and copper toxicity acted synergistically (Ditta *et al*., 2008). These TiO_2/CuO coatings are antiviral and may have applications in the food and healthcare industries (Yates *et al*., 2008; Foster *et al*., 2010). Recently, antimicrobial coatings containing a mixture of Cu and TiO_2 have been tested against *E. coli*. Despite the fact that a layer composed of mixed Cu or Ag and TiO_2 has an enhanced bactericidal effect under light conditions, this effect does not appear to be synergistic. Additionally, when treating *E. coli* in the dark, the bilayer composed of Cu and TiO_2 exhibited the best bactericidal effects of the coatings tested; nevertheless, its effect was limited to around a 2-$\log_{10}$ reduction (99%) in 30 hours (Foster *et al*., 2010).

4.6. Copper alloys

Environmental surfaces play an important role as reservoirs for microorganisms and as dispersal points for human pathogens (Kramer *et al*., 2006). In order to prevent the colonization and persistence of pathogens on surfaces, there has been a search for materials that pose a permanent toxicity to pathogens that can be applied on surfaces to prevent microbial spread. In 2008, copper alloys were first registered as official antimicrobial materials for use on fomites (inanimate surfaces) (U.S. Environmental

Protection Agency, 2008). The official registration of these copper alloys took place after the bactericidal properties of copper alloys against bacterial pathogens were tested by measuring the respective survival of *Enterobacter aerogenes*, *E. coli* O157:H7, MRSA, *P. aeruginosa*, and *S. aureus* on copper alloy and non-copper alloy surfaces. Various metal combinations included pure copper (99-90% Cu), bronze (95% Cu, 5% Sn, 0.2% Cr), copper-nickel (90% Cu, 10% Ni), brass (70% Cu, 30% Zn), copper-nickel-zinc (65% Cu, 17% Zn, 18% Ni), and stainless steel (Michels and Anderson, 2008).

Fixtures of EPA registered copper materials were recently assessed in a hospital. Brass tap handles (60% Cu, 40% Zn), a brass door push plate (70% Cu, 30% Zn), and a toilet seat coated with a mix of pure copper/resin composite (70% Cu) were sampled and the numbers of surviving bacteria were compared with those of equivalent fixtures covered with chrome-plated-, aluminum, or plastic-surfaces (Casey *et al*., 2010). Over the 10 week study period, all of the copper fixtures, with the exception of a brass tap handle on one occasion, had significantly lower ($P \leq 0.05$) bacterial levels than their non-copper counterparts (Casey *et al*., 2010).

4.7. Zinc and calcium

The food industry is interested in finding materials for creating antimicrobial films that, when applied to edible items, can prevent and/or delay their spoilage during their processing and transportation. Calcium alginate films, when loaded with ZnO

nanoparticles, displayed an antibacterial activity against *E. coli* using disk diffusion tests (Bajpai *et al*., 2011).

4.8. Zinc and titanium

Nanosize ZnO, when placed in a crystalline TiO_2 matrix, can be applied to surfaces for the prevention of microbial colonization. The antimicrobial action of the product is described as an electrostatic trap for microbes at a molecular level, whose destruction is achieved by photocatalytic oxidation. The coating uses the energy of light as a catalyst to create electrical activity at the molecular level. The positive charge of the coating attracts, traps, and then oxidizes the negatively charged virus, bacteria, mold, or spore (OxiTitan, 2011). It is worth noting that in the antimicrobial challenge tests, the coating did not achieve a 2-$\log_{10}$ reduction of vancomycin resistant enterococci, *Clostridium difficile*, MRSA, or MS-2 coliphage even after 24 hours.

4.9. Gold and silver

The antibacterial capabilities of nanocomposite coatings composed of Ag only or the combination of Ag and Au were evaluated against *Staphylococcus* spp. using the disk diffusion method. The greatest antibacterial effect was observed with the Ag/Au polymer nanocomposite, followed by the Ag polymer nanocomposite. The minor addition of Au nanoparticles to the Ag film enhances the release of Ag ions into the medium via a

galvanic effect (Zaporojtchenko *et al*., 2006) similar to that described with Ag and Cu combinations (Blenkinsopp *et al*., 1992; McLean *et al*., 1993).

4.10. Gold and titanium

Matrices of TiO_2 have been coated with Au nanoparticles by various thermal variable methodologies. Pure TiO_2 films had the best efficacy against *Bacillus subtilis* when exposed to light. The films composed of TiO_2 and Au nanoparticles also possessed notable antibacterial activity, but this was not greater than that displayed by films composed of TiO_2 alone. In this study, the application of gold had a negative effect on the TiO_2 antibacterial properties, which may be attributed to the following: 1). the gold nanoparticles are inert towards *B. subtilis* and the relatively large bacterial sizes in the nano-scale impede bacterial contact with the TiO_2, or 2). the gold nanoparticles cause an alteration of the TiO_2 surface charge, resulting in a less efficient interaction between the bacteria and the TiO_2 (Armelao *et al*., 2007) similar to the decreased effect observed with the combination of Ag and TiO_2 (Foster *et al*., 2010).

4.11. Gold and palladium

For both abdominal wall defects and inguinal hernias, non-absorbable mesh grafts cannot be used in the presence of an infection (Saygun *et al*., 2006). Consequently, there is an active search for materials that can be used to decontaminate the area of application

without causing an adverse reaction in the host. Polypropylene grafts coated with either Au alone or a mixture of Au and palladium (Pd) have shown potential in both *in vitro* and *in vivo* tests for their application on infected wounds. In the *in vitro* testing, *S. epidermidis* colony reduction was measured on grafts made of pure polypropylene coated with Au or with Au/Pd. After 24, 48, and 72 hours, the Au/Pd coated graft reduced the bacterial population significantly ($P < 0.05$) compared to the other two graft types. In the *in vivo* tests, the meshes were soaked in a solution containing *S. epidermidis* and inserted in the bodies of rats. No incisions were infected in rats with the Au/Pd grafts, 30% of the incisions of containing the Au grafts became infected, and 100% of the incisions containing the pure polypropylene grafts became infected (Saygun *et al*., 2006).

4.12. Mercury and other metals

Elemental mercury is widely used in dentistry to produce “silver” amalgams, which contain 50% mercury (Lorscheider *et al*., 1995). The antibacterial properties of dental amalgams, along with their metal composition ratio to achieve such properties, have not been sufficiently studied (Morrier *et al*., 1998). These amalgams aid in the prevention of gingivitis, pulpal inflammation, and secondary caries in restoration areas (Morrier *et al*., 1998; Nourollahi and Meryon, 1989). Morrier *et al*. (1998) tested the antimicrobial properties of single metal components in amalgams, a copper-silver alloy (28% Ag, 72% Cu), and six whole dental amalgams on *Streptococcus mutans* and *Actinomyces viscosus*, both dental caries related bacteria. When quantifying the population reductions of *S.*

mutans and *A. viscosus*, the amalgam named “Cupromuc” (70% Hg, 28.7% Cu, 0.6% Cd, 0.4% Ag, and 0.3% Zn) showed higher ($P < 0.05$) population reduction capabilities than any other amalgam tested, and the amalgam “Fluoralloy” (67.7% Ag, 27.8% Sn, 4.4% Cu, 1.5% Zn, and 1% F) was the second most effective (Morrier *et al*., 1998).

A particular amalgam may vary in its bactericidal effects against a particular bacterial type. The discrepancies between the antibacterial capabilities of two amalgams might be explained by slight differences in mercury and copper release. Morrier *et al*. (1998) suggested that the antimicrobial activity is higher for mercury, lower for copper, and the lowest for zinc. Thus, it is believed that mercury confers most of the antibacterial efficacy to the amalgam (Nies, 1999).

5. CONCLUSIONS

Metals and their combinations (e.g., zeolites containing multiple metals, copper alloys such as brass) have been used for anti-spoilage and antimicrobial purposes since ancient times. In recent years, the combination of metals is being re-explored as a means for microbial control, particularly on environmental surfaces (Bright *et al*., 2002; Gajjar *et al*., 2009; Foster *et al*., 2010; AgION, 2011; OxiTitan, 2011). It is believed that additive and perhaps sometimes synergistic antimicrobial effects can be achieved by combining two or more metals in a single application (Lin *et al*., 1996). Examples of antimicrobial synergy achieved by the combination of metals include the combination of TiO_2 and Cu against viruses (Ditta *et al*., 2008) and the combination of copper and silver ions for

treating *L. pneumophila* in water systems in hospitals (Lin *et al*., 1996). Additionally, enhanced antimicrobial effects have been described with combinations of silver and gold (Zaporojtchenko *et al*., 2006), silver and copper (McLean *et al*., 1993), and gold and palladium (Saygun *et al*., 2006). A desired property of a combined metal with enhanced or synergistic antimicrobial effects is the elimination of metal resistant bacteria, whose resistance may be mainly established against an antimicrobial based on one metal (McHugh *et al*., 1975; Marco and Stall, 1983; Andersen *et al*., 1991). Nevertheless, metal combinations may not always achieve enhanced antimicrobial effects when compared to materials with a single metal (Armelao et al., 2007; Foster et al., 2010).

Numerous heavy metals have been demonstrated to have broad spectrum antimicrobial activity; however, they do not act instantaneously, usually requiring several hours to reduce bacterial populations. These metals may therefore be used in applications in which the goal is to lower the overall contamination on a surface. The use of combined metal based antimicrobials or self-sanitizing surfaces coated with metals can be highly advantageous in many fields, particularly in medical and public settings (Copper Development Association, 2009), though their use should not replace the basic practices of good hygiene and regular cleaning of these surfaces.

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APPENDIX B

COPPER RESISTANT BACTERIA BETTER TOLERATE COMMERCIALLY AVAILABLE ANTIMICROBIAL TREATMENTS BASED ON SILVER AND SILVER-COPPER IONS.

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1. ABSTRACT

In the current study, the antibacterial efficacy of zeolites containing silver or copper ions or a combination of these metals was assessed against several diverse copper resistant (Cu^R) and copper sensitive (Cu^S) strains of clinically relevant bacterial species. Cu^R *Pseudomonas putida* was significantly reduced in comparison to the unamended zeolite control. Unexpectedly, a Cu^S *P. putida* strain with no reported metal resistance appeared to be more resistant to the zeolite containing either Ag or Ag/Cu than the Cu^R strain. Contrary to expectations, after three and six hours of exposure, the Cu^S *Escherichia coli* displayed significantly more resistance to the Ag/Cu and Cu treatments than the reportedly Cu^R *E. coli.* All three reportedly Cu^R strains of *Salmonella enterica* exhibited resistance to Cu and Ag, as well as to the combination of the two metals after three and six hours of exposure. The reductions observed after 24 hours for all three Cu^R strains with Cu alone were still statistically significant compared to that of the Cu^S *S. enterica* strain. In addition, two of the Cu^R strains were more resistant to silver after 24 hours of exposure, suggesting a shared resistance mechanism such a copper efflux pump that also removes silver ions from the cell. Both the Cu^R and Cu^S strains of *E. faecium* were highly resistant to all of the treatments. In general, after comparison of all the resistances with all the treatments, *E. faecium* was the most resistant species, *P. putida* was the least resistant species, and the *Salmonella* strains were more resistant than *E. coli* in most cases.

2. INTRODUCTION

Heavy metals are toxic to bacteria (Nies, 1999). As such, they have been used as antimicrobials in numerous applications for centuries (Marco and Stall, 1983; Ghandour *et al*., 1988; Andersen *et al*., 1991). Nonetheless, bacterial metal resistance mechanisms have been evolving since the existence of bacteria (Silver and Phung, 2005). It is unclear if the reported resistance in recent decades is due to human activities (Lutkenhaus, 1977; Marco and Stall, 1983; Andersen *et al*., 1991; Lin *et al*., 2002; Hasman, 2005; Espírito Santo *et al*., 2010). Metal resistant bacteria are those which are able to survive exposure to a metal concentration deemed lethal to their non-resistant counterparts. These bacteria display a higher than normal Minimal Inhibitory Concentration (MIC) to a specific metal (Chapman, 2003).

Bacterial resistance to copper (Cu) was observed as early as 1983 (Marco and Stall, 1983; Andersen *et al*., 1991). The copper resistance mechanisms are not well understood, but prior exposure to sub-lethal copper concentrations may be a contributing factor such as in the intestines of pigs when the pigs are given copper supplements (Aarestrup *et al*., 2002; Hasman, 2005), on crops when copper sulphate is applied as fungicide (Marco and Stall, 1983; Andersen *et al*., 1991) and on copper alloy coins (Espírito Santo *et al*., 2010). Long term disinfection with copper ionization systems (Lin *et al*., 2002) and mutations resulting in a degree of copper impermeability in bacterial cells (Lutkenhaus, 1977) have been implicated as precursors in the development of copper resistant (Cu^R) strains. Since copper alloys have now been registered as

antimicrobial materials (U.S. Environmental Protection Agency, 2008), the applications for copper coatings on general use fomites (inanimate surfaces) are expected to rise, particularly on fomites in hospitals (Copper Development Association, 2009). Such increased usage may select for Cu^R bacterial strains, leading to their proliferation (Lutkenhaus, 1977; Marco and Stall, 1983; Andersen *et al*., 1991; Aarestrup *et al*., 2002; Lin *et al*., 2002; Hasman, 2005; Espírito Santo *et al*., 2010).

Zeolites are ceramic powders (sodium aluminosilicates) with a three-dimensional crystalline structure in which metal ions can reside. Such zeolites can be used to coat hard surfaces or can be incorporated into plastics and textiles to create antimicrobial products. The zeolite acts an ion exchanger, exchanging the incorporated metal ions for other cations in the immediate environment. The released metal ions are then free to confer the desired antimicrobial effect. In the current study, the antibacterial efficacy of zeolites containing silver or copper ions or a combination of these metals was assessed against several diverse copper resistant (Cu^R) and copper sensitive (Cu^S) strains of clinically relevant bacterial species.

3. MATERIALS AND METHODS

Determination of metal ion concentration in solution

All glassware used in these and all subsequent experiments was submerged in 10% nitric acid for at least 16 hours to remove any contaminating metal ion residuals. The antimicrobial zeolites included in this work were provided by AgION® technologies (Wakefield, MA). The following formulations (wt/wt compositions with zeolite powder) were evaluated: 1) 2.5% Ag, 2) 10% Cu, and 3) a combination of 3.5% Ag and 6.5% Cu. For the zeolites containing Ag alone or a combination of Ag and Cu, a 10 mg sample of the zeolite was added to 100 ml of sterile physiological saline (0.85% NaCl) in a 250 ml Erlenmeyer flask. For the zeolite containing only Cu ions, 6.5 mg was used to reduce the amount of Cu in the final solution to a concentration similar to the amount of Cu found in the solution containing the Ag/Cu combination zeolite. This procedure resulted in 0.01% (wt/vol) solutions for the Ag and Ag/Cu zeolites and in a 0.0065% (wt/vol) solution for the 10% Cu zeolite.

The flasks were then placed on an orbital shaker (250 rpm) at room temperature (24°C). After 3, 6, and 24 hours, samples of the solutions were centrifuged (9,820 x *g* for 15 minutes at room temperature) to pellet the zeolite powders. After centrifugation, 5 ml of the supernatant was extracted and immediately added to 20 ml of 1% nitric acid. These samples were analyzed by inductively-coupled plasma mass spectrometry (ICP-MS) using the ELAN-DRC II spectrophotometer (Perkin-Elmer, Shelton, CT) to determine the

concentration of metal ions that been released into solution after each particular duration of time. These experiments and subsequent assays were conducted with triplicate samples/replicates. The solutions containing only Cu were measured with seven replicates for the 24-hour concentration.

Bacterial strains

The bacterial strains *Enterococcus faecium* 75-30733-5, *Escherichia coli* 77-30013-2 and *Pseudomonas putida* 08991 were obtained from Dr. Christopher Rensing at the University of Arizona in Tucson, AZ. The strains *Salmonella enterica* S9, *S. enterica* S19, and *S. enterica* S20 were obtained from Dr. Sadhana Ravishankar, also from the University of Arizona. All of these strains have been previously reported to be Cu^R strains (Cooksey *et al*., 1990; Ravishankar *et al*., 2010; Elguindi *et al*., 2011). The presumably Cu^S strains employed in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and included *E. faecium* 19579, *E. coli* 25922, *P. putida* 31483, and *S. enterica* 23564.

Experimental procedure

Prior to the start of an experiment, an Erlenmeyer flask containing either 250 ml of brain heart infusion (BHI; Becton, Dickinson and Company, Sparks, MD) for *E. faecium* or 100 ml of tryptic soy broth (TSB; EMD Chemicals, Darmstadt, Germany) for the other species was inoculated with a colony from a fresh culturete. The flask was incubated on

an orbital shaker (250 rpm) for 16 hours at 24°C for the *P. putida* strains and 37°C for all other strains. After incubation, the bacteria were pelleted via centrifugation (9,820 x *g*, 15 min, 20°C). The pelleted cells were washed by resuspension in 100 ml of physiological saline and centrifuged again as before. Following the second centrifugation step, the bacterial pellet was resuspended in 10 ml of physiological saline. From this 10 ml bacterial suspension, small volumes were added to 10 ml of sterile physiological saline to achieve an optical turbidity (measured using a BIOLOG turbidimeter; BIOLOG, Hayward, CA) equivalent to a MacFarland's number 0.5 optical density standard [= 1.5 x 10^8 colony-forming units (CFU)/ml].

A 10-ml volume of this bacterial stock solution was then added to Erlenmeyer flasks containing 90 ml of sterile saline and 0.01% or 0.0065% (wt/vol) zeolite powder as described previously. In addition to these antibacterial treatment flasks, the following two controls were also included: 1) a control with 10 ml of bacterial stock added to physiological saline, and 2) a control with 10 ml of bacterial stock added to physiological saline containing unamended zeolite powder (did not contain any metals).

All of the inoculated flasks (treatment and control flasks) were then incubated at room temperature (24°C) on an orbital shaker (250 rpm). The experiment was performed with triplicate flasks for each of the treatments (zeolite with Ag alone, zeolite with Cu alone, or zeolite with Ag and Cu combination) and the control with unamended zeolite powder. Duplicate flasks were used for the control that did not contain any zeolite. After 0, 3, 6 and 24 hours, a 100 µl sample was removed and placed into 900 µl of Dey Engley neutralizing broth (D/E neutralizer; Becton, Dickinson and Company, Sparks, MD). From

this neutralized sample, serial ten-fold dilutions were performed in saline and these dilutions were used to inoculate selective agar plates (with duplicate plates) via the spread plate technique. The recovered *E. faecium* was cultured on KF Streptococcus Agar (Becton, Dickinson and Company, Sparks, MD), *E. coli* on Levine Eosin Methylene Blue Agar (EMB agar; EMD Chemicals, Darmstadt, Germany), *P. putida* on *Pseudomonas* Isolation Agar (PI agar; Becton, Dickinson and Company, Sparks, MD), and *Salmonella* strains on Xylose Lysine Deoxycholate Agar (XLD Agar; EMD Chemicals, Darmstadt, Germany). *E. coli* and *Salmonella* strains were incubated for 24 hours and *E. faecium* for 48 hours at 37°C. The *P. putida* strains were incubated for 48 hours at 26°C for strain 31483 and 30°C for strain 08991.

Data analyses

The numbers of surviving bacteria and the bacterial logarithmic reductions were used to determine the germicidal efficacy. A logarithmic reduction is represented by the formula $\log_{10} N_o/N_t$, where N_o is the original bacterial concentration at time zero, and N_t is the surviving bacteria at time *t*. A two-tailed Student *t* test (assuming unequal variances) was used to determine if there was a significant difference ($P \leq 0.05$) between the reductions observed between the treatments and controls and between bacterial strains.

4. RESULTS

Copper and silver ions released into solution

For all of the treatments, there was no statistical difference between the concentrations of metal ions found in the supernatant between any of the samples after different incubation periods (Table 1). Nevertheless, the amount of Cu released from the Ag/Cu combination zeolite (average of 39.2 ± 9.4 µg/L for all time intervals) was significantly lower ($P \leq 0.05$) than that released by the zeolite containing only Cu (average of 425.3 ± 157.3 µg/L for all time intervals). There was no difference between the amount of Ag released from the zeolite containing only Ag versus the zeolite containing both Ag and Cu. The overall average for Ag ion release from all samples at all time intervals was 32.2 (± 7.2) µg/L (ppb).

Efficacy of zeolites against copper resistant and copper sensitive strains

The reductions observed for both Cu^R and Cu^S strains of *P. putida*, *E. coli*, *S. enterica*, and *E. faecium* are shown in Tables 2, 3, 4, and 5, respectively.

Pseudomonas putida strains. The copper resistant (Cu^R) *P. putida* populations were significantly reduced at all time points in comparison to the unamended zeolite control with all of the treatments; both the Ag/Cu and the Cu treatments achieved very similar bacterial reductions with a >3-$\log_{10}$ reduction shortly after three hours of exposure (Table

2). The Ag formulation resulted in a >3-$\log_{10}$ only achieved after 24 hours of exposure. On the other hand, despite the fact that the Cu treatment rapidly reduced the copper sensitive (Cu^S) *P. putida*, with a 4.8-$\log_{10}$ reduction after three hours, only after 24 hours was a comparable level of reduction achieved with either the Ag or the Ag/Cu zeolite. Unexpectedly, the Cu^S *P. putida* strain with no reported metal resistance (ATCC, 2010) appeared to be more resistant to the zeolite containing either Ag or Ag/Cu than the Cu^R strain.

Escherichia coli strains. Most of the reductions observed with both *E. coli* strains were statistically significant ($P \leq 0.05$) in comparison to the unamended zeolite control within six hours of exposure (Table 3). The Cu alone and Ag/Cu treatments significantly reduced the Cu^R *E. coli* after three hours with a ≥1.0-$\log_{10}$ reduction. There was no statistical difference between these two treatments at any of the exposure times. Conversely, no significant reductions were observed with the Ag treatment at either three or six hours of exposure. Thus, there was a difference found between the Ag treatment and the two treatments containing Cu at these earlier time intervals. Nevertheless, by 24 hours, significant reductions of >4-log were achieved by all the treatments.

Contrary to expectations, after three and six hours of exposure, the Cu^S *E. coli* displayed significantly more resistance to the Ag/Cu and Cu treatments than the reportedly Cu^R *E. coli*. This Cu^S *E. coli* strain was more sensitive to Ag at both the three and six hour time intervals. However, by 24 hours, there was no difference observed between any of the treatments or between strains.

Salmonella enterica strains. All three reportedly Cu^R strains of *S. enterica* exhibited resistance to Cu and Ag, as well as to the combination of the two metals after three and six hours of exposure (Table 4). The reductions observed after 24 hours for all three strains with the Cu alone treatment were still statistically significant compared to that of the Cu^S *S. enterica* strain (2.2-, 0.3-, and 1.3-$\log_{10}$ reductions for strains S9, S19, and S20, respectively vs. a >4.9-$\log_{10}$ reduction for the Cu^S strain). In addition, two of the strains (S19 and S20) were more resistant to silver after 24 hours of exposure as well.

Strain S19 was the most Cu-resistant. Alternatively, strain S9 was more sensitive to Ag than the other two copper resistant *S. enterica* strains. In general for the Cu^R strains, the efficacy of all the treatments followed the order of Ag/Cu > Ag > Cu, though not all of these differences were significant.

The copper sensitive strain, although considerably more sensitive to Cu than the Cu^R strains ($P \leq 0.05$), had only been reduced by 1.5-$\log_{10}$ after six hours of exposure. In contrast, this strain was highly sensitive to Ag, with a >5.6-$\log_{10}$ reduction within the same exposure time.

Enterococcus faecium strains. Both the Cu^R and Cu^S strains of *E. faecium* were highly resistant to Ag, Cu, and a combination of Ag and Cu (Table 5). Very small reductions (usually 0.1 to 0.3-$\log_{10}$) were observed at times with both strains that were statistically significant in comparison to the unamended zeolite control; nevertheless, these reductions are not meaningful in a practical sense. Despite this, the Cu^S strain did appear to be slightly more sensitive to the Cu treatment than the Cu^R strain at each time exposure.

Using a combination of Ag and Cu did not appear to have any increased antibacterial effect with either strain.

Comparison between copper resistant strains. The relative resistances to both metals were compared between the reportedly copper resistant strains (Table 6). The bacterial resistance to the individual metals and the metal combination were in the following orders from most resistant to least resistant (only considering differences that were statistically significant):

Cu: *E. faecium* = *S. enterica* S19 > *S. enterica* S20 = *S. enterica* S9 > *E. coli* > *P. putida*

Ag: *E. faecium* > *S. enterica* S20 = *S. enterica* S19 = *E. coli* > *S. enterica* S9 = *P. putida*

Cu/Ag: *E. faecium* > *S. enterica* S20 > *S. enterica* S19 > *S. enterica* S9 > *E. coli* > *P. putida*

In general, *E. faecium* was the most resistant species, *P. putida* was the least resistant species, and the *Salmonella* strains were more resistant than *E. coli* in most cases.

5. DISCUSSION

In past studies with zeolites containing 2 metals, there has been little effort to determine the antimicrobial capacity being provided by each of the metals; rather, the efforts have been to compare the antimicrobial properties of fomites (inanimate surface) containing or coated with the combined metal zeolite in comparison to the same type of fomites

without any zeolite (Bright *et al*., 2002; Takai *et al*., 2002; Galeano *et al*., 2003; Rusin *et al*., 2003; Bright *et al*., 2009; De Muynck *et al*., 2010). For instance, a zeolite supplemented with a combination of Ag and Cu exhibited greater bactericidal efficacy than one containing Ag alone (Bright *et al*., 2002); nonetheless, the antibacterial effect provided by the Cu alone was not determined.

The Cu^R *E. faecium* strain (75-30733-5) was slightly more resistant to Cu than the Cu^S strain (19579); nevertheless, both strains were found to be quite resistant to Ag and Cu ions both individually and in combination. This suggests that this species is inherently resistant to these metals. This was the only Gram-positive bacterial species included in the current study.

As one would expect, the Cu^S strain of *S. enterica* (23564) was found to be more sensitive to Cu than the Cu^R strains. In addition, this strain was far more sensitive to Ag ions than the Cu^R strains. This suggests that copper resistance in this organism also confers resistance to Ag, perhaps through a shared mechanism such as an efflux pump that is able to remove both ions from the cell. The Cu^R strain S9 was more sensitive to both Cu and Ag ions than the other two copper resistant strains.

The molecular mechanisms involved in Cu homeostasis may also provide Ag extrusion. In a two component copper regulatory system in *E. coli*, the copper-sensory protein CueR, which regulates the main copper extrusion component (*copA* gene), is also activated by the presence of intracellular Ag^{1+} (Stoyanov *et al*., 2001). The *cop* operon is responsible for copper homeostasis in *Enterococcus hirae* (Odermatt *et al*., 1993; Odermatt *et al*., 1994; Odermatt and Solioz, 1995). A product of this operon, CopB, is a

Cu^{1+}/Ag^{1+} cell extruding P-type ATPase (Odermatt *et al*., 1994; Solioz and Odermatt, 1995). *E. hirae* wild-type strains are as vulnerable as CopB deletion mutants when challenged with increasing silver concentrations (Odermatt *et al*., 1993). In wild-type cells, CopA Ag^{1+} influx exceeds CopB Ag^{1+} extrusion (Wunderli-Ye and Solioz, 1999); however, if these conditions were reversed so that the CopB Ag efflux surpasses that of the CopA influx, resistance to Ag could result.

The *P. putida* Cu^S strain (31483) was also more sensitive to Cu ions than the Cu^R strain (08991), although the Cu^R strain was surprisingly also quite sensitive with a reduction of 3.1-$\log_{10}$ within three hours of exposure. Interestingly though, the copper sensitive strain was more resistant to Ag ions than the Cu^R strain. In addition, the combination of metals was far less effective than Cu ions alone against this strain. This was likely due to the lower amount of Cu ions released from the zeolite containing the two metals. This theory is supported by the results from a previous study in which a sodium-based zeolite (clinoptilolite) was found to release metals with different efficiencies ($Ag^{1+} > Zn^{2+} > Cu^{2+}$). In the same study, the greatest bactericidal activity was achieved by Ag. This may have been due to its faster release rate from the zeolite and consequently higher Ag concentration in contact with the bacteria (Top and Ülkü, 2004), or it may be due to Ag ions being more effective than Cu ions at low (ppb) concentrations (unpublished data).

The putatively Cu-sensitive *E. coli* strain (25922) was more resistant to Cu, but more sensitive to Ag than the Cu-resistant strain (77-30013-2). This might indicate a

different mechanism for copper resistance than the one found in the *Salmonella* strains. The combination of metals also had no enhanced effect against *E. coli*.

Several of these reportedly Cu resistant strains did not appear to possess significant resistance to Cu, and in the case of *E. coli* strain 77-30013-2, were even decidedly more sensitive to Cu in comparison to a reference strain. The protocols used to determine bacterial resistances to metals have typically been conducted by adding various concentrations of the metal ion to nutrient-rich agar plates or to nutrient-rich broths and assessing the ability of the bacteria to grow in comparison to a wild-type strain. These are similar to the types of assays used to assess bacterial antibiotic resistance. The organic matter in such media can interact with metals such as copper (Wunderli-Ye and Solioz, 2001; Lin *et al*., 2002), diminishing its antibacterial efficacy. These assays therefore likely overestimate the bacterial metal resistance (Calomiris *et al*., 1984; Andersen *et al*., 1991; Harrison *et al*., 2005; Schmidt *et al*., 2007). Experiments conducted in a nutrient-deprived environment such as those typically encountered by bacteria in the environment are therefore a much more accurate assessment of bactericidal efficacy. To illustrate this point, the reportedly Cu^R *E. coli* strain used in this study was grown in both organic-rich and organic-free media (Luria Bertani [LB] broth and 0.85% NaCl, respectively) containing various $CuSO_4$ concentrations for 16 hours. The Cu^R *E. coli* survived a maximum concentration of 254.2 ppm $CuSO_4$ in the LB broth, yet only survived 4 ppm $CuSO_4$ in the NaCl solution (data not shown). The maximum Cu concentration allowing for bacterial survival was thus decreased 63-fold in an organic-free medium.

Silver and copper are effective, broad-spectrum antibacterial agents; however, they do not act instantaneously, usually requiring at least several hours to reduce bacterial populations. These metals may therefore be used in applications in which the goal is to lower the overall contamination on a surface such as on environmental surfaces in a hospital. These metals have also been shown to have a synergistic effect when used in combination (Lin *et al*., 1996; Silvestry-Rodriguez *et al.* 2007, unpublished data). In the current study, with the exception of the *E. faecium* strains, the zeolite containing both Ag and Cu proved to be effective against the putative Cu^R strains. Nevertheless, it appears that adding the two metals simultaneously to the zeolite actually inhibits the release of the copper ions. Therefore, it would likely be a better approach to create a zeolite containing Ag ions and a second zeolite containing Cu ions and then mix these two powders together before applying to materials.

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Table 1. Ag and Cu concentrations released from the zeolite matrix into the saline supernatant at various time intervals.

Zeolite	Time (hrs)	Ag (ppb ± SD)	Cu (ppb ± SD)
2.5% Ag	3	37.9 ± 14.1	
	6	25.6 ± 1.3	
	24	34.2 ± 2.4	
6.5% Cu	3		326.3 ± 44.6
	6		317.0 ± 52.2
	24		514.1 ± 167.2
3.5% Ag / 6.5% Cu	3	28.8 ± 5.6	65.6 ± 37.1
	6	31.5 ± 1.3	36.4 ± 2.0
	24	34.4 ± 0.6	49.9 ± 0.3

SD = Standard deviation.

Table 2. Log_{10} reduction (± standard deviation)[a] of copper sensitive and copper resistant strains of *Pseudomonas putida* after exposure to silver (Ag) and copper (Cu) zeolite powders.

***P. putida* strain**	**Time (hrs)**	**Saline control**	**Unamended zeolite**	**Zeolite treatments**		
				Ag	**Cu**	**Ag/Cu**
Cu sensitive 31483	3	0.0 ± 0.0	0.0 ± 0.0	[b]0.2 ± 0.0	[b]4.8 ± 0.6	[b]0.2 ± 0.1
	6	0.0 ± 0.0	0.1 ± 0.2	0.5 ± 0.4	[b]>5.2 ± 0.2	0.5 ± 0.3
	24	0.0 ± 0.0	0.9 ± 0.1	[b]4.3 ± 0.6	[b]4.1 ± 0.0	[b]4.1 ± 0.2
Cu resistant 08991	3	0.0 ± 0.0	0.1 ± 0.1	[b]0.3 ± 0.1	[b]3.0 ± 1.2	*[b]3.2 ± 0.5
	6	0.0 ± 0.0	0.0 ± 0.0	*[b]1.4 ± 0.3	[b]>4.3 ± 0.9	*[b]>4.6 ± 1.1
	24	0.9 ± 0.3	0.8 ± 0.5	[b]5.1 ± 0.3	[b]>4.8 ± 0.8	*[b]>5.2 ± 0.0

[a] The initial titer was 1.0 x 10^7 CFU/ml for strain 31483 and 8.7 x 10^6 CFU/ml for strain 08991.

[b] Significant reduction ($P \leq 0.05$) in comparison to the unamended zeolite control.

* The difference between the copper sensitive and resistant strains was statistically significant.

Table 3. Log_{10} reduction (± standard deviation)[a] of copper sensitive and copper resistant strains of *Escherichia coli* after exposure to silver (Ag) and copper (Cu) zeolite powders.

E. coli strain	Time (hrs)	Saline control	Unamended zeolite	Zeolite treatments		
				Ag	Cu	Ag/Cu
Cu sensitive 25922	3	0.0 ± 0.0	0.0 ± 0.0	[b]0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
	6	0.0 ± 0.0	0.0 ± 0.0	[b]0.8 ± 0.1	[b]0.2 ± 0.0	0.4 ± 0.2
	24	0.1 ± 0.2	0.0 ± 0.0	[b]>5.3 ± 0.4	[b]3.2 ± 1.6	[b]5.1 ± 0.2
Cu resistant 77-30013-2	3	0.0 ± 0.0	0.0 ± 0.0	*0.0 ± 0.0	[b]1.0 ± 0.6	*[b]1.0 ± 0.1
	6	0.0 ± 0.0	0.0 ± 0.0	*0.1 ± 0.2	*[b]1.9 ± 0.9	*[b]1.2 ± 0.1
	24	0.0 ± 0.0	0.0 ± 0.0	[b]>4.6 ± 0.8	[b]>4.2 ± 1.4	[b]>5.1 ± 0.0

[a] The initial titer was 2.8 x 10^7 CFU/ml for strain 25922 and 5.7 x 10^6 CFU/ml for strain 77-30013-2.

[b] Significant reduction ($P \leq 0.05$) in comparison to the unamended zeolite control.

* The difference between the copper sensitive and resistant strains was statistically significant.

Table 4. Log_{10} reduction (± standard deviation)[a] of copper sensitive and copper resistant strains of *Salmonella enterica* after exposure to silver (Ag) and copper (Cu) zeolite powders.

S. enterica strain	Time (hrs)	Saline control	Unamended zeolite	Zeolite treatments		
				Ag	Cu	Ag/Cu
Cu sensitive 23564	3	0.0 ± 0.0	0.4 ± 0.4	[b]1.9 ± 0.2	[b]1.4 ± 0.6	[b]1.8 ± 0.6
	6	0.1 ± 0.2	0.4 ± 0.4	[b]>5.6 ± 0.0	[b]1.5 ± 0.2	[b]>5.2 ± 0.4
	24	0.0 ± 0.0	0.2 ± 0.2	[b]>5.6 ± 0.0	[b]>4.9 ± 1.1	[b]>5.6 ± 0.0
Cu resistant S9	3	0.6 ± 0.1	0.2 ± 0.2	*0.5 ± 0.1	*0.1 ± 0.1	*0.0 ± 0.0
	6	0.2 ± 0.3	0.5 ± 0.2	*0.6 ± 0.3	*0.1 ± 0.0	*[b]1.3 ± 0.3
	24	0.0 ± 0.0	0.4 ± 0.0	[b]>5.0 ± 0.7	*[b]2.2 ± 0.9	[b]>5.1 ± 0.5
Cu resistant S19	3	0.1 ± 0.0	0.2 ± 0.1	*0.0 ± 0.0	*0.0 ± 0.0	*0.1 ± 0.0
	6	0.0 ± 0.0	0.0 ± 0.0	*0.1 ± 0.1	*[b]0.1 ± 0.0	*[b]0.3 ± 0.1
	24	0.1 ± 0.1	0.2 ± 0.0	*[b]2.4 ± 0.1	*0.3 ± 0.2	*[b]3.2 ± 0.6
Cu resistant S20	3	0.0 ± 0.0	0.0 ± 0.0	*0.0 ± 0.0	*0.0 ± 0.0	*0.0 ± 0.0
	6	0.0 ± 0.0	0.1 ± 0.1	*0.1 ± 0.0	*0.1 ± 0.0	*0.0 ± 0.0
	24	0.3 ± 0.0	0.2 ± 0.1	*[b]2.2 ± 0.4	*[b]1.3 ± 0.1	[b]4.3 ± 1.1

[a] The initial titer was 1.8 x 10^7 CFU/ml for strain 23564, 1.4 x 10^7 CFU/ml for strain S9, 2.8 x 10^7 CFU/ml for strain S19, and 1.8 x 10^7 CFU/ml for strain S20.

[b] Significant reduction ($P \leq 0.05$) in comparison to the unamended zeolite control.

* The difference between the copper sensitive and resistant strains was statistically significant.

Table 5. Log_{10} reduction (± standard deviation)[a] of copper sensitive and copper resistant strains of *Enterococcus faecium* after exposure to silver (Ag) and copper (Cu) zeolite powders.

E. faecium strain	Time (hr)	Saline control	Unamended zeolite	Zeolite treatments		
				Ag	Cu	Ag/Cu
Cu sensitive 19579	3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	[b]0.3 ± 0.0	0.0 ± 0.0
	6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	[b]0.3 ± 0.0	0.0 ± 0.0
	24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	[b]1.3 ± 0.9	[b]0.2 ± 0.0
Cu resistant 75-30733-5	3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	*[b]0.1 ± 0.0	0.0 ± 0.0
	6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	*0.0 ± 0.0	0.0 ± 0.0
	24	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	[b]0.1 ± 0.0	[b]0.2 ± 0.1

[a] The initial titer was 1.6 x 10^7 CFU/ml for strain 19579 and 2.1 x 10^7 CFU/ml for strain 75-30733-5.

[b] Significant reduction ($P \leq 0.05$) in comparison to the unamended zeolite control.

* The difference between the copper sensitive and resistant strains was statistically significant.

Table 6. Comparison of metal resistances among Cu resistant strains upon exposure to Ag and/or Cu zeolites[a].

Copper resistant Bacterial Strain		Treatment	Copper resistant Bacterial Strain																	
			E. coli			*E. faecium*			*P. putida*			*Salmonella* S9			*Salmonella* S19			*Salmonella* S20		
		Time (hrs)	3	6	24	3	6	24	3	6	24	3	6	24	3	6	24	3	6	24
Copper resistant Bacterial Strain	***E. coli***	Ag				*	*	↑	←	←	*	←	*	*	*	*	*	*	*	*
		Cu				*	↑	↑	*	←	*	↑	↑	*	↑	↑	↑	↑	↑	↑
		Ag+Cu				↑	↑	↑	←	←	*	↑	*	*	↑	↑	↑	↑	↑	*
	E. faecium	Ag	*	*	←				←	←	←	←	*	←	*	*	←	*	*	←
		Cu	*	←	←				←	←	←	↑	*	←	*	*	*	*	*	←
		Ag+Cu	←	←	←				←	←	←	*	←	←	←	←	←	*	*	←
	P. putida	Ag	↑	↑	*	↑	↑	↑				*	*	*	↑	↑	↑	↑	↑	↑
		Cu	*	↑	*	↑	↑	↑				↑	↑	↑	↑	↑	↑	↑	↑	↑
		Ag+Cu	↑	↑	*	↑	↑	↑				↑	↑	*	↑	↑	↑	↑	↑	*
	***Salmonella* S9**	Ag	↑	*	*	↑	*	↑	*	*	*				↑	*	↑	↑	*	↑
		Cu	←	←	*	←	*	↑	←	←	←				*	*	↑	*	*	*
		Ag+Cu	←	*	*	*	↑	↑	←	←	*				←	↑	↑	*	↑	*
	***Salmonella* S19**	Ag	*	*	*	*	*	↑	←	←	←	←	*	←				*	*	*
		Cu	←	←	←	*	*	*	←	←	←	*	*	←				*	*	←
		Ag+Cu	←	←	←	↑	↑	↑	←	←	←	↑	←	←				↑	↑	*
	***Salmonella* S20**	Ag	*	*	*	*	*	↑	←	←	←	←	*	←	*	*	*			
		Cu	←	←	←	*	*	↑	←	←	←	*	*	*	*	*	↑			
		Ag+Cu	←	←	*	*	*	↑	←	←	*	*	←	*	←	←	*			

[a] For each treatment and exposure time, an arrow points to the strain that had statistically significantly ($P \leq 0.05$) more survivors (and thus had a greater level of resistance). An asterisk denotes no significant difference between the numbers of bacteria recovered for the 2 strains