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Resistance to Metals Used in Agricultural Production

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ABSTRACT Metals and metalloids have been used alongside antibiotics in livestock production for a long time. The potential and acute negative impact on the environment and human health of these livestock feed supplements has prompted lawmakers to ban or discourage the use of some or all of these supplements. This article provides an overview of current use in the European Union and the United States, detected metal resistance determinants, and the proteins and mechanisms responsible for conferring copper and zinc resistance in bacteria. A detailed description of the most common copper and zinc metal resistance determinants is given to illustrate not only the potential danger of coselecting antibiotic resistance genes but also the potential to generate bacterial strains with an increased potential to be pathogenic to humans. For example, the presence of a 20-gene copper pathogenicity island is highlighted since bacteria containing this gene cluster could be readily isolated from copper-fed pigs, and many pathogenic strains, including *Escherichia coli* O104:H4, contain this potential virulence factor, suggesting a potential link between copper supplements in livestock and the evolution of pathogens.

INTRODUCTION

Metal compounds are widely used in livestock production because they are necessary supplements and play a very important role as essential trace elements that are part of the nutritional requirements of most animal species. However, copper and zinc compounds are also added to feed in larger concentrations for achieving additional beneficial effects. Therefore, we will focus this chapter on copper and zinc; their use and indications; the concerns related to the selection of resistance, toxicity, and environmental pollution and policies; and the alternatives and new developments regarding their use. Compounds derived from the nonessential metal arsenic, such as roxarsone, which have been used in livestock for feed supplementation in some countries around the world, will not be discussed in great detail in this chapter.

USE OF METAL COMPOUNDS IN AGRICULTURAL PRODUCTION: REQUIREMENTS AND RECOMMENDATIONS

Copper

Copper is an essential trace element that is necessary for the human body because it has major functions regarding fetal growth and early postnatal development, hemoglobin synthesis, maturation of the connective

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tissues, proper nerve function and bone development, and inflammatory processes. Therefore, most animal species are required to have copper in their feed and exhibit copper deficiency symptoms if copper is insufficient. Copper is also expected to interact with other compounds in the feed (phytates, fiber fraction, zinc, iron, calcium, ascorbic acid, molybdenum, and sulfur), and therefore the concentrations added to feed are increased to ensure that the necessary amount is available for absorption by the target animal species. This is also dependent on the animal's intestinal tract, and these interactions influence the absorption, as extensively reviewed by Suttle (1).

An excess of copper can also be detrimental and lead to toxicity manifested as anemia and liver dysfunction. Release of copper from the liver may lead to vascular hemolysis and death. The toxicity of copper depends on speciation, with Cu(I) being more toxic then Cu(II). Certain animal species such as sheep and young calves are sensitive to excess copper (2). Copper might also exert a selection pressure on the gut microbiota and influence the composition of the gut flora. Furthermore, the use of therapeutic concentrations might select for resistance, which is addressed later in this chapter. Requirements for copper in animal feed depend on the target species, the age group and the feed composition (2).

Copper is contained in a number of plants and seeds in varying concentrations and may become concentrated with the use of copper compounds in agriculture, but the availability of copper in feed is not well known. For food-producing animals and small pet animals such as rabbits, the copper background levels in complete feeds are rather low and in the range of 5 (salmon feed) to 15 (rabbit feed) mg Cu/kg feed, and higher values are observed in feed for dairy cows, containing about 20 mg Cu/kg feed. Supplementation of animal feed is common practice, and the current limits for supplementation (3) were revised by the European Union in 2016 (Table 1) (4).

Copper additives

The list of copper compounds authorized for copper supplementation in feed is extensive and includes cupric

TABLE 1 New proposed maximum limits of copper in complete animal feed[b]

Target species, animal category	R[a]	1.5 × R	Background	NPMC[b]	CAMC[b]
Chickens for fattening, reared for laying	8	12	10	25	25
Laying hens, breeder hens	8	12	10	25	25
Turkeys for fattening, 0–8 weeks of age	10	15	10	25	25
Turkeys for fattening, from 8 weeks of age onward	6	9	10	25	25
Other poultry	8	12	10	25	25
Piglets, weaned	8	12	10	25	170
Pigs for fattening	6	9	10	25	25
Sows	10	15	10	25	25
Calves, milk replacer	10	15	5	15[c]	15
Cattle for fattening	8.8[h]	13.2	10	30[d]	35
Dairy cows	8.8–13.2[h]	13.2–19.8	10	30	35
Sheep	7[h]	10.6	10	15[e]	15
Goats	7–22[h]	10.6–33	10	35	25
Horses	8.8[h]	13.2	10	25	25
Rabbits	5	7.5	10	25	25
Salmonids	5	7.5	10	25	25
Other fish	8	12	10	25	25
Crustaceans				50	50
Dogs	12	15.6[f]	10	25	25
Cats	10	13[f]	10	25	25

[a]R, requirement.
[b]NPMC, newly proposed total maximum contents of copper in complete feed (4); CAMC, currently authorized maximum content of copper in complete feed (3).
[c]Because copper in milk-based diets shows a high bioavailability and does not contain substantial amounts of copper antagonists, the background level (5 mg/kg) is not added to the allowance (15 mg/kg).
[d]Considering cattle feeding on pasture, a potentially high amount of copper antagonists in forage is taken into account.
[e]NPMC is limited by the Mineral Tolerance of Animals (MTL); dietary concentrations above 15 mg/kg could provoke chronic copper poisoning (CCP).
[f]No requirement data available; the allowance (12 and 10 mg/kg for dogs and cats, respectively) is therefore only multiplied by 1.3 to consider different bioavailability of copper supplements.
[g]Adjusted from dry matter to complete feed with 88% dry matter.
[h]Source: reference 4.

acetate, monohydrate; basic cupric carbonate, monohydrate; cupric chloride, dihydrate; cupric methionate; cupric oxide; cupric sulfate, pentahydrate; cupric chelate of amino acid hydrate; copper lysine sulfate; cupric chelate of glycine hydrate; copper chelate of hydroxy analogue of methionine; dicopper chloride trihydroxide; and copper bislysinate. These authorized compounds fall under European Economic Community (EEC) number E4 (3). Most copper-containing compounds show bioavailability similar to copper sulfate for all animal species. However, cupric oxide and cupric carbonate (and cuprous iodide in poultry) showed a lower and more variable relative bioavailability (4).

Therapeutic use of copper

Copper may be prescribed for copper deficiency and is normally administered orally using premixes in feed, or it may be administrated using injectable solutions. Additionally, copper has antimicrobial properties, and copper sulfate may be used in footbaths as a fungicide for the control of foot-rot in cattle and sheep (5 to 10% solution) (5). Copper has also been used as a growth promoter, even though the efficacy of this practice has not been very well demonstrated. Growth promotion might depend on the feed antagonists present and relate to improved appetite or digestibility in pigs and poultry (1).

Zinc

Similar to copper, zinc is also an essential trace element and therefore is necessary to comply with the nutritional requirements of livestock. Zinc is part of 10% of all proteins and contributes to their tertiary structure and the function of enzymes, and therefore its role is essential in diverse organs and systems. For example, zinc is needed for glucose and lipid metabolism, cell proliferation, embryogenesis, and systems related to the nervous and immune systems.

Symptoms of zinc deficiency can be observed in most animal species (6). Known examples of this are parakeratosis in swine (7) and growth deficiencies in feathering and skeletal development in poultry (8). Zinc is an essential trace element, and zinc deficiency might be related to malabsorption of this metal compound. Similarly, Friesian cows and bull terrier dogs have been shown to have genetic syndromes related to zinc malabsorption (9, 10).

Most of the absorption of zinc occurs in the small intestine, and feed components such as phytates may hamper the absorption in monogastric species, while low molecular weight binding ligands such as citrate, picolinate, EDTA, and amino acids such as histidine and glutamate were shown to increase the absorption of zinc (11).

Zinc is mainly metabolized in the liver, and the main zinc deposit is in bone. Measurement of zinc in bone may be used to determine zinc status or utilization, while measurement of zinc in hair may be used for diagnostic purposes. Zinc concentrations in plasma are not reliable because zinc binds extensively to proteins.

Since zinc is mainly excreted in feces, it is the variable zinc excretion that together with absorption maintains zinc homeostasis in the body. These processes also influence the seepage of zinc from manure into the environment. Zinc absorption and bioavailability are influenced by the feed composition; not only the plant component but also high concentrations of other metals such as copper, nickel, and iron may lead to zinc deficiency. Therefore, formulations of feed need to take into account other feed components that might interfere with zinc absorption and bioavailability.

Toxicity to zinc is low in general, and tolerance to high levels of zinc has been observed. However, ruminants, especially young and pregnant animals, are more susceptible to the effects of excess zinc compared to pigs or poultry. Zinc toxicity has been observed in steers and heifers, but tolerance seems to be higher in dairy cows. Sheep may develop symptoms of excess zinc in their diet, manifesting as symptoms ranging from weakness and jaundice to abortions and stillbirth. Horses, especially young and pregnant animals, are among the most sensitive to excess zinc in the diet and react with lameness and osteochondrosis. Pigs are among the most zinc-tolerant animal species, and it has been shown that even though they can exhibit symptoms of toxicity, it depends on the exposure time to excess zinc diet and the relation of other components of the diet such as calcium. Therefore, in some countries, high zinc concentrations are allowed for therapeutic purposes (12).

Zinc is naturally occurring in agricultural produce, but the levels vary greatly. The content of zinc in natural products depends on the zinc concentration in the soil and the conditions that might influence zinc uptake by plants. Some zinc may also be added due to deposition on plants, soil contamination, and processing. Similar to copper, the available concentrations in raw materials vary and are relatively low in relation to the animal requirements. Therefore, zinc supplementation to animal feed is common (12). However, supplementation has been at relatively high levels, and several organizations have indicated that these levels could be lowered to reduce the impact on environment (12, 13).

For requirements in different animal species and age groups as well as current and proposed supplementation levels, please consult the data in Table 2 (12).

Zinc additives

For supplementation of feed, a number of additives are utilized, including zinc oxide, zinc chloride monohydrate, zinc sulfate monohydrate, zinc sulfate heptahydrate, zinc chelate of amino acids hydrate, zinc acetate dihydrate, and zinc lactate trihydrate, which fall under EEC number E6 (3). In regard to therapeutic use, the products used are similar but require a prescription if regulation permits. In the European Union, this usage has been allowed in some countries but it will be discontinued because the Committee for Veterinary Medicine Products at the European Medicines Agency has recommended that market authorizations be withdrawn for existing products, and new products will be refused largely due to environmental risks and acknowledgement of the risk for of coselection (14).

Therapeutic use of zinc

Zinc may be used in some countries under prescription for treatment and metaphylaxis of diarrheal disease in young animals such as postweaning piglets. Zinc supplements are normally given in the form of premixes used in feed or drinking water. The most relevant zinc forms for this practice are zinc oxide, zinc chloride, and zinc sulfate. For example, in Denmark and Belgium, veterinarians have until recently prescribed zinc oxide supplementation of up to 2,500 ppm for weaning pigs for up to 14 days after weaning, which is much more than the maximum concentration allowed in pig feed—250 ppm in the European Union, with the usual concentrations ranging between 50 and 125 ppm (15–17). Similarly, in other countries around the world, the therapeutic use of zinc is relatively widespread (18), and doses for therapeutic use are between 1,000 and 3,000 ppm for up to 2 to 3 weeks (19). This is contradictory to the fact that these high doses given over a long period may cause toxicity. The use of therapeutic concentrations of zinc has been considered beneficial for the reduction and prevention of postweaning diarrhea in weaners and postweaning scouring (20). However, the growth promotion effects are not well understood (12).

According to the risk assessments released at the end of 2016, the benefits of the therapeutic use of zinc compounds in animal production do not outweigh the

TABLE 2 New proposed maximum limits of zinc in complete feed[f]

Target species, animal category	R[a]	1.5 × R	Background	NPMC[b]	CAMC[c]
Chickens for fattening, reared for laying	40–50	60–75	30	100	150
Laying hens, breeder hens	45	67.5	30	100	150
Turkeys for fattening, 0–8 weeks of age	70	105	30	120	150
Turkeys for fattening, from 8 weeks of age onward	50	75	30	120	150
Other poultry	40, 60, 60	80	30	100	150
Piglet below 11 kg weight	100	150	30	150	150
Piglet, weaned above 11 kg weight	80	120	30	150	150
Pigs for fattening	60	90	30	100	150
Sows	50–100	75–150	30	150	150
Calves, milk replacer	40	60	30	100	200
Cattle for fattening	35[d]	53	30	100	150
Dairy cows, dairy heifer	44[d]	66	30	100	150
Sheep	40[d]	60	30	100	150
Goats	31[d]	47	30	100	150
Horses	44[d]	66	30	100	150
Rabbits	70	105	50	150	150
Salmonids	50	75	60	150	200
Other fish	20	30	60	100	200
Dogs		100[e]	70	150	250
Cats		75[e]	70	150	250

[a]R, requirement.
[b]NPMC, newly proposed total maximum contents of zinc in complete feed (13).
[c]CAMC, currently authorized maximum content of zinc in complete feed (3).
[d]Adjusted from dry matter to complete feed with 88% dry matter.
[e]Allowance, taken as 1.5 times the requirement.
[f]Source: reference 13.

environmental risks, and additionally, there is an acknowledged risk for coselection of antibiotic resistance; therefore the Committee for Veterinary Medicine Products has recommended the withdrawal of existing market authorizations for veterinary products containing zinc and refusal of approval for new formulations. This recommendation has been backed by the EU Commission, and phase-out is expected in the coming years. Therefore, such products will not be available for therapeutic purposes in the European Union member states in the future (14).

Other Metallic Compounds for Therapeutic Purposes

Arsenic is a metalloid compound that is naturally present in rocks but is present in low amounts in soil and water. The presence of arsenic in the environment may also occur due to human industrial activity (mining, burning nonferrous metals, burning fossil fuel, or use in fertilizers, pesticides, insecticides, etc.). Unlike copper and zinc, arsenic is not thought to be an essential trace element, and therefore supplementation in feed is not considered for nutritional purposes. Arsenic and inorganic arsenic compounds are considered toxic and carcinogenic, while other arsenic-derived compounds are considered either "possibly carcinogenic to humans" or "not classifiable as to their carcinogenicity to humans" (21). However, there have been reports of beneficial effects in cancer therapy, and therefore arsenic might have some unexpected beneficial effects (22). Additionally, exposure to inorganic arsenic in drinking water has been linked to a reduction in breast cancer (23).

Organic arsenical compounds are active against coccidia and other parasites, so they have been used in poultry production to reduce coccidial infection and promote growth. However, even though roxarsone, arsanilic acid, and nitarsone are organic compounds and are thought to be less toxic, it was noticed that their usage led to the increased occurrence of inorganic arsenic residues in liver detected by improved analytical methods, and they have therefore been under scrutiny. These compounds were widely used in the United States and have only recently been banned, starting with a ban on roxarsone, carbasone, and arsanilic acid in 2013, which was followed by a ban on the use of nitarsone for histomoniasis in turkeys in 2015. However, the widespread use and subsequent exposure to arsenic in humans was determined through a significant association between arsenic content in urine and poultry intake in a study performed in the United States between 2003 and 2010 (24). Because these compounds are now banned in the United States and the European Union, it is important to focus on other places in the world where this type of supplementation is still ongoing (25, 26), both to avoid consumption of arsenic by humans and to avoid pollution of the environment around farms (27).

Effect of Supplementation on Microflora and Organs

Copper

It has been generally assumed that copper does not affect the normal bacterial flora, but an extensive literature study found that in piglets and growing pigs, low copper concentrations (<50 mg/kg feed) affected the microbiota in the gastrointestinal tract. Similarly, 100 to 250 mg/kg copper sulfate was found to significantly change the microbial community structure. In poultry (broilers), supplementation with copper, even at low concentrations (<50 mg/kg feed), appeared to affect the microbiota in the gastrointestinal tract. In particular, the *Clostridia* population seemed to be affected even at low concentrations. At higher concentrations (>200 mg/kg feed), inorganic or organic bound copper also appeared to affect the population of lactobacilli and coliforms in broilers (28). Furthermore, the reduction of the pH of the gizzard content may cause severe gizzard erosion (4).

The risk assessments made for copper salt supplements (including copper carbonate) in relation to human consumption have concluded that a maximum residue level is not needed because it seems unlikely that copper salts, when used according to the specifications in veterinary medicine settings, are likely to cause harm in humans through consumption of residues contained in food of animal origin (29).

Zinc

The main consensus regarding zinc supplementation is that it does not appear to have major consequences on the microflora and organs. However, recent studies demonstrated that the effect observed might be due to the regulation of factors related to the immune response leading to a reduction of stem cell factor expression in the small intestine and causing a reduction in the number of mast cells and histamine release. These findings may have important implications for the prevention of weaning-associated diarrhea in piglets (30). However, a number of studies suggest that changes in the stress response may also indicate a more profound effect of therapeutic zinc supplementation that might affect the immune response (31).

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maximum residue level is not needed because it seems unlikely that zinc salts, when used according to the specifications in veterinary medicine settings, are likely to cause harm in humans through consumption of residues contained in food of animal origin (32).

Usage Data and Distribution

High amounts of zinc additives are used for medical purposes in piglet production in Europe. As an example: European pig production was 248 million heads in 2008 (EURO-25 in 2008); 30% of these were produced in Denmark and Belgium. A percentage piglets were most likely given feed containing 2.5 g Zn/kg in the first 14 days after weaning. If daily feed consumption is 0.4 kg in the first week and 0.5 kg in the second week, the zinc consumed amounts to approximately 1,312 tons per year (13).

A similar scenario can be found in the United States. Here, as in Europe, zinc is fed to newly weaned pigs at high dietary levels of 2.0 to 3.0 g/kg feed (19). Assuming a mean of 2.5 g/kg and the same feed intake during the first 14 days after weaning as stated above for Europe, the 113.8 million pigs raised for slaughter in 2016 (33) would have consumed approximately 1,800 tons of zinc.

Taking Denmark as an example of a major pig-producing country using zinc by prescription, it was observed that the consumption of zinc oxide prescribed by Danish veterinarians has increased over the past 10 years, from ~150 tons in 2005 to ~500 tons in 2014. In this time period, the total consumption of zinc oxide tripled, while pig production increased by only 14% (15, 20). Another example of a European country using zinc oxide for therapeutic purposes is Belgium, where the use of zinc oxide in therapeutic doses in piglets administered for 2 weeks after weaning has been allowed only since September 2013. In Belgium, zinc oxide has become an alternative for colistin, which was previously used in weaned piglets. The data for 2014 and 2015 show a substantial increase in the use of this metal compound, with 81,964 kg and 87,199 kg of zinc oxide, respectively, consumed in Belgium. Simultaneously, the Belgian authorities noticed a decrease in the use of polymyxins (colistin) from 5.8 mg/PCU (population correction unit, which is defined as a measure of biomass, where 1 PCU is equivalent to 1 kg of biomass of livestock or slaughtered animals) in 2012, when zinc oxide was not available, to 3.3 mg/PCU in 2014, and a further 51% decrease was noticed in 2015 (16, 34). As mentioned above, the market authorizations for zinc products will be withdrawn for use for therapeutic purposes following the decision taken by European Medicines Agency, and therefore we expect a sharp drop in the consumption of this compound in the European Union. Because the use of zinc compounds is not exclusive to Europe and is quite widespread in North America (18) and other regions, some therapeutic use might remain, depending on local regulations. Reducing the amount of zinc to a dietary requirement of 100 mg/kg for newly weaned pigs would reduce the yearly zinc consumption in pig farming by over 90% (35).

It has been shown that high zinc supplementation after weaning until pigs reached 12 kg, followed by high copper supplementation until they reached about 25 kg in body weight was both cost-effective and promoted the best growth. Suggested therapeutic copper levels of 100 to 250 mg/kg are 95 to 98% above the dietary requirement for this age group (19, 35).

Environmental Concerns from Use of Metals in Agriculture: Metal Ecotoxicity

The extensive supplementation of feed with these compounds and the additional practices of therapeutic and/or growth promoter usage are not innocuous. Most of these compounds are excreted by the animals and end up in the environment, where their accumulation can have major consequences (36) because these are not degradable and will increase the concentration in the environment, especially in soils amended with slurry/manure from animal production (37). For example, Denmark, which is one of the largest pig-producing countries in the world and uses metals in animal production, has maintained a national monitoring program of heavy metals in the environment for the last 28 years to better understand the effects of these practices on the environment. The values and analyses published in 2016 indicate that the use of pig slurry has led to a significant increase in the concentrations of copper and zinc in soil (38).

Furthermore, the data show that 45% of the analyzed soils have reached zinc levels above the predicted no-effect concentrations (PNEC), while for copper, only one soil sample was above the PNEC. Continued agricultural use of zinc is expected to increase the levels, and larger areas will have soil exceeding the PNEC. Furthermore, the authors noted that the current situation raises concerns regarding the aquatic environment because the current use of zinc and copper in agriculture might lead to leaching of these metals into water and negatively affect aquatic species (37, 38).

In addition, at the European level, zinc is one of the compounds studied as part of risk assessments regarding the environmental pollution of heavy metals. These reports focus on aspects related to manufacturing of zinc products. A number of studies looking at the accumu-

lation of zinc in soils due to agricultural use are also mentioned, and although these studies are relatively different in scope and methodology, they all agree that zinc will accumulate in soils in a significant manner, and increasing areas will exceed the critical concentrations. However, the time this will take varies and therefore needs to be assessed at the regional level (39). Taking into account these aspects and also acknowledging the risk for coselection, the European authorities have been alerted and are conducting ongoing collection of information for environmental risk assessment of heavy metals as part of veterinary medicinal products. The Committee for Veterinary Medicine Products has therefore concluded that the benefits do not outweigh the risks, and the risk for the environment justifies withdrawing the market authorizations for the therapeutic usage of zinc compounds (34).

Alternatives

As mentioned above, zinc and copper cannot be replaced since they are essential trace elements, but the concentrations can be reduced and adjusted to the essential requirements to avoid overuse. Similarly, in the future, the use of those metals at high concentrations for therapeutic purposes will be discontinued at least in the European Union, not only because of the added pressure leading to selection of bacterial resistance to copper and zinc and subsequently coselection of antibiotic-resistance genes, but mainly because of environmental concerns. Antimicrobials are alternatives for this therapeutic use but are not viable options since there are a number of ongoing efforts to reduce antimicrobial consumption in animals. Replacement should go hand in hand with improvement of management practices to reduce the need for the use of metals or other compounds during the weaning period.

DETECTION OF HEAVY METAL RESISTANCE

In recent years, the identification and/or characterization of strains of bacteria of animal origin that have reduced susceptibility or increased tolerance to heavy metals has gained a lot of attention, and therefore, an increasing number of studies include heavy metal susceptibility or tolerance testing. The Web of Science database was searched systematically for articles between January 2005 and December 2016 using the search terms "heavy + metal+ resistance+ animal" and "heavy+ metal+ resistance+ bacteria+ MIC". This resulted in the retrieval of 278 articles, of which only 23 reported MIC data on bacterial isolates of animal origin, including fish. These studies were reviewed and revealed a number of weaknesses, which can impact the quality of the results or the conclusions made. Here, we will highlight the major pitfalls that make comparisons of studies challenging.

Several methods, such as disc diffusion, agar dilution, and microbroth dilution, have been used in metal tolerance assays. Disc diffusion, the use of paper discs impregnated with a specific heavy metal salt concentration placed on agar plates spread with bacteria, was rarely used (only two studies [40, 41]). Agar and microbroth dilution were the most common methods to determine an isolate's MIC to a particular heavy metal. Both methods are based on a dilution series of the heavy metal that is incorporated into either molten agar or liquid broth. The methodology is similar to that used for antibiotic susceptibility testing, where the dilution series is typically 2-fold dilutions rather an exponential serial dilution. While most studies use the lowest concentration at which there is inhibition of visible growth, a few studies use optical density measurements to determine the presence of bacterial growth (42, 43).

As previously described by Hasman et al. (44), the choice of media and the pH of that media are crucial factors that can influence the detection of metal resistance levels. For example, certain components in complex media can sequester free metal ions, reducing the concentration available for the bacteria. Therefore, it is advisable to use standardized media such as Mueller Hinton, which has been extensively validated for use in antibiotic susceptibility testing. Of the 23 studies included in this analysis and described using agar dilution (n = 18), up to six different media were used. While Mueller Hinton agar was the most common, brain heart infusion agar (45), Luria Bertani agar (46), nutrient agar (47, 48), and tryptic soy agar (49, 50) were also used. As previously mentioned, the pH of the medium can influence the detection of metal resistance. For example, pH values above 5.5 can cause zinc sulfate in complex media to precipitate, thereby reducing the concentration of the metal that the bacteria have to encounter. Furthermore, addition of metals into the medium can change the final pH, which can affect bacterial growth. Therefore, it is strongly recommended that after supplementation of the metal, the media is adjusted accordingly to ensure optimal bacterial growth and avoid misleading results. Only seven articles noted that pH adjustments were performed. However, even in studies where adjustments were reported, the pH value was not always consistent; e.g., for $CuSO_4$ in Mueller-Hinton agar, a range of pH values were used by the different studies (pH 7, 7.2, and 7.4) (51–55). It is recommended

that when using Mueller Hinton agar or broth, the adjusted pH should be as follows: zinc chloride, pH = 5.5; copper sulfate, pH = 7.2; sodium arsenate, cadmium acetate, and silver nitrate, pH = 7.4 (51, 56).

Studies wanting to categorize bacterial isolates as susceptible or resistant to a particular heavy metal on the basis of their MICs or inhibition zone diameters require approved interpretive criteria. There are no clinical breakpoints for heavy metals. Therefore, epidemiological cutoff values are based on previous studies reporting the MIC distributions for a particular bacterial species. Five studies used a control strain, e.g., the laboratory strain *Escherichia coli* K12 or C600, and strains were considered resistant if the MIC values exceeded that of the control strain (57–61). While this could be a reasonable approach to characterize reduced heavy metal susceptibility among bacterial isolates, the control strain should belong to the same bacterial species as the test isolates. Akinbowale et al. (59) used *E. coli* K12 to describe resistance in *Aeromonas* spp. and *Pseudomonas* spp. While all isolates are Gram negative, there are intrinsic differences between the species that can affect the interpretation of the results.

Increased metal tolerance to zinc and copper has been described in many bacterial species. Initially, these resistant bacterial species were detected among environmental bacteria isolated from areas with high concentrations of heavy metals. Due to the use of heavy metals in food-producing animals and the association of heavy metal resistance with antimicrobial resistance as a result of co-selection, many studies have investigated the levels of reduced susceptibility (Table 3) and the associated genes in a number of bacterial species isolated from food-producing animals (Table 4).

MECHANISMS OF METAL HOMEOSTASIS AND RESISTANCE

Metal homeostasis is a delicate balance of ensuring that a cell's requirement for essential metals is met to ensure proper cell function while at the same time limiting the amount of metal in the cell to avoid toxicity. The ability to balance the influx and efflux of metals, metal homeostasis, is determined by the presence or absence of metal transport systems, which can depend on an organism's living environment and therefore its exposure to metals.

Copper

As in other soft metals, the bioavailability of copper changed with the appearance of oxygen in the environment during the Great Oxidation Event. Copper became available due to the increased oxidation of copper sulfides and the higher solubility of Cu(II) (62, 63). With

TABLE 3 Bacteria of animal origin for which copper and zinc resistance has been described

Metal	Bacterial isolate(s)	Animal origin	References
Copper	Actinomycetes	Goat	236
	Aeromonas hydrophilia	Fish including shellfish	48
	Citrobacter spp.	Fish, pigs	47, 144
	Enterococcus faecalis, E. faecium	Chicken, cattle, pigs	56
	Escherichia coli	Chicken, cattle, pigs	56, 144, 145, 155
	Klebsiella pneumoniae	Prawns	237
	Lactobacillus spp.	Pigs, cattle, poultry	238
	Salmonella spp.	Chicken, cattle, pigs	56, 144, 156
	Serratia marcescens	Fish including shellfish	48
	Staphylococcus aureus, S. hyicus	Chicken, cattle, pigs	56
	Streptomycetes	Goats	236
Zinc	*A. hydrophilia*	Fish including shellfish	48
	E. faecalis, E. faecium	Chicken, cattle, pigs	56
	E. coli	Chicken, cattle, pigs	56
	K. pneumoniae	Prawns	237
	Lactobacillus sp.	Pigs, cattle, poultry	238
	P. mirabilis	Fish including shellfish	48
	P. aeruginosa	Various	49
	Salmonella spp.	Chicken, cattle, pigs	56
	S. marcescens	Fish including shellfish	48
	S. aureus, S. hyicus	Chicken, cattle, pigs	56
	Vibrio cambelli, V. harveyi, V. mimicus, V. ordelli	Fish including shellfish	48

TABLE 4 Copper- and zinc-resistance genes identified in bacteria from livestock

Heavy metal	Heavy metal-resistance gene	Bacterial species	Animal origin	References
Copper	*pcoA*, *pcoD*	*Salmonella enterica* serovar Mbandaka *S. enterica* serovar Derby *S. enterica* serovar Heidelberg *S. enterica* serovar Typhimurium *S. enterica* serovar Worthington *S. enterica* serovar Rissen *S. enterica* serovar Agona *S. enterica* serovar Senftenberg *S. enterica* serovar London *S. enterica* serovar Ohio *Escherichia coli* *Histophilius somni*	Pigs, cattle, poultry	53, 146, 239–242
	copB	*Staphylococcus aureus* *Staphylococcus epidermidis* *Staphylococcus haemolyticus* *Staphylococcus hominis* *Staphylococcus lentus* *Staphylococcus pasteuri* *Staphylococcus rostri* *Staphylococcus sciuri* *E. coli*	Pigs, cattle, poultry	199, 240, 243
	cueO	*E. coli*	Poultry	240
	cusC	*E. coli*	Poultry	240
	mco	*S. aureus*	Pigs	243
Zinc	*czrC*	Mainly associated with methicillin-resistant staphylococci: *S. aureus* *Staphylococcus hyicus* *Staphylococcus epidermidis* *S. haemolyticus* *S. hominis* *S. lentus*	Pigs, cattle, poultry, meat from these animal species	51, 199–203, 244, 245
	czcD	*S.* Typhimurium *S. enterica* serovar Infantis	Pigs	53
	zntA	*E. coli*	Poultry	240

life initially evolving under anoxic conditions and the limited availability of copper for cells, prokaryotes utilized a limited number of copper-containing enzymes. Copper homeostasis mechanisms evolved to protect cells from increasing copper availability and its cytotoxicity due to Cu(I) attacking Fe-S clusters and the generation of reactive oxygen species under aerobic conditions (64–66).

Copper uptake

In contrast to eukaryotes, prokaryotes use a small number of copper-containing enzymes that are, with the exception of plastocyanin and caa_3-type cytochrome oxidase, located in the periplasm and cytoplasmic membrane. Therefore, very few specific copper uptake systems have been identified in bacteria. In *Bacillus subtilis*, the protein YcnJ shows homology to CopCD, appearing to be a fusion of the two proteins, and was shown to be involved in copper uptake. Expression of *ycnJ* is regulated by the repressor YcnK (67, 68). While common in the genus *Bacillus*, *ycnJ* does not seem to be present in other members of the *Firmicutes*. *Bacillus cereus* produces a copper-chelator (coproporphyrin III) that might be involved in copper uptake (69).

Gram-negative bacteria face the additional challenge of an outer membrane to take up required nutrients. In *Methylosinus trichosporium*, *E. coli* and *Pseudomonas aeruginosa* porins have been found to play an important role in copper uptake, allowing the metal ion to enter the periplasm and therefore being able to interact with the transporters in the cytoplasmic membrane (70–72). From the periplasm, copper can enter the cell via several mechanisms. ZupT, a member of the ZIP family in *E. coli*, was shown to have a broad substrate spectrum also taking up Cu(II) (73, 74). Since members of this protein family are ubiquitously present, it can be

assumed that they can also allow copper uptake in other species as well (75). Similar to YcnJ in *B. subtilis*, some members of the *Proteobacteria* have CopCD, with CopC being a periplasmic copper-binding protein and CopD a cytoplasmic membrane transporter. Overexpression of *copCD* increases the cells' copper sensitivity, indicating an uptake system (76). In several Gram-negative bacteria, copper chelators (chalkophore) have been identified, and *Cyanobacteria* utilize siderophores for both copper and iron uptake (70, 77–82).

Chromosomally encoded copper defense systems

Due to the increased bioavailability of copper during Earth's history and the capability of Cu(I) to damage iron-sulfur clusters and catalyze production of reactive oxygen species, organisms had to develop strategies to deal with the toxic effects of copper to cells. While the targets of copper toxicity are mainly restricted to the cytoplasm in Gram-positive bacteria, Gram-negative bacteria face the additional challenge of protecting their periplasm.

Since copper often enters the bacterial cell in an unspecific manner (utilizing other metal-uptake systems), bacteria cannot limit the amount of copper that enters the cytoplasm. Therefore, they had to develop mechanisms to expel excess cytoplasmic copper.

Widespread transporters utilized for copper efflux are members of the P_{1B-1}-ATPase subfamily [Cu(I) transporters] of P_{1B}-ATPases (formerly CPx-ATPases for the CPx-sequence motif in transmembrane helix VI) (83–90). In bacteria, the first copper-transporting ATPases were identified in *Enterococcus hirae* (91–95). *E. hirae* encodes two proteins, CopA and CopB, with one, CopB, belonging to the "classical" P_{1B-1}-ATPases removing excess Cu(I) from the cytoplasm, while CopA belongs to the FixI/CopA2-like ATPases that are important for proper assembly of membrane-bound Cu-containing enzymes (85, 87, 96). P_{1B}-ATPases have been identified to be involved in copper resistance in several Gram-positive bacteria (97, 98).

Similarly, in Gram-negative bacteria, P_{1B-1}-ATPases have been shown to be involved in expelling excess copper from the cytoplasm. The best characterized are the chromosomally encoded copper homeostasis systems from *E. coli*, including *copA* encoding the P_{1B}-ATPase CopA (99–101). In *E. coli*, *copA* is part of the CueR-regulon. The repressor CueR binds Cu(I) at zeptomolar concentrations and regulates the expression of *copA* and *cueO* (100, 102–105). While CopA in *E. coli* expels Cu(I) from the cytoplasm (106, 107), Cu(I) would now still be in the periplasm to cause damage to the cell. This necessitates CueO, which is a periplasmic multicopper oxidase that has been shown to oxidize Cu(I) into the less toxic Cu(II). CueO also oxidized enterobactin, enabling further sequestration of copper (108–116). The presence of genes encoding homologs of CopA and CueO is widespread among members of the *Enterobacteriaceae*, and this two-pronged approach appears to be a common strategy for copper detoxification (under "normal" aerobic circumstances) (49, 117–121). However, for CueO to be active, the organism requires the presence of oxygen. In the digestive tract of humans and/or animals, limited or no oxygen is available [and Cu(I) is also more stable than in an oxygenated environment]. Therefore, other strategies to keep the periplasm safe from copper toxicity need to be in place (99, 122). In *E. coli*, the CBA transport complex CusCBA is involved in Cu detoxification of the periplasm in the absence of CueO (99, 100, 123). Expression of *cusCFBA* is regulated by the two-component regulatory system CusRS, with CusS being a membrane-bound sensor kinase that detects periplasmic copper and relays the information to the cytoplasmic response regulator CusR, regulating *cusCFBA* expression (124, 125). It was found that in an anaerobic environment, expression was induced at much lower copper concentrations than in the presence of oxygen (100). The CusCBA complex is composed of the resistance nodulation cell division (RND)-protein CusA in the cytoplasmic membrane, the outer membrane factor (OMF) CusC in the outer membrane, and the membrane fusion protein CusB stabilizing the complex. Three molecules of CusA form a complex creating a "vestibule," which forms a funnel to the channel formed by the CusC trimer. Cu(I) enters the funnel in the periplasm and via CusC is expelled into the surrounding medium. Cu(I) transport is energized via the proton gradient across the cytoplasmic membrane (101, 126–131). While CBA transport systems have been described for different types of heavy metals and organic compounds (including antibiotics), the presence of the periplasmic protein CusF is unique to transport systems involved in copper and silver transport (123). CusF could either act as a periplasmic chaperon delivering the metal ion to the CBA transport complex or function as a regulator of the transport (132–139). An alternative system for protection of the periplasm against copper-mediated toxicity under anaerobic conditions can be found in *Salmonella*. Here, the copper tolerance is linked to the presence of the periplasmic protein CueP. Expression of *cueP* in a *cus*-deletion strain of *E. coli* can partially restore its copper resistance under anaerobic

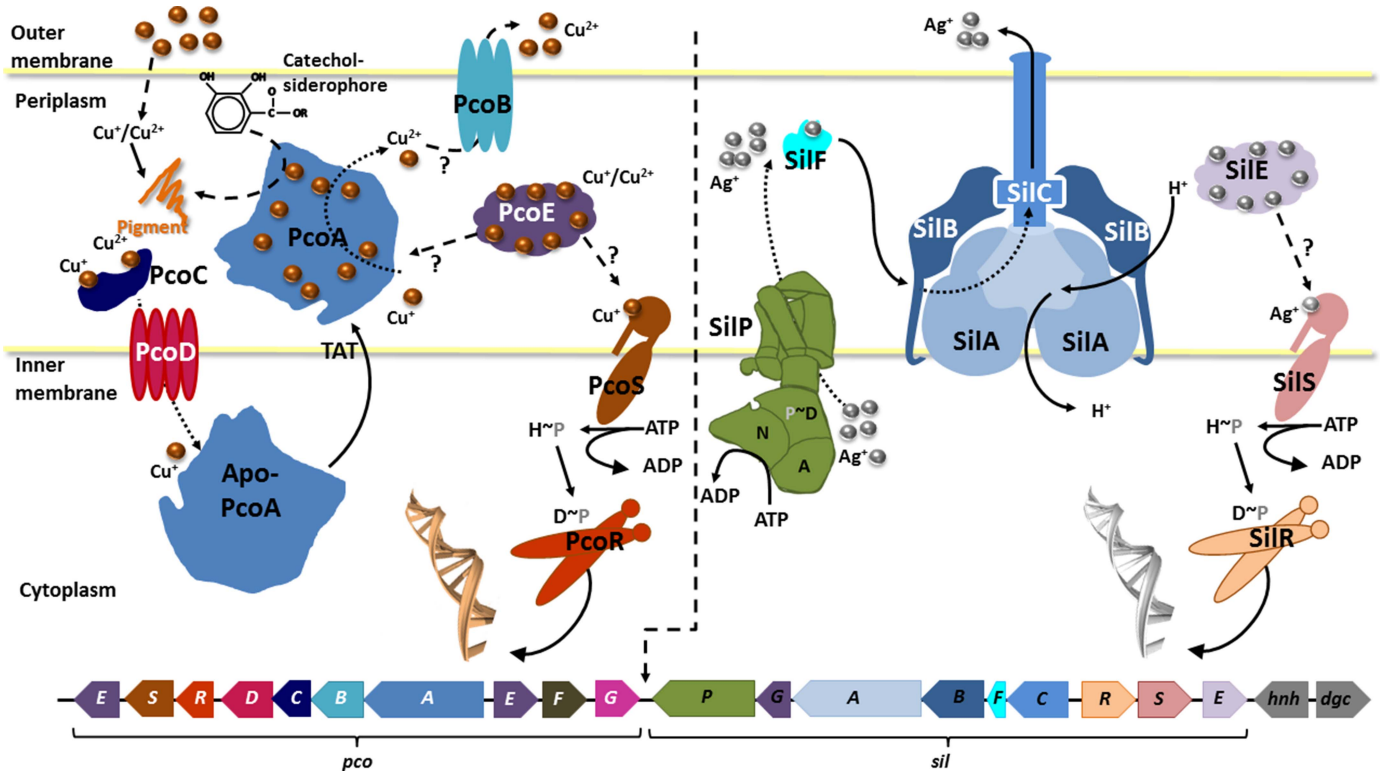
conditions (120, 140–143). CueP seems to be confined to members of the genera *Citrobacter* and *Salmonella*.

Copper resistance systems encoded on mobile elements

Genomic islands involved in copper resistance of *Enterobacteria*

A plasmid-bound copper resistance determinant in *Enterobacteria* was first described on plasmid pRJ1004 from *E. coli* (144, 145). Copper resistance encoded on this plasmid is linked to the presence of the *pco* system, which encodes a two-component regulatory system, PcoRS, as well as to the structural proteins involved in copper resistance: PcoA, a periplasmic multicopper oxidase, the outer membrane protein PcoB, the inner membrane protein PcoD, and two periplasmic proteins, PcoC and PcoE (146–149). Expression of *pcoE* is regulated via a chromosomally encoded system, CusRS (124, 149), while expression of *pcoABCD* is regulated by PcoRS (147). Periplasmic PcoE is able to sequester excess copper, giving the cell temporary protection from the toxic effects of copper (149). Periplasmic PcoC also binds copper but is able to transfer it to membrane-bound PcoD to catalyze Cu(I) uptake into the cell. Once in the cytoplasm, Cu(I) is incorporated into the multicopper oxidase PcoA, which is then exported into the periplasm via the twin-arginine translocation pathway. Once in the periplasm, PcoA detoxifies Cu(I) by either oxidation of catechol siderophores and subsequent sequestration of Cu(I) or oxidation of Cu(II) to Cu(II). Cu(II) might be removed from the periplasm via PcoB (Fig. 1) (101, 146, 148, 150, 151). Located adjacent to *pco* on pRJ1004 is a *sil* determinant (150, 151). The *sil* determinant was first identified on plasmid pMG101 from *Salmonella enterica* serovar Typhimurium and was shown to confer silver resistance (152, 153). Expression of the structural genes of the *sil* determinant is regulated by the two-component regulatory system SilRS. In the presence of silver, *silCFBA* is expressed forming the SilCBA efflux complex (similar to CusCBA), allowing for the export of Ag(I) and Cu(I) from the periplasm. SilF is a periplasmic protein (like CusF) that is able to bind

FIGURE 1 Copper fitness (or pathogenicity) island in *Enterobacteriaceae*. Genes and protein products of the enterobacterial copper fitness island composed of the *pco*- and *sil*-determinants. The genes, including their transcriptional/translational direction, are indicated below the illustration of the proposed or experimentally determined function of the proteins encoded by the *pco/sil* system. Refer to text for details. (Reprinted with permission [171].)



Ag(I) and either shuttles Ag(I) to the transport complex as substrate for export or could act as a regulatory protein of the function of SilCBA. In addition to the RND-dependent transporter SilCBA, the P_{1B-1}-ATPases SilP, the periplasmic protein SilE, and a periplasmic protein, SilG, of unknown function are encoded as part of the *sil* determinant. Like PcoE, SilE is able to bind metal ions [Ag(I) and Cu(I)] and protect the periplasm from short-term metal stress. The P_{1B-1}-ATPase SilP transports silver ions from the cytoplasm into the periplasm, from where they can be removed via SilCBA (150, 151, 153, 154). In contrast to copper, silver ions cannot undergo redox reactions, and therefore oxidation of Ag(I) as a detoxification mechanism is not an option for the cell. However, the entire *sil* resistance determinant also confers resistance to Cu(I). The overall 20-gene cluster *pco/sil* has been referred to as a copper-pathogenicity island (150) or a copper homeostasis and silver resistance island (151) (Fig. 1). The entire gene cluster has been identified in isolates of *E. coli* and *S.* Typhimurium, including *E. coli* O104:H4 from pigs fed a high-copper diet (155, 156).

Analysis of the available completed genome and plasmid sequences of *Enterobacteria* (Table 5) revealed that the *pco/sil* gene cluster is frequently flanked by *Tn*7-like elements, allowing for transfer of the gene cluster (154, 157). Recent sequencing of *E. coli* J53 (pMG101) (NCTC 50110) showed that the *pco/sil* cluster has integrated from pMG101 (152, 153) into the bacterium's genome (154). Interestingly, in some genera (*Citrobacter*, *Kosakonia*, and *Raoultella*), all of the identified *pco/sil* sequences are chromosomally encoded, while in others (*Klyvera* and *Serratia*), the sequences are carried only on plasmids. In most of the genera containing the *pco/sil* determinant, it can be carried on plasmids as well as on the bacterial chromosome, including strains that

TABLE 5 Distribution of *pco/sil* and yersiniabactin biosynthesis genes among *Enterobacteriaceae*

Genus[a]	Number of sequences analyzed[b]	Occurrence of copper/silver tolerance determinants				
		pco[c]	*sil*[d]	*pco/sil*[e]	Yersiniabactin synthesis[f]	*pco/silP* and yersiniabactin synthesis[g]
Citrobacter	60 genomes 50 plasmids	10 0	10 0	10 0	3 0	0
Cronobacter	29 genomes 22 plasmids	1 6	1 6	1 6	0 0	0
Enterobacter	167 genomes 121 plasmids	22 6	28 7	21 6	0 0	0
Escherichia	946 genomes 901 plasmids	33 7	31 11	30 7	121 0	18
Klebsiella	674 genomes 628 plasmids	2 92	3 92	2 91	82 2	30
Kluyvera	6 genomes 4 plasmids	0 1	0 1	0 1	0 0	0
Kosakonia	10 genomes 4 plasmids	1 0	1 0	1 0	0 0	0
Pantoea	66 genomes 61 plasmids	0 0	0 1	0 0	0 0	0
Raoultella	9 genomes 8 plasmids	1 0	1 0	1 0	4 0	1
Salmonella	602 genomes 369 plasmids	14 4	13 6	13 4	5 7	0
Serratia	64 genomes 27 plasmids	0 2	0 2	0 2	0 0	0
Yersinia	192 genomes 153 plasmids	0 0	0 0	0 0	33 0	0

[a]Genera of *Enterobacteriaceae* harboring *pco*, *sil*, and/or *ybt*.
[b]Number of completed genomic and plasmid sequences of respective genera available for Microbial Genome BLAST (http://blast.ncbi.nlm.nih.gov; accessed 13 September 2017).
[c]Analysis (blastn) using *pco* from pRJ1004 (GenBank accession number X83541.1 [146]) as query.
[d]Analysis (blastn) using *sil* from pMG101 (GenBank accession number NG_035131.1 -[153]) as query.
[e]Analysis (blastn) using *pco* (GenBank accession number X83541.1; [146]) and *sil* (accession number KC146966.1 [151]) from pRJ1004 as query.
[f]Analysis (tblastn) using Ybt peptide/polyketide synthetase HMWP1 (GenBank accession number AAC69588.1 [246]) as query.
[g]Number of strains harboring *pco/sil* and *ybt* with determinant being located on chromosome and/or plasmid, respectively.

harbor a copy on each location. While in most cases the entire 20-gene cluster *pco/sil* is present, some strains of *Enterobacter*, *Escherichia*, *Klebsiella*, *Pantoea*, and *Salmonella* contain only one of the two resistance determinants, *pco* or *sil* (Table 5).

Another strategy of *Enterobacteriaceae* to protect against the toxic effects of copper is the use of siderophores, especially yersiniabactin (158). The presence of the genes encoding the yersiniabactin synthesis pathway has previously been described as a virulence factor, but its presence in copper-resistant isolates indicates its importance in protection from copper toxicity. Yersiniabactin biosynthesis has been found in strains of *E. coli*, *Salmonella*, *Yersinia pestis*, and *Klebsiella pneumoniae*, including *E. coli* isolates from livestock fed high-copper diets (155, 159–163). Analysis of available completed genome and plasmid sequences of *Enterobacteriaceae* revealed the presence of the yersiniabactin biosynthesis pathways in the genera *Citrobacter* and *Raoultella*, in addition to the previously known genera. While commonly encoded on the bacterial chromosome, in some genera the yersiniabactin biosynthesis genes are located on plasmids as well (Table 5). Some strains of *E. coli* (including strains of enterohemorrhagic *E. coli* O104:H4), *K. pneumoniae*, and *Raoultella* have both the *pco/sil* gene cluster and the yersiniabactin biosynthesis determinant (Table 5).

Genomic islands involved in copper resistance of *Enterococcus*

In addition to Gram-negative bacteria, Gram-positive bacteria of the genus *Enterococcus* have frequently been isolated from livestock and shown to have high copper resistance (52, 121, 164, 165). Copper resistance in *Enterococcus* has been shown to be plasmid encoded and can be transferred via conjugation (121, 166–169). The first gene that could be linked to the plasmid-encoded copper resistance determinant in *Enterococcus* was *tcrB*. TcrB is a member of the P_{1B}-ATPases of copper transporters, but due to the presence of a histidine-rich cytoplasmic N-terminus, a CPH-motif in transmembrane helix VI, and an MSXST-motif, it would be predicted to belong to the P_{1B-3}-ATPase subfamily utilizing Cu(II) as substrate. TcrB is encoded as part of the *tcrYAZB* operon, which encodes TcrA, an additional P_{1B}-ATPase of the P_{1B-1}-ATPase subfamily with Cu(I) as the transported substrate, a cytoplasmic copper chaperon TcrZ, and TcrY, a copper-dependent regulator (150, 166, 170). Approximately 62% of *Enterococcus* strains that harbor transferrable copper resistance contained not only *tcrYAZB* but also *cueO*. This is in contrast to the strains analyzed in another study, where 22% only had *tcrB* and 16% encoded for CueO (121).

Sequence analysis of copper-resistant *Enterococcus faecalis* showed the presence of *tcrZAYB* and *cueO* as part of a larger gene cluster that also encoded a two-component regulatory system, CusRS, and an additional transcriptional regulator, CopY. Directly adjacent to *copY*, an additional P_{1B-1}-ATPase named CopA is encoded, leading to the prediction of *copA* expression being regulated by CopY. Genes encoding several putative metal-binding proteins possibly serving as metal chaperones in the copper detoxification process are also in close proximity. The genes *cusRS* are directly downstream of *cueO*, suggesting regulation by this two-component regulatory system. Since the P_{1B}-ATPases TcrA, TcrB, and CopA export copper ions from the cytoplasm, regulation of the respective genes by cytoplasmic copper-dependent regulators, CopY and TcrY, seems logical. In contrast, CueO, located just outside the cytoplasmic membrane, oxidizing Cu(I) to the less toxic Cu(II), appears to be regulated by CusRS sensing environmental copper concentrations (Fig. 2) (42, 150, 171). Other strains of *E. faecalis* and *Enterococcus faecium* contain related copper-resistance islands that vary slightly in the presence and arrangement of some of the genes in the copper-resistance island. These changes are probably at least in part due to the large number of putative transposons encoded in this region. BLAST analysis of the *E. faecium* HF50105 genome revealed a similar gene region in 5 of the 149 completed plasmid sequences of members of the genus *Enterococcus*, two belonging to *Enterococcus durans*, two to *E. faecium*, and one to *Enterococcus gallinarum*. No chromosomally encoded copper-resistance island could be identified. Further sequence analysis showed that this gene cluster is only present in the genus *Enterococcus* but not in other *Firmicutes*.

Zinc

Zinc bioavailability has changed drastically in course of Earth's history. The greater bioavailability of zinc after the Great Oxidation Event is reflected in the increased use of zinc in eukaryotes (62, 63, 172). Zinc serves as a structural or catalytic component in hundreds of proteins and is one of the most abundant transition metals in a cell. The total amount of zinc in an *E. coli* cell was determined to be in a range similar to the cellular concentrations of calcium and iron (173). However, free Zn(II) in the cytoplasm is essentially nonexistent, since the Zn(II) regulators ZntR (efflux) and Zur (uptake) respond to free Zn(II) concentrations of 10^{-16} M in

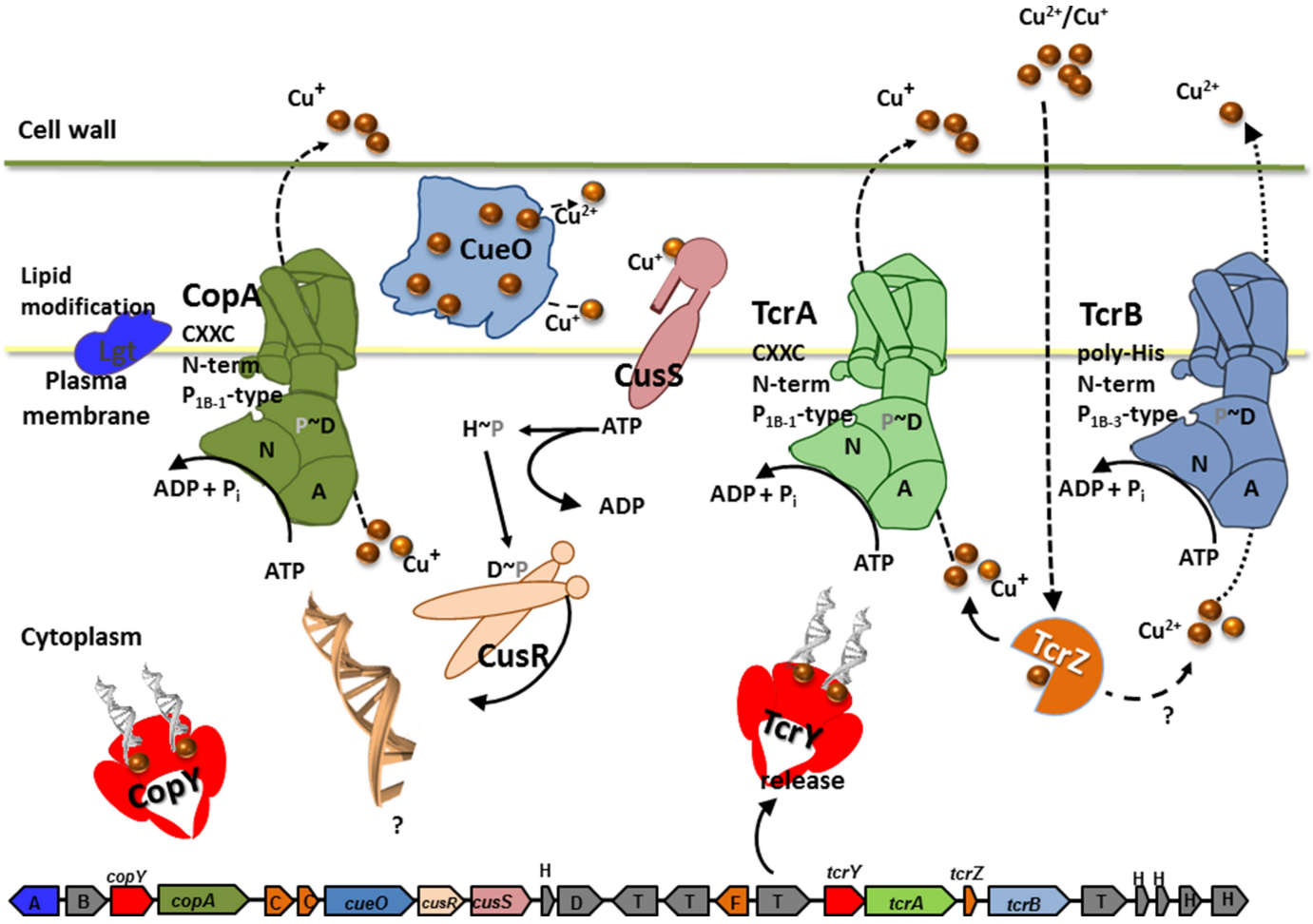


FIGURE 2 Copper fitness island in *Enterococcus*. Genes and proposed protein products of the copper island in *Enterococcus faecium* HF50105 (GenBank accession number AITS01000024). The genes, including their transcriptional/translational direction, are indicated below the illustration of the proposed function of the proteins. (Refer to text for details.) Adjacent to and separating the genes involved in copper resistance are genes encoding prolipoprotein diacylglyceryl transferase (A), integral membrane protein (B), predicted metal-binding protein/chaperone (C), hypothetical protein (H), transposase (T), and disrupted P-type ATPase (F) that have been identified. (Reprinted with permission [171].)

E. coli (173). This shows that bacteria very tightly control the uptake and efflux of Zn(II) to ensure proper cellular function while avoiding metal toxicity.

Zinc uptake

At least three systems (ABC-transporter, ZIP transporter, and phosphate-bound uptake) have been described to be involved in bacterial zinc uptake. Best understood are the systems involved in zinc uptake of *E. coli*. Under zinc-limiting conditions, *E. coli* utilizes the ABC (ATP-binding cassette) transporter ZnuABC. ZnuA is a zinc-binding protein located in the periplasm. ZnuB spans the cytoplasmic membrane and forms the channel to transport Zn(II) into the cytoplasm. ZnuC is bound to the cytoplasmic site on ZnuB, providing the energy for Zn(II)-transport via ATP hydrolysis (174, 175). Expression of the *znu* operon is regulated by Zur, which binds cytoplasmic Zn(II) and acts as repressor of *znu* by blocking the RNA polymerase from binding to the –10 region of the *znuC* promoter. Half-maximal repression by Zur *in vitro* has been shown to occur at a concentration of 2.0 ($\pm$ 0.1) $\times 10^{-16}$ M free Zn(II), indicating that the presence of any free Zn(II) in the cell effectively turns off Zn(II) uptake by ZnuABC (173, 176–178). While genes encoding ZnuABC homologs have been identified in the genomes of many bacteria, it is absent in the genome of

Cupriavidus metallidurans CH34, a bacterium originally isolated from a zinc decantation tank (179–181).

Under nonlimiting zinc conditions, *E. coli* utilizes a different uptake system, ZupT, the first identified bacterial member of the ZIP (ZRT-, IRT-like protein [ZRT, zinc-regulated transporter; IRT, iron-regulated transporter]) family of transport proteins (75, 182). Genes encoding ZupT have been identified in members of many bacterial phyla. In *E. coli*, *zupT* is expressed constitutively at a low level, and expression is therefore independent of external zinc availability. In contrast, in *C. metallidurans*, *zupT* expression is regulated by FurC, decreasing the amount of transporter under high zinc conditions and thereby limiting Zn(II) uptake (74, 181). It is not known if or how *zupT* expression is regulated in other microorganisms. While initially described as a zinc uptake system in *E. coli*, ZupT has broad substrate specificity and is also able to take up other metals, including Fe(II), Co(II), and Mn(II). Cadmium and copper also cross the cytoplasmic membrane via ZupT-mediated unspecific uptake (74). Several amino acids have been identified to contribute to ZupT's substrate specificity (73). In uropathogenic *E. coli* as well as *S. enterica*, ZupT plays an important role in bacterial virulence, especially in the absence of *znu* (175, 183).

Under nonlimiting conditions, zinc can also enter *E. coli* as a metal-phosphate complex via PitA (inorganic phosphate uptake system) (184, 185). Magnesium uptake systems have been shown to have a broad substrate spectrum and are able to transport Zn(II) in addition to Mg(II) (180, 186–188). The presence of both zinc uptake systems is very common among bacteria.

Zinc efflux

Zinc resistance in bacteria is facilitated via efflux (189, 190). At least four systems (P_{1B}-type ATPases, CDF transporters, 2-TM-GxN transporters, and CBA efflux systems) involved in transporting Zn(II) out of the cell have been identified. These systems can be encoded chromosomally or on plasmids.

The most effective transporters that export Zn(II) out of the cell are soft-metal P_{1B}-type ATPases. Members of this family of membrane proteins contain six to eight transmembrane helices and cytoplasmic domains involved in ATP binding and hydrolysis as well as metal-binding domains. Based on structural features and the resulting substrate spectra, several subfamilies can be differentiated. Zn(II) is exported by members of the P_{1B-2}-ATPase subgroup (83, 86). The best-characterized member of this group is ZntA from *E. coli* (191, 192). Members of this subgroup possess eight transmembrane helices, with a conserved CPC motif and Asp as transmembrane metal-binding sites. ZntA has a broad substrate specificity and can also transport Pb(II) and Cd(II), as can other members of this P_{1B-2}-ATPase subgroup. Additionally, other metals such as Co(II), Ni(II), and Cu(II) bind to the transmembrane metal-binding domain, However, when these metals are bound, no ATP hydrolysis or transport takes place (193). Zn(II)-transporting ATPases can be chromosomally encoded, such as ZntA from *E. coli*, or encoded on plasmids, such as CadA from *Staphylococcus aureus* (191, 194, 195). Expression of *zntA* of *E. coli* is regulated by the MerR-like regulator ZntR. In the absence of Zn(II), apo-ZntR binds to the *zntA* promoter, repressing transcription. In the presence of Zn(II) in the cytoplasm, Zn-ZntR acts as a transcriptional activator and allows for transcription of *zntA*. *In vitro* analysis of ZntR affinity to Zn(II) revealed that concentration in the femtomolar range is required for ZntR to bind. However, *in vivo* studies identified ZntR responding to cytoplasmic free Zn(II) concentrations in the nanomolar range (173, 196). On the *E. coli* chromosome, *zntR* and *zntA* are not located in the same gene cluster (197). In contrast, on plasmid pI258 of *S. aureus*, the genes encoding the P_{1B-2}-ATPase and its regulator are organized in an operon, *cadCA*. CadC is a member of the ArsR/SmtB-family and represses transcription in the absence of Zn(II), Cd(II), or Pb(II) (198). P_{1B-2}-ATPases are highly efficient at removing Zn(II) from the cytoplasm, and deletion of the respective genes usually results in Zn(II) sensitivity of the organism (189, 191). Another P_{1B-2}-ATPase of *Staphylococcus* is CzrC, which can be found in some methicillin-resistant strains encoded as part of SCC*mec* (staphylococcal cassette chromosome *mec*) (51, 199, 200). While often associated with SCC*mec*, *czrC* has also been identified in *mecA*-negative strains and on plasmids of *S. aureus* (201). Initially identified in *S. aureus*, *czrC* has also been found in isolates of other *Staphylococcus* species: *S. haemolyticus*, *S. epidermitis*, *S. lentus*, *S. hominis*, and *S. hyicus* (51, 199, 201–204). Out of the 489 completed *Staphylococcus* genomes in the NCBI database (as of 1 November 2017), 15 carry *czrC* (9 *S. aureus*, 1 *S. condimenti*, 3 *S. epidermis*, and 2 *S. simulans*), while the gene is not found on any of the 483 *Staphylococcus* plasmid sequences. While to date, the regulation of *czrC* expression has not yet been studied, it is likely regulated by an ArsR-like regulator encoded directly upstream of *czrC*.

A second family of membrane transporters linked to bacterial zinc export are cation diffusion facilitator (CDF) proteins, which contain six transmembrane helices with a C- and N-terminus located in the cytoplasm.

E. coli contains two genes encoding CDF proteins. Both transporters, ZitB and FieF, have been shown to be involved in Zn(II) transport (205, 206). While deletion of *zitB* alone does not impact *E. coli*'s ability to handle elevated concentrations of Zn(II), a double mutant defective in both ZitB and the P_{1B-2}-ATPase ZntA exhibits higher zinc sensitivity than a mutant strain defective only in ZntA (205). While no increase in zinc sensitivity was observed after deletion of *fieF*, everted membrane vesicles of *E. coli* GR362 (deficient in ZnuABC, ZupT, ZntA, ZntB, and ZitB) expressing *fieF* accumulated ^{65}Zn(II). This transport was energized via the proton gradient, as has been shown for other CDF proteins (206). Using reporter gene fusion, expression of both genes, *zitB* and *fieF*, was found to be induced by Zn(II) and to a lesser extent by Cd(II). However, later analysis showed that mRNA levels of *zitB* remain constant after addition of Zn(II) and are elevated, but still independent of Zn(II) concentration, in a *zntA* deletion strain (196, 205, 206). It has been suggested that ZitB is involved in maintaining zinc homeostasis under "normal" conditions, while ZntA confers zinc resistance. CDF proteins involved in Zn(II) transport have been identified in many bacterial species. In *S. aureus*, the chromosomally encoded CDF transporter has been named ZntA and is encoded in an operon encoding ZntR in addition to ZntA. ZntA-null mutants were zinc sensitive, and expression of the operon was Zn(II) dependent and regulated by ZntR (207, 208).

First identified in *S.* Typhimurium, ZntB is a member of the 2-TM-GxN family of membrane transporters. This transporter family is widely found in bacteria, and ZntB-like proteins have been identified in many *Proteobacteria*. Deletion of *zntB* rendered *S.* Typhimurium more sensitive to Zn(II) and Cd(II). ZntB has two transmembrane helixes with the ~270-amino acid N-terminus and the small C-terminus located in the cytoplasm. ZntB forms a pentamer, forming a cytoplasmic funnel. Each soluble domain monomer of ZntB binds three Zn^{2+}, one in the funnel, while full-length ZntB binds four Zn^{2+} (209–213). In *Agrobacterium tumefaciens*, *zntB* is constitutively expressed but does not seem to contribute to the bacterium's metal tolerance (214).

In contrast to the zinc efflux systems described so far, CBA efflux systems are protein complexes composed of a central transporter of the RND family, a membrane fusion protein, and an OMF. CBA efflux systems are involved in the transport of mono- and divalent heavy metals as well as organic compounds. The RND protein has 12 transmembrane helixes and two large periplasmic domains, which interact with the OMF. Both the RND and the OMF function as homotrimers, forming a channel starting in the periplasmic vestibule of the RND and reaching across the outer membrane via the OMF. The membrane fusion protein (hexamer) interacts with the RND protein and the OMF (190, 215, 216).

Of interest here are members of the heavy metal efflux (HME) family. Members of the HME-RND family are involved in the export of heavy metals, and several subfamilies can be differentiated based on sequence motifs and resulting metal substrates. Zn(II) transport has been credited to the HME1 subfamily (217). The best-characterized member of this family is CzcA from *C. metallidurans* CH34. The *czc* gene cluster is encoded on plasmid pMOL30 of *C. metallidurans* CH34 and allows growth in the presence of Co(II), Zn(II), and Cd (II), increasing the MIC by factors of 10, 25, and 100, respectively, compared to the plasmid-free strain AE104 (218). While export of the metal ions from the cytoplasm was assumed and data indicate transport by CzcA across the cytoplasmic membrane, the fact that a CDF protein as well as a P_{1B-4}-ATPase are encoded as part of the *czc* gene cluster in addition to the two-component regulatory system CzcRS, which monitors the metal content within the periplasm, points toward export of the majority of metal ions from the periplasm into the surrounding environment by CzcCBA. As indicated, the CzcCBA system from *C. metallidurans* CH34 is the best-characterized zinc transporting CBA system to date. The structure of CzcCBA has not yet been solved (189, 215, 216, 219–224). The first structure of an RND protein was solved for AcrB, a multidrug transporter from *E. coli* (225, 226). To date, additional structures of RND transporters have been solved, including ZneA from *C. metallidurans* CH34. ZneA was shown to actively transport Zn(II) across the membrane via conformational changes within the three ZneA protomers. ZneA interacts with ZneB (membrane fusion protein) and ZneC (OMF) to form the membrane-spanning transport complex. The structure of ZneB was also shown to undergo conformational changes during export of the metal ions, reminiscent of CusB. However, the involvement of ZneCAB in zinc homeostasis of *C. metallidurans* CH34 is not understood (227, 228). Compared to CDF and P_{1B}-type ATPases, CBA efflux systems are not as frequently present on bacterial genomes in bacterial handling of excess zinc.

Copper and Zinc Resistance Determinants: Link to Bacterial Virulence

In addition to these specific metal resistance determinants, there are numerous other genes that improve survival under elevated copper or zinc concentrations.

This overview does not go into detail regarding these rather unspecific genes, but we refer the reader to a recent excellent study as an example (229).

The involvement of zinc and copper in bacterial killing is a recent important finding that is of relevance to understanding potential public health challenges arising from feeding livestock copper and zinc supplements. Professional phagocytes such as macrophages engulf pathogens and destroy them through elevation of Zn^{2+} and Cu^{+} concentrations in the phagosome, along with other mechanisms (oxidative burst, induction of Fe^{2+}, and Mn^{2+} efflux) (230).

However, it is probable that the mechanisms macrophages use to kill infectious agents evolved in protozoa long before multicellular life arose and the need for macrophages appeared (231). This hypothesis was strengthened showing that copper (and probably zinc) poisoning is used in the model amoeba *Dictyostelium discoideum* to kill bacterial prey (232). Bacteria, in turn, were not just inert prey, and some succeeded in bypassing and resisting phagocytic cells by developing a number of strategies to avoid the protozoan killing mechanisms such as counteracting resistance systems specific for Zn and Cu and possibly other antimicrobial metals. This main concept of ongoing evolution driven by protozoan predation could explain the ongoing evolution of pathogens such as methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, and extended spectrum beta-lactamase-producing *E. coli*, all of which pose a very important public health problem (233). Farmed animals such as pigs and poultry receive additional Zn and Cu in their diets due to supplementing elements in compound feed as well as medical remedies. Enteral bacteria in farmed animals have been shown to develop resistance to trace elements such as Zn and Cu. Resistance to Zn is often linked with resistance to methicillin in staphylococci, and Zn supplementation to animal feed may increase the proportion of multiresistant *E. coli* in the gut. Resistance to Cu in bacteria, enterococci in particular, is often associated with resistance to antimicrobial drugs such as macrolides and glycopeptides (e.g., vancomycin). Since Cu and Zn have an important role in protozoan predation, these metal resistances could make survival of these bacteria much more likely. These strains could then become pathogens after transfer to humans (234, 235).

CONCLUSIONS

Copper and zinc have been widely used in livestock feed both as growth promoters and as necessary supplements. In many countries the use of these metals appears to have increased due to a ban on the use of antibiotics. They are effective growth promoters, but it is not entirely clear how this is achieved. The potential negative impacts include zinc and copper contamination in the environment but more importantly the potential of coselecting antibiotic-resistance genes and possibly generating more pathogenic strains of medically relevant bacteria.

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