Norwegian University
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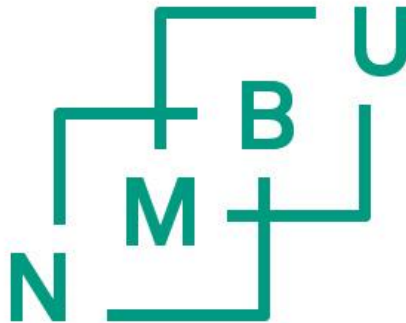
# The role of zinc and copper in the conjugative spread of ESBL-genes between *Escherichia coli in vitro*

May Linn Buberg

**The role of zinc and copper in the conjugative spread of ESBL-genes between *Escherichia coli in vitro***

by May Linn Buberg

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# Contents

## Acknowledgements

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AMR: Antimicrobial Resistance

Cu: Copper

Zn: Zinc

ESBL: Extended spectrum betalactamase

NMBU: Norwegian University of Life Sciences

WHO: World health organization

WP: Work package

HGT: Horizontal gene transfer

MGE: Mobile genetic elements

*E. coli: Escherichia coli*

MIC: Minimum inhibitory concentrations

LB-broth: Luria-Bertani broth

## Sammendrag Norsk

Antibiotikaresistens er ansett som en økende trussel for både human- og dyrehelse. Kylling i Norge blir ikke rutinemessig behandlet med cefalosporiner, likevel finner vi cefalosporinresistente bakterier i tarmen hos kylling og på kyllingkjøtt i butikkene. Norsk kylling fôres med fôr som inneholder høyere nivåer av sink og kobber enn de biologisk behøver. Flere metaller har vist å ha drivende effekt for utvikling av mikrobiell resistens. Effekten av dette har ikke blitt undersøkt nøye, og vi besitter derfor lite kunnskap om hva disse overflødige metallene gjør med spredningen av antimikrobiell resistens. Målet med denne forskerlinjeoppgaven var å undersøke effekten av sink og kobber på den konjugative spredningen av resistensgener. To *Escherichia coli* (*E. coli*) isolater samlet inn fra kyllingkjøtt gjennom NORM-VET ble brukt som plasmid-donorer da de begge er bærere av ESBL (Extended spectrum beta-lactamase) plasmider. Konsentrasjonene brukt i eksperimentene ble bestemt på bakgrunn av MIC-verdier og vekstkurver. Ved å bruke en standard konjugasjonsmodell i LB-buljong fant vi at sink og kobber reduserer konjugasjonsfrekvensen mellom *E. coli* ved økende konsentrasjoner av metaller. Stabiliteten til plasmidene ble vurdert, og vi konkluderte med at plasmidene forble stabile i mottakerbakterien gjennom flere generasjoner uten påvirkning av metallene. Til slutt analyserte vi effekten av sink og kobber på to gener som er involvert i konjugasjonsmekanismen ved bruk av qPCR. Denne studien demonstrerer at sink og kobber begrenser konjugasjonsfrekvensen mellom bakterier, sannsynligvis ved å påvirke genutrykket involvert i konjugasjonen.

## List of publications

**Buberg May Linn, Witsø Ingun Lund, L'Abée-Lund Trine Marie, Wasteson Yngvild**.

Zinc and copper reduce conjugative transfer of resistance plasmids from ESBL-producing *E. coli*

Submitted October 2019 – Under review

## Summary of the paper

Poultry in Norway are not treated with cephalosporins, yet cephalosporin resistant bacteria have been found in the chicken intestines and on retail chicken meat. Chicken receive higher amounts of Zn and Cu in their feed than what they biologically require. Various metals are shown to act like drivers for antimicrobial resistance; however, little research has been done to investigate the effect of these excess metals on the spread of antimicrobial resistance. The aim of our study was to investigate the effect of Zn and Cu on the conjugational spread of resistance genes. Two *Escherichia coli* (*E. coli*) isolates from retail chicken meat carrying extended spectrum beta-lactamase (ESBL) encoding plasmids were used as plasmid donors, and the experimental concentrations of Zn and Cu were determined by MIC testing and growth curves. By using a standard conjugation method in LB broth, we found that Zn and Cu reduced the conjugation frequency between *E. coli* in a concentration dependent manner. Plasmid stability was estimated by propagating monocultures over several generations, and we found that the plasmids remained stable in the host without any interference of the metals. Finally, we analyzed the effect of Zn and Cu on genes involved in plasmid transfer by real time qPCR. Our study demonstrate that Zn and Cu inhibit the bacterial conjugation frequency, possibly by interfering with expression genes involved in conjugation. These findings provide further insights about the conjugational spread of resistance genes in the fight against antimicrobial resistance.

## Introduction

### Background

The work presented in this thesis has been a part of the project NoResist at the Norwegian University of Life Sciences, in cooperation with the Norwegian Veterinary Institute, Nofima and Norwegian Institute of Public Health. In addition to myself, two postdoc students have been assigned to the main project. The aim of NoResist is to "Obtain knowledge on persistence and spread of antimicrobial resistance in the Norwegian food production chain which can be used to prevent, reduce or inhibit such resistance", and is divided into 5 work packages, where NMBU is responsible for WP2. Objectives are given under "Aims".

The following gives an introduction to, and a background for the topics covered by my thesis.

### Norwegian chicken production chain

Broiler production amounted 28% of total amount of meat produced in Norway in 2017 (1). Norwegian chicken live inside isolated and heated houses. They walk around freely on bedding consisting of wood shavings and have free access to food and water. The lightening is adapted to the animal`s needs, assuring that they have adequate amounts of light during the day. Normally they are slaughtered between 28-32 days of age and have a living weight of approximately 1.2 kg. They are not fed any growth promotors or antimicrobials. In 2014, the industry decided to stop using the coccidiostatic Narasin, and replaced it with a vaccine by the end of 2016. Chicken are now only fed commercially produced pelleted feed consisting only of essential nutrients (2).

### Antimicrobial resistance

Antimicrobial resistance is defined as "the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial (such as antibiotics, antivirals and antimalarials) from working against it. As a result, standard treatment become ineffective, infections persist and may spread to others." (3). The spread of antimicrobial resistance (AMR) has become a threat against human and animal health (4). Part of this resistance has its origin within the agriculture sector, and dissemination of resistant bacteria from the food production chains may be one out of several routes in which consumers can be exposed to AMR bacteria (5-9). Several risk factors have been

discovered, such as overuse and misuse, but there are still a lot of unanswered questions that needs to be addressed.

The NORM-VET monitoring program for antimicrobial resistance in the veterinary and food production sectors was established in 2000 as a part of the Norwegian governments action plan against antimicrobial resistance and has since then been coordinated by the Norwegian Veterinary Institute. Their goal is to collect information about the occurrence of AMR-bacteria from feed, animals and foodstuffs, and to overlook the trends over time, compared to the development in other countries and in relation to the situation within human medicine (10). NORM-VET detected in 2012 cephalosporin resistant *Escherichia coli* in 43% of the Norwegian broiler flocks. In addition, 32.2% of *E. coli* from retail chicken meat were categorized as cephalosporin resistant (11), and even higher numbers were registered in Denmark and Sweden (12). The NORM-VET findings were surprising as the use of antimicrobial agents in the poultry production in Norway is limited, and among the lowest in Europe (11, 13).

Third and fourth generation cephalosporin have been defined as critically important antimicrobials by the WHO (14), however, extended spectrum beta-lactamase (ESBL) producing bacteria have previously been isolated from a variety of animal species in different European countries (15-19). In 2015, the Norwegian Scientific Committee for Food and Environment concluded in their "Assessment of antimicrobial resistance in the food chains in Norway" that the probability of human exposure of ESBL-producing *Enterobacteriaceae*, and their respective corresponding genes, from live poultry and poultry meat was considered non-negligible (20). Such exposure of resistant bacteria may result in consumers becoming carriers of resistant strains of *E. coli,* if these bacteria colonize the human gut (6, 21-23). In the case that they at later occasions cause disease, their resistance characteristics may lead to failure of treatment and increased mortality (24, 25).

The two plasmids included in this work both holds the $bla_{CMY-2}$ gene. This encodes a *betalactamase*, which is an enzyme providing resistance against third generation cephalosporins and most commonly occurs on plasmids. Bacteria carrying this will express an AmpC phenotype, meaning that they can hydrolyze penicillins, as well as broad-spectrum cephalosporins such as ceftazidime or cefotaxime (26).

## Zinc and Copper

Zinc (Zn) and copper (Cu) are commonly found in soil, water, plants and in manure from various farm animals, including chicken (27). The latter may have beneficial fertilizing properties. Zn and Cu are important trace elements for plants but may be of environmental concern in larger quantities affecting groundwater, surface water and aquatic animals (28-31). Zn and Cu are also important elements in the cellular metabolism; they allow many critical enzymes to function properly and are essential for wound healing, protein synthesis, and maintaining the strength of the skin, blood vessels, and various tissues in the organism (32). Chicken, as other mammals, need to consume small amounts of metals through their diet. However, broiler chicken receive feed containing larger amounts of Zn and Cu than what they biologically require (33). The excess amount of metals gets disposed in the chicken manure and may end up in soil if this manure is used as fertilizer. Zn and Cu have antimicrobial effects; the bacterial toxicity of Zn may be due to their chemical affinity for thiol groups in biomolecules and Cu toxicity is based on production of hyperoxide radicals and their interactions with cell membranes. However, little is known about the effects of these trace elements on the spread of other antimicrobial resistance traits within the food production chain (34, 35). The presence of Zn and Cu may act as drives for development of AMR in exposed bacteria, but data on the required dose and time exposure are lacking (36).

## Horizontal gene transfer – conjugation

In addition to the vertical evolvement of genetic material by division, bacteria exhibit the benefits of exchanging genetic information horizontally. These mechanisms are known as transduction, transformation and conjugation. Conjugation allows bacteria to spread genetic information across diverse bacterial phyla by the use of mobile genetic elements (MGE) (37). Examples of MGEs include transposons, bacteriophages and plasmids (38). Hansen et al showed that plasmid horizontal transfer is more important than clonal dissemination for transmission of CMY-2 mediated cephalosporin resistance between animals and humans (39).

Bacteria acquire resistance genes against antimicrobials and metals on mobile genetic elements (40).  This facilitates a mechanism of co-selection, commonly divided into co-resistance (two or more resistance genes present on the same genetic element) and cross-resistance (the same mechanism providing resistance against several substances). These are mechanisms where

selection pressure from metals may cause further dissemination of other antimicrobial resistance genes in the absence of antimicrobials (41, 42).

In Norway, conjugative ESBL encoding plasmids have been frequently discovered in bacteria isolated from broiler chicken, implicating that these animals are a potential reservoir for cephalosporin-resistant *E. coli* (43). The factors involved in the maintenance of resistance plasmids through the food chain are still unknown.

## Knowledge gaps

This thesis covers a broad spectrum of topics and therefore presents several knowledge gaps. For example; little is known about the effects of trace elements on the spread of antimicrobial resistance within the food production chain. Previously, the stability of the described plasmids encoding plasmid stability systems had not been confirmed. Zn and Cu may act as drivers for spread of AMR in exposed bacteria, independent of co- or cross-resistance, but data on the required concentration and time exposure for this effect are lacking.

## Aims

The Research track program thesis has been a part of the project «NoResist – Combating antimicrobial resistance in the Norwegian food production chain». NoResist address the need for more knowledge based countermeasures against the development and dissemination of antimicrobial resistance in Norwegian food production chain (44). The focus has been on the poultry production chain, which internationally has been associated with a challenge of antimicrobial resistant (AMR) bacteria.

The aim of my project was to study the influence of zinc and copper on the transfer of AMR plasmids by addressing the question; "Do zinc, copper influence the transfer of AMR genes?"

The aim was accomplished through the following objective: study the effect of Zn and Cu on conjugation *in vitro* of $bla_{CMY-2}$ carrying plasmids in *E. coli* collected from retail chicken meat.

## Hypothesis

We hypothesized that excess levels of Zn and Cu in the gut environment acts as resistance drivers by promoting transfer of resistance plasmids in *E. coli* by increasing their conjugation rate.

## Materials and methods

This section gives a very brief summary of the sample materials and methods used in the study. Methodological considerations will be addressed in the discussion. The submitted manuscript provides a detailed description of materials and methods, including manufacturers, temperatures and metal-concentrations.

## Bacterial strains

Two *E. coli* isolates from retail chicken meat were used as plasmid donors in the conjugation experiments. The strains were collected through the NORM-VET monitoring program for antimicrobial resistance in the veterinary and food production sectors in Norway in 2012 (11). Both strains harbored a plasmid carrying the *bla*$_{CMY-2}$ gene (45):

- *E. coli* 2012-01-1292 (pNVI1292/IncK)
- *E. coli* 2012-01-2798 (pNVI2798/IncI1)

The *E. coli* DH5α strain, resistant to nalidixic acid (Nal$^R$), was used as recipient.

## Cecum samples

To determine the *in vivo* concentrations of Zn and Cu in the chicken cecum intestine, ten 25-day old chicken were collected from a Norwegian conventional chicken farm. The chickens were euthanized, cecum-content collected, and analyzed for Zn and Cu content at Eurofins Food and Feed Testing Norway (Moss, Norway)

## Laboratory methods

Laboratory methods included stepwise dilution in broth for MIC determination of Zn and Cu, and growth curves using a Tecan platereader to determine the influence of Zn and Cu on planktonic growth. Conjugation experiments were conducted in LB broth, conjugation frequencies calculated and transconjugants confirmed both on agar and with PCR. The transconjugants from the conjugation assay and the recipient strain was propagated by serial transfers over 300 generations to confirm the stabile integration of the conjugative plasmids. Plasmid presence was confirmed by colony PCR targeting *bla*$_{CMY-2,}$ and plating on transconjugant-selective agar. The analysis of expression of genes involved in conjugation was performed on *E. coli* 2012-01-1292 (pNVI1292/IncK) by real-time qPCR.

## Statistical methods

All experiments were performed as at least three independent experiments, with three technical replicates. As data were not normally distributed, we used a nonparametric regression through the quantile regression technique in Stata (Stata MP/16 for Windows), to evaluate the effect of Zn and Cu levels on the experiments. We adjusted for the impact of strain and biological replicate. Results were reported as coefficients with corresponding p-values as compared to the control. One-way ANOVA was used in the comparison of differences between samples with Zn and Cu and the control samples in the plasmid stability experiment and the analysis of gene expression. The level of statistical significance was set to $p < 0.05$.

## Results

All results are included in the article, and summarized in the following figures and tables:

## Tables:

**Table 1**: Primers used in this study for qPCR for evaluation of expression of genes involved in conjugation.

**Primer sequences**

| Gene | Forward (5`- 3`) | Reverse (5`- 3`) | Slope[a] | %Eff[b] | Gene description[c] |
|------|------------------|------------------|----------|---------|---------------------|
| *nikB* | CGCCTGATAATGGCTGCTTT | CGCTGTTTTGCGCACAATA | -3.44 | 95.05 | Conjugal transfer relaxase protein NikB |
| *rpoA* | GGCACAATCGATCCTGAAGAG | TTCCAGTTGTTCAGCCAGAATG | -3.37 | 97.85 | DNA-directed RNA polymerase, alpha subunit |
| *traB* | GGCAAAAACCGCGAACAT | TCCAGGGAAGGACGTGTTG | -3.4 | 96.75 | Type IV secretion/conjugal transfer ATPase, VirB4 family |

[a] The slope was calculated from the regression line in the standard curve.

[b] The efficiency was calculated using the slope of the regression line in the standard curve.

[c] According to UniProt Database.

**Table 2:** Determined minimum inhibitory concentrations of Zn and Cu for donor and recipient strains.

| Metal/Strain | *E. coli* 1292 (IncK) | *E. coli* 2798 (IncI1) | *E. coli* DH5α |
|---|---|---|---|
| Zn (ZnCl⁻) | 0.4 mg/mL | 0.4 mg/mL | 0.3 mg/mL |
| Cu (CuSO⁴) | 0.9 mg/mL | 1 mg/mL | 0.75 mg/mL |

**Table 3:** Conjugation frequencies in response to different concentrations of Zn and Cu.

| Strain | Additive | mg/mL | Conjugation frequency[a] | SD +/- |
|---|---|---|---|---|
| *E.coli* 2798 (IncI1) | Cu | 0.01 | 1.26E-04 | 1.03E-04 |
| | | 0.255 | 3.95E-06 | 9.12E-06 |
| | | 0.5 | NTD[b] | 0.00E+00 |
| | Zn | 0.05 | 9.31E-05 | 6.00E-05 |
| | | 0.125 | 1.69E-05 | 6.85E-06 |
| | | 0.2 | NTD[b] | 0.00E+00 |
| | Control | 0 | 2.04E-04 | 1.31E-04 |
| *E.coli* 1292 (IncK) | Cu | 0.01 | 3.37E-05 | 4.57E-05 |
| | | 0.255 | 2.37E-06 | 2.28E-06 |
| | | 0.5 | NTD[b] | 0.00E+00 |
| | Zn | 0.05 | 1.04E-06 | 1.35E-06 |
| | | 0.125 | NTD[b] | 0.00E+00 |
| | | 0.2 | NTD[b] | 0.00E+00 |
| | Control | 0 | 9.21E-05 | 5.31E-05 |

[a]Conjugation frequencies were calculated as the mean number of transconjugants divided by the mean number of recipients from all replicates, for each combination.

[b]NTD = No transfer detected (no colonies detected) on transconjugant selective plate.

**A**



*E. coli* 1292 (IncK)

**B**



*E. coli* 2798 (IncI1)

**C**
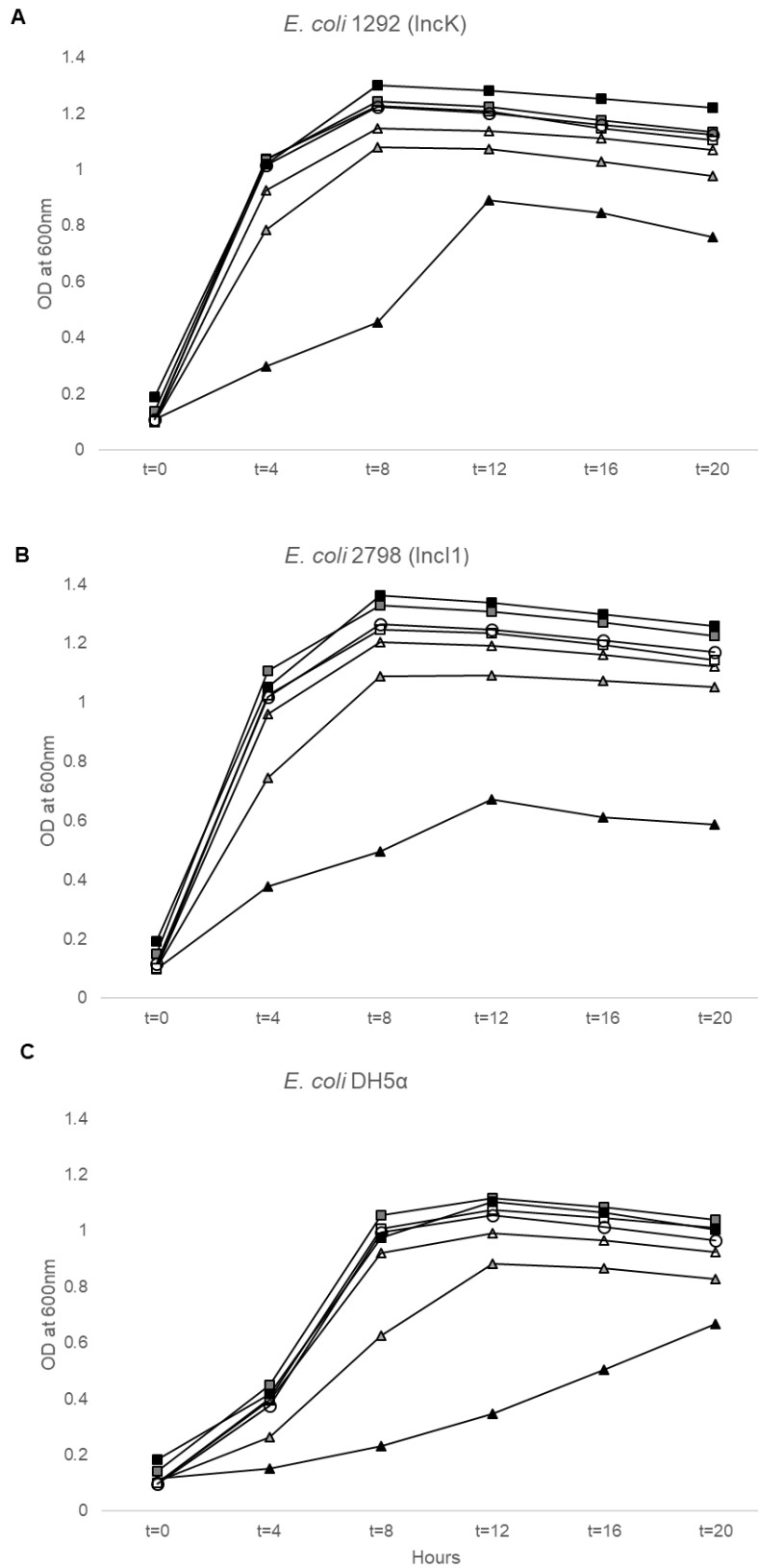


*E. coli* DH5α

**Figure 1:** Growth curves. (A) *E. coli* 1292 (IncK), (B) *E. coli* 2798 (IncI1), (C) *E. coli* DH5α (△) 0.05 mg/mL ZnCl,(▲) 0.125 mg/mL ZnCl, (▲) 0.2 mg/mL ZnCl, (□) 0.01 mg/mL CuSO₄, (▣) 0.255 mg/mL CuSO₄, (■) 0.5 mg/mL Cu SO₄, (o) Control. The data is based on three biological replicates with three technical replicates each.
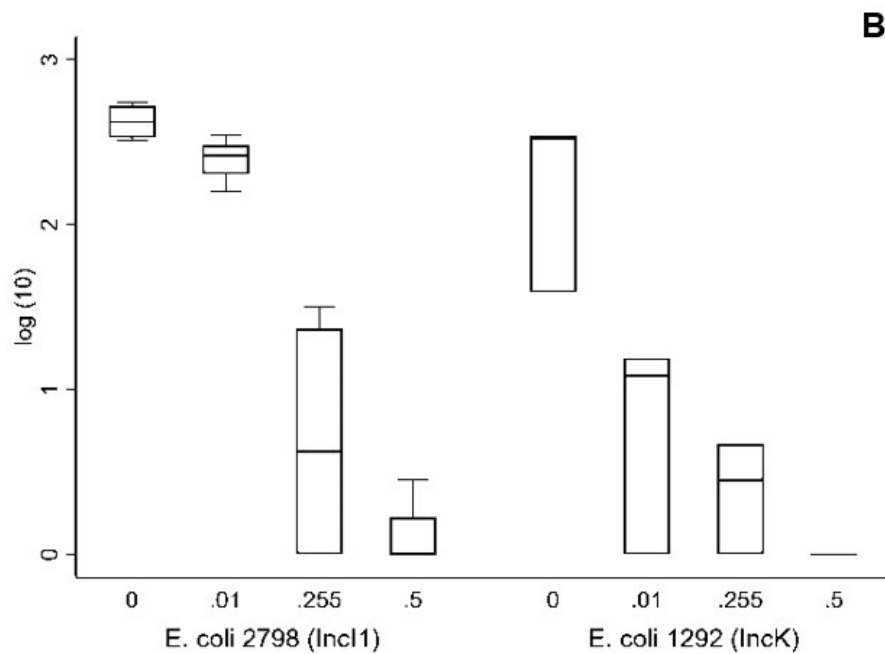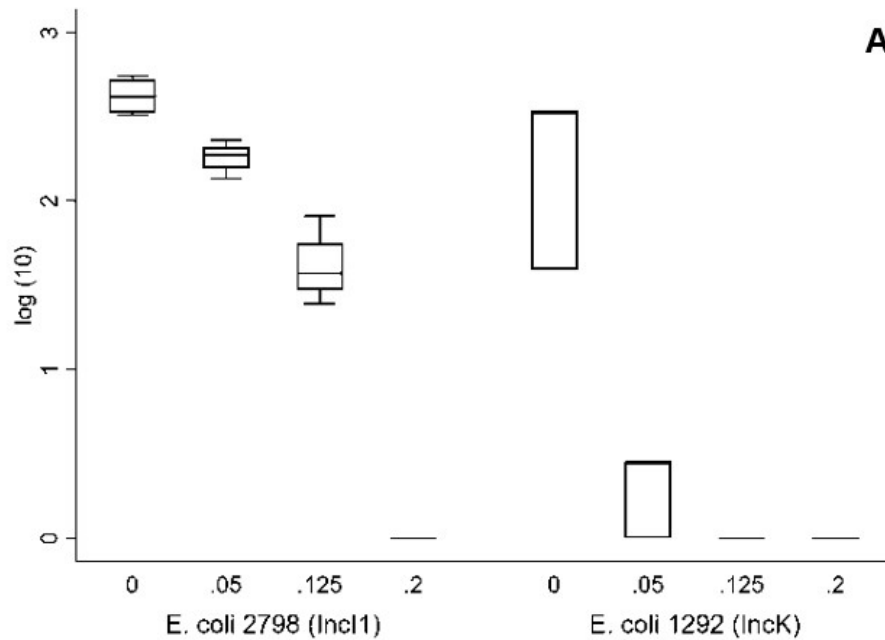
**Figure 2:** Distribution of transconjugants demonstrating a dose-dependent reduction of transconjugants in the presence of Zn (A) and Cu (B). The horizontal line within the box represents the mean. Boxes represents the first to third quartile and the whiskers the highest and lowest values, while dots represent outliers. The data is based on four biological replicates with three technical replicates each.
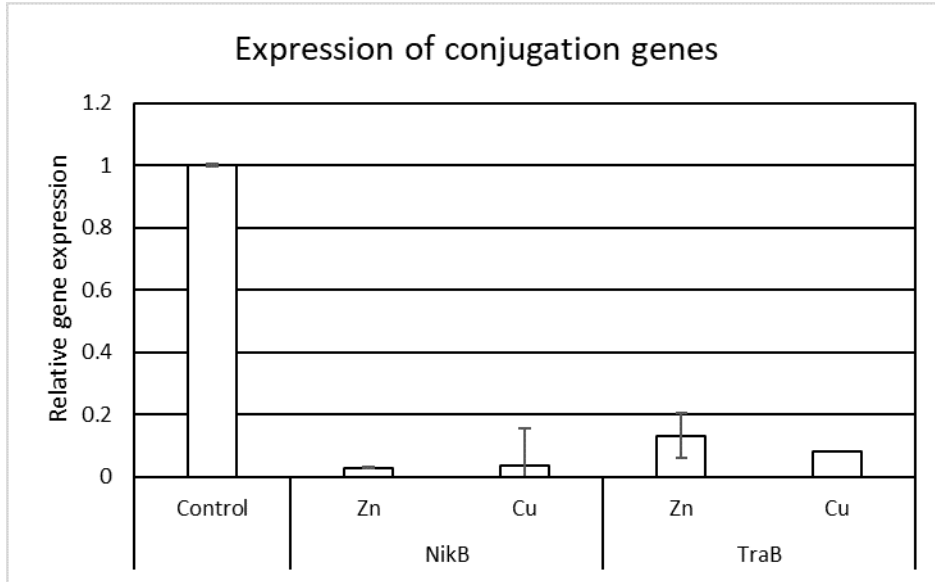


**Figure 3**: Expression of genes involved in conjugation in *E. coli* 1292 (IncK) in response to ZnCl and CuSO$_4$. The data is presented as mean values ± SD (n = 6).

## My contributions

My contributions to this paper included establishing a conjugation assay with added Zn/Cu and establishing *in vivo* concentrations of Zn and Cu from chicken intestines. I performed growth curves, MIC and plasmid confirmation with PCR. Analysis and preparation of results, in addition to writing were also included in my work.

## Other contributors

Work provided by Ingun Lund Witsø includes qPCR, growth curves and the plasmid stability assay.

In this section I will discuss and evaluate my work, while the results and the scientific literature is discussed in the article itself.

**Evaluation of materials and methods**

The chosen isolates were collected from retail chicken meat in Norway and has been whole genome sequenced and characterized by Mo et al. (45). They carry one plasmid each, but with the same resistance profile, and are therefore considered representative for the *in vivo E. coli* population in poultry in Norway from 2012 and chosen as study organisms for my project. However, NORM-VET has recently reported a shift in the genotypes detected, which may be due to the stagnated use of Narasin, or other changes that has been done further up in the poultry production chain. A larger group of study isolates would have been beneficial to strengthen our study, in addition to comparison of conjugation in different medium, and with Narasin in addition to Zn and Cu.

The reason for only measuring up-/down-regulation of expression of nikB and traB genes in *E. coli* 1292 is that this isolate contained the plasmid that had the broadest dissemination in the samples from NORM-VET in 2012, and therefore considered the most representative one.

**Study design**

The study was designed as an experimental study where we manipulated the growth medium of an organism and observed the changes. Performance of a power analysis prior to implementation could have been beneficial. Other changes in the design that could have been done to increase the validity of the work is use of a larger group of isolates, several additives and evaluation of several conjugation genes.

**Information bias**

We expected the conjugation frequency to increase in the presence of metals as this is a phenomenon described in the literature (46-48). It is known that bacteria easily adapt to changes in the environment, and that much of the bacterial success of persistence can be traced back to horizontal gene exchange. We therefore spent a lot of time evaluating our methods to ensure that the observed results were an actual reduction of conjugation.

**Strengths and weaknesses of the protocols:**

| Protocol | Strengths | Weaknesses |
|---|---|---|
| *In vivo* concentrations | Gives insight to the amount of Zn and Cu that bacteria encounter in the intestines.<br><br>Homogenous production, same food, same housing, same deliver of chicken. | Only 10 samples<br>Only one farm<br>Only one replicate |
| Conjugation study | WGS strains – A lot of information available<br><br>Confirmed conjugation to various strains with different methods (ref Mo et al)<br><br>Already established method with minor changes | Only 2 strains<br>Only one mating media<br>Only one recipient<br>Laboratory recipient strain |
| MIC | Background for selection of working concentrations of metals<br><br>Performed in the same broth as the mating was done.<br><br>Ensures that we do not use concentrations that inhibit growth | Only evaluated in broth<br>Evaluated as visible growth |
| Growth curves | Background information<br>Repeated in replicates | Biological variation |
| Gene expression | Investigation of the reason for reduction of conjugation | Only evaluated a few genes<br>Only performed on one of the strains |

**Confounding factors**

There may be uninvestigated confounding factors contributing to our conclusion of the effect of Zn and Cu. The strains used in these experiments have the genes encoding a plasmid stability system (49) which prevents the recipient strain from eliminating the plasmid once present in the cell. *E. coli* is also known for producing colicins, enzymes that inhibit growth of other strains of *E. coli*. This has not been evaluated in this work, but similar mechanisms may play a role in the transfer of genes in broth.

The combination of Zn and Cu joined may also have presented additional information and would need to be investigated together with other metals found in chicken manure, as bacteria live in intricate environments.

**External validity**

This research mainly provides information about the situation in Norway but may be of interest for other countries where the $bla_{CMY-2}$ gene is found in the chicken population. is prevalent. However, our findings indicate that excess levels of Zn and Cu in poultry feed is not acting as a driver of antimicrobial resistance by increasing the conjugation rate of *E. coli*. This is a controversial finding, as both affirmative and contradicting results are published in the literature.

**Challenges and personal reflections**

The learning process as a research track student has been rewarding, and as my knowledge and understanding grew, evaluation of my previous work fell naturally. There was information I retrospectively would like to have had in advance of my experiments. For example, a power analysis in advance when choosing my working concentrations of Zn and Cu would have made defending the selected concentrations easier during the writing stage. It would also have saved me a lot of troubleshooting and extra work to have had a deeper understanding of the statistics earlier in this process. This may be two-sided, as the easiest way to understand statistics as a biologist appears to be by using it with self-retrieved results, whilst one of the most useful times to use the statistics is prior to implementation of the experiments.

During my work a few challenges arose. In the early beginning while establishing the method, a lot of time was spent getting the donor/recipient ratio for the conjugation protocol right. This was

due to both a typing error in the original protocol and absence of direct supervision in the laboratory in the beginning, which cost me weeks of failing experiments due to my limited experience in the lab and my limited knowledge about the methods used. However, this forced me to ask questions, work independently and find solutions, which eventually provided me with a thorough understanding of the protocols and deeper insights in good common laboratory customs. This is knowledge I value retrospectively and was a process that allowed me to bond with my coworkers in the lab, and that taught me the value of asking for others guidance and experience.

Other experiment related issues include a problem with precipitation of the metal salts in the mating medium. The salts used were ZnCl and CuSO$_4$ which are considered highly soluble salts, but we saw that by leaving them over night in room temperature they ended up precipitating to the bottom of the mating tubes, especially ZnCl. As a result of this the concentration of Zn in the medium ended up as a gradient where we had no way of knowing if the observed transconjugants were made in the higher or lower layers of the mating broth. This problem was solved by incubating the medium with shaking overnight to ensure 100% dissolving in the medium, which was successful.

Another thing that has been important for my research track program has been our antimicrobial resistance colloquiums. This was a group consisting of research track students, PhD- students and professors that met up to learn more about, and discuss topics related to antimicrobial resistance. This gathering both served as an important arena for learning, but also provided motivation and a social network.

Conclusion

We found that Zn and Cu reduced the conjugation frequency between *E. coli* in a concentration dependent manner, while plasmids remained stable in the host without any interference of the metals. A down-regulation of conjugational genes in response to Zn and Cu may be involved in a decrease of bacterial conjugation frequency. Based on these *in vitro* studies it is less likely that Zn and Cu in poultry feed alone is responsible for conjugational spread of antimicrobial resistance. These findings were unexpected, and the opposite of our hypothesis. This gives an indication that conjugational spread of AMR may not be a black and white picture, there may be confounding factors that remains unknown, or that the *in vivo* circumstances alter up-/down-

regulation of genes involved in the complex conjugational machinery so that the end-result differs.

The regulation of the conjugation mechanism is complex, and in this study, we have addressed two genes which we consider of major importance for conjugation. It would be interesting to investigate the effect of Zn and Cu in combination, in addition to the interaction with Narasin. Other genes related to regulation of conjugation could also be of relevance for further work. However, by using more advanced molecular and bioinformatic methods, the regulation mechanisms may be studied in more depth and detail. Examples of this include *ex vivo* models for more accurate evaluation of what is happening *in vivo*, or transcriptomics studies.

Altogether I feel privileged for being granted the experience of the research track program. It has been a couple of highly rewarding and exciting years, that has left me with new knowledge, valuable acquaintances, and inspiration to pursue a future career within research.

Vedlegg
- Article
- Declaration co-author
- Declaration supervisor

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# Zinc and copper reduce conjugative transfer of resistance plasmids from ESBL-producing E. coli

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| Abstract: | The present work addresses the effect of excess levels of Zn and Cu in the growth medium on the conjugative transfer of plasmids carrying the antibiotic resistance gene blaCMY-2 from extended spectrum beta-lactamase (ESBL)-producing E. coli. Norwegian poultry are not treated with antibiotics, but still, ESBL-producing E. coli are found in the chicken populations. Chicken receive higher amounts of Zn and Cu than their biological need, and several metals have been shown to act as drivers of antimicrobial resistance.<br>In the present study, ESBL-producing E. coli strains collected from retail chicken meat, were mated in broth containing various concentrations of Zn and Cu. Manual counting of transconjugants showed that Zn and Cu reduced the conjugation frequency between E. coli strains in a concentration dependent manner. Quantitative real time PCR analyses showed that the presence of Zn and Cu in the growth media reduced expression of the conjugation genes traB and nikB. By propagating monocultures over several generations, it was found that the blaCMY-2 plasmids remained stable in the recipient strains. Together the results show that exposure of ESBL-producing E. coli to Zn and Cu reduce horizontal transfer of the blaCMY-2 resistance plasmid by reducing expression of genes involved in conjugation in the plasmid donor strain. |

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18    **Running title:** Zn and Cu reduce plasmid transfer

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1

**Abstract**

The present work addresses the effect of excess levels of Zn and Cu in the growth medium on the conjugative transfer of plasmids carrying the antibiotic resistance gene $bla_{CMY-2}$ from extended spectrum beta-lactamase (ESBL)-producing *E. coli*. Norwegian poultry are not treated with antibiotics, but still, ESBL-producing *E. coli* are found in the chicken populations. Chicken receive higher amounts of Zn and Cu than their biological need, and several metals have been shown to act as drivers of antimicrobial resistance. In the present study, ESBL-producing *E. coli* strains collected from retail chicken meat, were mated in broth containing various concentrations of Zn and Cu. Manual counting of transconjugants showed that Zn and Cu reduced the conjugation frequency between *E. coli* strains in a concentration dependent manner. Quantitative real time PCR analyses showed that the presence of Zn and Cu in the growth media reduced expression of the conjugation genes *traB* and *nikB*. By propagating monocultures over several generations, it was found that the $bla_{CMY-2}$ plasmids remained stable in the recipient strains. Together the results show that exposure of ESBL-producing *E. coli* to Zn and Cu reduce horizontal transfer of the $bla_{CMY-2}$ resistance plasmid by reducing expression of genes involved in conjugation in the plasmid donor strain.

2

**Introduction**

47

48 Spread of antimicrobial resistance (AMR) has become a significant threat against human

49 and animal health[1]. Parts of this resistance has its origin within the agriculture sector and

50 dissemination of resistant bacteria from the food production chains may be one out of

51 several routes by which consumers can be exposed to AMR bacteria[2-6]. In 2012, the

52 *NORM-VET monitoring program for antimicrobial resistance in the veterinary and food*

53 *production sectors* detected cephalosporin resistant *Escherichia coli* in 43% of the

54 Norwegian broiler flocks[7]. In addition, 32% of *E. coli* from retail chicken meat were

55 categorized as cephalosporin resistant, but even higher numbers were reported from

56 Denmark and Sweden[8]. The NORM-VET findings were surprising as the use of

57 antimicrobial agents in the poultry production in Norway is limited, and among the lowest

58 in Europe[7, 9]. In Norway, conjugative ESBL encoding plasmids have frequently been

59 discovered in bacteria isolated from broiler chicken, implicating that these animals are a

60 potential reservoir for cephalosporin-resistant *E. coli*[10].

61

62 Third and fourth generation cephalosporins have been defined as critically important

63 antimicrobials by the World Health Organization. However, extended spectrum beta-

64 lactamase (ESBL) producing bacteria have been isolated from a variety of animal species

65 in different European countries which could represent a major threat to public health[11-15].

66 In 2015, the Norwegian Scientific Committee for Food and Environment concluded that:

67 "The probability of human exposure of ESBL-producing *Enterobacteriaceae*, and their

68 respective corresponding genes, from live poultry and poultry meat was considered non-

69 negligible"[16]. Exposure to ESBL-producing *Enterobacteriaceae* may result in consumers

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70 becoming carriers of resistant bacteria, if these bacteria establish themselves as part of

71 the human gut microbiota[3, 17-19]. In situations where these bacteria cause disease, or

72 spread their resistance genes to other pathogenic bacteria, their resistance

73 characteristics may lead to treatment failure and increased mortality[20, 21].

74

75 Conjugation allows bacteria to spread genetic information across diverse bacterial phyla

76 by the use of mobile genetic elements[22]. Inter- and intraspecies dissemination of

77 resistance plasmids is the main mechanism of horizontal gene transfer of AMR between

78 bacteria and is mediated by the type IV secretion system (T4SS)[23]. The plasmid-located

79 *tra* operon encodes the genes important for transport of the plasmid from the donor to the

80 recipient cell. The TraB protein exhibit ATPase activity thought to provide energy for the

81 assembly of the T4SS machinery and is known to play a major role in the conjugative

82 transfer of plasmid DNA[24, 25]. Transfer of plasmid DNA is initiated and terminated at the

83 origin of transfer, oriT. *NikB* encodes a relaxase, responsible for site- and strand specific

84 cleaving and rejoining of oriT at the nick site of the plasmid[26]. Hansen *et al.*[27] showed that

85 horizontal transfer of plasmids is more important than clonal dissemination for

86 transmission of *bla*$_{CMY-2}$ mediated cephalosporin resistance between animals and

87 humans.

88

89 Mo *et al.*[28] described two *bla*$_{CMY-2}$ encoding plasmids (pNVI1292/IncK and

90 pNVI2798/IncI1), which were found in *E. coli* strains isolated from retail chicken meat in

91 Norway. They further showed that the plasmids could spread to a variety of

92 *Enterobacteriaceae* species by conjugation. These plasmids encode two plasmid stability

4

93 systems, namely relBE/stbDE and pndAC, which presumably facilitate dissemination and

94 stability of the $bla_{CMY-2}$ encoding plasmids. However, the importance of this stability

95 system is not well studied.

96

97 Metals like Zn and Cu have antimicrobial effects; the bacterial toxicity of Zn may be due

98 to the chemical affinity for thiol groups of biomolecules and Cu toxicity is based on

99 production of hyperoxide radicals and interactions with cell membranes[29]. Bacteria

100 acquire resistance genes against antimicrobials and metals with antibacterial properties

101 on mobile genetic elements[30]. When two or more resistance genes are present on the

102 same genetic element, or the same mechanism provides resistance against several

103 substances, it may result in co-selection of genes conferring metal and antibiotic

104 resistance. By these mechanisms, selection for resistance to zinc (Zn), copper (Cu) and

105 other potentially toxic metals may act as drivers for spread of AMR[31, 32]. However, data

106 on the required concentration and time exposure for this effect to occur is still lacking[33].

107

108 Zn and Cu are important elements in the cellular metabolism; they allow many critical

109 enzymes to function properly and are also essential for wound healing, protein synthesis,

110 and maintaining the strength of the skin, blood vessels, and various tissues in the

111 organism[34]. Copper and zinc are routinely used as additives in animal feed in livestock

112 farming, however, when animals receive feed containing larger amounts of Zn and Cu

113 than what they biologically require the excess of metals are thereby released into the

114 environment[35]. Zn and Cu are therefore commonly found in soil, water, plants and in

115 manure from various farm animals, including chickens[36]. The occurrence of Zn and Cu

5

116 may have beneficial fertilizing properties, as these are important trace elements for plants,

117 but may also be of environmental concern when present in large quantities, affecting

118 groundwater, surface water and aquatic animals[37-40].

119

120 We hypothesize that excess levels of Zn and Cu promote transfer of resistance plasmids

121 from ESBL-producing *E. coli*. The aim of our study was to assess the effect of Zn and Cu

122 on conjugation of $bla_{CMY-2}$ carrying plasmids from ESBL producing *E. coli* collected from

123 retail chicken meat.

124

125 **Materials and methods**

126 *Bacterial strains and growth media*

127 Two *E. coli* strains isolated from retail chicken meat were used as plasmid donors in the

128 conjugation experiments; *E. coli* 2012-01-1292 (pNVI1292/IncK) and *E. coli* 2012-01-

129 2798 (pNVI2798/IncI1), hereafter named *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1),

130 respectively. The strains were collected through NORM-VET in 2012 , and harbored the

131 $bla_{CMY-2}$ gene[41], the most common plasmid-mediated AmpC-beta-lactamase in *E. coli*[42].

132 Both strains have recently been whole genome sequenced (Mo and coworkers,

133 unpublished data)[41]. The *E. coli* DH5α strain, resistant to nalidixic acid (Nal[R]), was used

134 as recipient. Both donors ferment lactose, while the recipient does not. The bacteria were

135 cultured in Luria-Bertani (LB) broth (Sigma-Aldrich, Germany) or Brain Heart Infusion

136 (BHI) broth (Sigma-Aldrich, Germany) throughout the whole study unless otherwise

137 stated. ZnCl (Sigma-Aldrich, Germany) and $CuSO_4$ (Merck, Germany) were used as

138 sources of Zn and Cu throughout the whole study.

139

*In vivo and experimental concentrations of Zn and Cu*

141 The NORM-VET collects AMR-bacteria from the cecum of chickens. To determine the *in*

142 *vivo* concentrations of Zn and Cu in chicken cecum, ten 25-day old chicken were collected

143 from a commercial chicken farm in Norway. The chickens were euthanized, cecum-

144 content collected, and analyzed for Zn and Cu content at Eurofins Food and Feed Testing

145 Norway (Moss, Norway).

146

147 Experimental concentrations for ZnCl and $CuSO_4$ to be used in the conjugation assays

148 were based on the measured *in vivo* concentrations in the cecum samples and the content

149 of Zn and Cu in poultry feed, without exceeding their minimum inhibitory concentrations.

150

*Minimum inhibitory concentrations (MIC)*

152 MICs for ZnCl and $CuSO_4$ were determined for all strains by serial dilutions

153 (Supplementary Tab. S1) in LB-broth[43]. The concentrations tested ranged from 0.03

154 µg/mL – 3 mg/mL for ZnCl and 0.01 µg/mL – 1 mg/mL for $CuSO_4$.

155

*Growth curves of donor and recipient strains*

157 Overnight cultures of each strain were diluted 1:1000 in fresh LB-broth containing the

158 selected experimental concentrations of ZnCl and $CuSO_4$. A control without

159 supplemented metals was included in each experiment. A volume of 200 µL of each

160 sample was transferred to a 96 well microtiter plate (Greiner, Sigma-Aldrich, Germany)

161 and incubated at 37°C in Tecan platereader. The optical density ($OD_{600}$) was measured

7

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162 in the cultures every 10 min for 24 h. Each experiment was performed in three biological

163 replicates, with three replicates of each sample.

164

*Conjugation study*

166 Conjugation experiments were conducted in LB-broth according to Sunde *et al*.[44], with

167 minor modifications. Briefly, the donor and recipient strains were grown overnight in LB-

168 broth at 37°C and subsequently diluted to an $OD_{600}$ equivalent to a 1 McFarland standard.

169 A volume of 500 µL of the recipient strain culture and 10 µL of the donor strain culture

170 was mixed in 4 mL LB-broth containing the selected experimental concentrations of ZnCl

171 or $CuSO_4$, respectively, including a control without supplements. All cultures were

172 incubated for 4 h at 37°C. LB-broth supplemented with Zn or Cu were prepared one day

173 prior to the experiment and incubated at 37°C overnight under agitation to prevent

174 precipitation of the added metal. Dilutions of each mating culture were plated on Mueller-

175 Hinton agar plates (Sigma-Aldrich, Germany) supplemented with 20 mg/L nalidixic acid

176 and/or 0.5 mg/L cefotaxime, and incubated for 24 h and 48 h at 37°C. In order to quantify

177 the conjugation and to test for toxic effects of the metals on the donor and recipient strains

178 individually, we plated the mating cultures on donor-, recipient- and transconjugant

179 selective plates. The conjugation frequency was determined by manual counting of colony

180 forming units (CFU) and dividing the number of transconjugants with the number of

181 recipients.

182

183 Representative colonies from each transconjugant-selective plate was plated on

184 bromothymol lactose blue agar (Sigma-Aldrich, Germany) in order to distinguish

8

185  transconjugants from spontaneously mutated donors. In addition to different abilities to

186  ferment lactose, the transconjugants and mutated donors are distinguishable by colony

187  morphology. PCR analysis of bacterial colonies was conducted to confirm that the

188  transconjugants harbored the $bla_{CMY-2}$ gene[45].

189

190  *Plasmid stability*

191  The transconjugants from the conjugation assay (DH5α with pNVI1292/IncK or

192  pNVI2798/Incl1) were propagated by serial transfers as previously described[46, 47], with

193  minor modifications. Briefly, 10 μL of stationary phase culture was transferred into 990 μL

194  of fresh LB-broth supplemented with 0.05 mg/mL ZnCl or 0.01 mg/mL $CuSO_4$ every 12 h

195  for 5 days, corresponding to approximately 300 generations. Cultures in LB-broth without

196  metal supplements were used as controls. The bacteria were grown at 37°C under

197  agitation (180 rpm). To confirm the presence or absence of plasmids pNVI1292/IncK and

198  pNVI2798/Incl1, serial diluted samples from each transfer were plated on Mueller-Hinton

199  agar plates with or without antibiotics (20 mg/mL nalidixic acid and 0.5 mg/mL

200  cefotaxime). Plates without antibiotics were incubated at 37°C for 24 h and plates

201  containing antibiotics were incubated for 48 h. The number of CFU was counted manually.

202  The presence of the $bla_{CMY-2}$ plasmid was confirmed by colony PCR as described above.

203

204  *Sample preparation, RNA isolation and quantitative PCR*

205  The transcriptional analysis of genes involved in conjugation, *nikB* and *traB*, was

206  performed in *E. coli* 1292 (IncK). An overnight culture was inoculated in fresh LB-broth

207  with ZnCl or $CuSO_4$. Bacteria cultured in plain LB media were used for comparison. The

9

208   samples were incubated for 4 h at 37°C. Total RNA was isolated from harvested *E. coli*

209   using PureLink RNA Mini Kit (Life technologies; Carlsbad, USA) according to the

210   manufacturer`s protocol. On-column PureLink DNAse (Life technologies; Carlsbad, USA)

211   treatment was performed according to the protocol. An amount of 100 ng of total RNA

212   was used to synthesize cDNA using High Capacity cDNA Reverse Transcriptase kit

213   (Applied Biosystems, California, USA) according to the manufacturer`s protocol. Primers

214   were designed by Primer Express software, and the sequences are listed in Table 1. A

215   standard curve using serial dilution of DNA from *E. coli* 1292 was made to calculate PCR

216   efficiency for each primer pair. Real time reactions were performed using Power SYBR

217   Green PCR Master Mix (Life Technologies) and real time amplification was carried out

218   using Step One Real Time PCR system (Applied Biosystems, California, USA). The data

219   was collected and analyzed by normalization against the endogenous control gene *rpoA*

220   using StepOne Software v2.3.

221

222   *Statistical analysis*

223   All experiments were performed as at least three independent experiments, with three

224   technical replicates. As data were not normally distributed, we used a nonparametric

225   regression through the quantile regression technique in Stata (Stata MP/16 for Windows),

226   to evaluate the effect of Zn and Cu levels on the experiments. We adjusted for the impact

227   of strain and biological replicate. Results were reported as coefficients with corresponding

228   p-values as compared to the control. One-way ANOVA was used in the comparison of

229   differences between samples with Zn and Cu and the control samples in the plasmid

230  stability experiment and the analysis of gene expression. The level of statistical

231  significance was set to p < 0.05.

232

233  **Results**

234  *Selected Zn and Cu concentrations*

235  The poultry feed contained 100 mg/kg Zn and 15 mg/kg Cu, and the results from the

236  poultry cecum-content ranged from 8.52 - 83.5 mg/kg Cu and 71.9 - 225 mg/kg Zn. MIC

237  data for *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) is shown Table 2. Thus, the

238  experimental concentrations were selected as follows: 0.05 mg/mL, 0.125 mg/mL and 0.2

239  mg/mL for Zn, and 0.01 mg/mL, 0.255 mg/mL and 0.5 mg/mL for Cu.

240

241  *Growth curves*

242  Growth curves for the donor and recipient strains are shown in Figure 1. The

243  concentrations of Zn and Cu tested did not have any effect on planktonic growth, except

244  a delayed growth rate at the highest concentrations of Zn (0.125 mg/mL and 0.2 mg/mL).

245  None of the concentrations of Cu tested had any effect on the planktonic growth of the

246  strains. The two donor strains *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) showed higher

247  growth rates compared to the recipient *E. coli* DH5α strain.

248

249  *Conjugation study*

250  The conjugation frequency was first determined in a pilot study were samples were taken

251  after 4 and 24 h of mating. No difference in the conjugation frequency was observed

252  between the two time points (data not shown). Therefore, 4 h was chosen for further

11

253   conjugation and transcriptional studies. The mating cultures were plated on donor-,

254   recipient- and transconjugant selective plates to calculate the conjugation frequency

255   (Table 3) and to evaluate any inhibiting effect of the metals in the mating cultures. Neither

256   the donors nor the recipient showed any reduction in CFU compared to the control (data

257   not shown).

258

259   As shown in Figure 2 the effect of increasing levels of Zn and Cu were clear. These

260   findings were supported by the non-parametric regression analysis, where strong effects

261   of Zn and Cu levels were found ($p < 0.001$). No statistical effects of strain were seen for

262   the recipients for Zn ($p = 31$) and Cu ($p = 0.55$) (Supplementary Fig. S2). No statistical

263   effect of replicate could be observed in the data. Our results show that conjugation of the

264   IncK plasmid was reduced by more than 98% at all concentrations of Zn tested compared

265   to the control. This was also observed for the two highest concentrations of Cu while 0.01

266   mg/mL Cu gave an 90% reduction of conjugation for the IncK plasmid. At the two highest

267   concentrations of Zn and Cu there was a more than 90% reduced conjugation of the IncI1

268   plasmid, while the lowest concentrations of Zn and Cu gave a reduction of 58% and 41%,

269   respectively. A representative selection of colonies was picked for further confirmation

270   and all of them were confirmed PCR-positive for the $bla_{CMY-2}$ gene. Furthermore, all tested

271   transconjugants gave a negative result on the bromomethyl lactose agar, confirming the

272   recipient phenotype.

273

274   *Plasmid stability*

275    Both plasmids carry genes encoding stability systems (*relBE/stbDE* and *pndAC*). We

276    wanted to investigate whether these stability systems promote plasmid maintenance, and

277    if Zn and Cu had any impact on the stability of the plasmids. By propagating the

278    transconjugants in monocultures for approximately 300 generations and calculate CFU

279    after plating on transconjugant selective plates, we could show that both plasmids were

280    maintained within the transconjugants (Supplementary Fig. S1). Furthermore, the results

281    showed that there was no difference in plasmid stability between the IncK- and the IncI1

282    plasmid and that sub-inhibitory concentrations of Zn and Cu did not have any influence

283    on the maintenance of the plasmids ($p < 0.05$).

284

285    *Effect of Zn and Cu on expression of conjugative-related genes*

286    Qualitative PCR analysis showed that genes involved in transfer of the IncK plasmid had

287    a significantly reduced expression following exposure to 0.05 mg/mL Zn or 0.01 mg/mL

288    Cu compared to the control ($p < 0.05$) (Figure 3). Specifically, the expression of *traB* was

289    reduced by 87% and 92% in the samples with Zn and Cu compared to the control without

290    Zn or Cu. The expression of *nikB* was reduced by 97% and 96% in response to Zn and

291    Cu compared to the control.

292

293    **Discussion**

294    Conjugation is a complex mechanism that allows bacteria to spread genes encoding

295    beneficial traits that will increase bacterial survival. A previous study has shown that the

296    two plasmids included in this study are inter- and intraspecies transferable at different

297    conditions, indicating that they may contribute to the maintenance of antibiotic resistant

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298  genes in the environment[28]. In contrast to earlier studies, which show that Zn and Cu are

299  associated with increased conjugation[48-50], we found that Zn and Cu reduced the

300  conjugation frequency between *E. coli* strains in a concentration dependent manner.

301  There was also no difference in the number of colonies on the donor- and recipient-

302  selective plates from the mating cultures which confirms that that Zn and Cu does not

303  affect growth of donor- or recipient strains. Toxic effects of the metals can therefore not

304  explain the observed reduction in conjugation frequencies. A pilot study was performed

305  at both 4 and 24 h of mating (data not shown). No difference in conjugation rate was

306  observed between the two time points which is consistent with a previous report  by Mo

307  et al[28]. Altogether, this justifies our choice of using the 4 h time point in the mating

308  experiments. The growth curves showed that the recipient strain grew slightly slower than

309  the donor strain, however, this was compensated for by using larger amounts of recipient

310  cells in the conjugation experiments.

311

312  The plasmids used in this study did not contain any known Zn or Cu resistance genes[41],

313  which rules out co-selective mechanisms. Our results are consistent with a recent study,

314  which showed that metal stress (Zn and Cu included) decreased plasmid transfer

315  frequencies to bacterial communities independent of metal-resistance[51]. Suzuki *et al.*[52]

316  also showed a reduction in horizontal transfer of the tetracycline resistance gene *tet*(M)

317  in response to Zn and Cu exposure. Reduced plasmid transfer in response to metal stress

318  could be a consequence of changes in metabolic status, decrease in plasmid replication,

319  activation of the SOS-response or a combination of different mechanisms. This study also

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320 showed that both the IncK and IncI1 plasmids remained stable in their host throughout

321 several generations, independent of presence of Zn or Cu.

322

323 The molecular mechanisms of heavy metals on conjugative transfer of resistance genes,

324 with exception of co-selective mechanisms, have rarely been investigated[53]. In order to

325 understand the effect of Zn and Cu on conjugation in our study, we performed a real-time

326 transcriptional analysis which showed a reduction in expression of conjugation-

327 associated genes in response to sub-inhibitory concentrations of Zn and Cu. However,

328 our findings contrast the report by Zhang *et al*.[50], who found that different concentrations

329 of heavy metals increased conjugation and upregulated the expression of genes involved

330 in conjugation of plasmids. Strain background, concentrations of the metals and

331 experimental conditions might explain the contradictory results.

332

333 Conjugative transfer of plasmids are controlled by a wide range of genes[54]. A failure to

334 form a functional relaxosome, mediated by the NikB protein, can result in an incapability

335 of the plasmid to mobilize[55]. Our results from the transcriptional analysis could therefore

336 indicate that the reduced expression of *nikB* in response to Zn and Cu, could lead to a

337 dysfunctional relaxosome, and the incapability of the plasmid to transfer from the donor

338 to the recipient strain. The expression of the *traB* gene involved in plasmid transfer was

339 also significantly reduced, which indicates that metals disturb the function of the

340 conjugation machinery.

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342   Our goal was to use concentrations of Zn and Cu that mimic the conditions found in the

343   chicken intestines as closely as possible. However, due to the natural characteristics of

344   the metals, the MIC and the working concentrations of Zn and Cu are lower than what

345   was found *in vivo.* We focused on the effect of heavy metals on the plasmid encoded

346   genes by using an experimental setup that excluded the effect of co-selection. However,

347   we cannot exclude that Zn and Cu interfere with expression of chromosomal genes or

348   genes located in the recipient strain. The effect of Zn and Cu on the SOS-response may

349   be of future interest, in addition to investigate the combined effect of Zn and Cu on

350   conjugation. The expression of other conjugational genes needs to be studied in order to

351   learn more about how metals interfere with conjugation at a transcriptional level. It would

352   also be beneficial to study horizontal gene transfer in more complex models than the ones

353   used in the present study; preferable models that to a larger degree resembles the "real

354   life" conditions in the chicken intestinal environment. Nevertheless, our results indicate

355   altogether that Zn and Cu interfere with genes involved in conjugation, and thereby

356   decrease the frequency of conjugational transfer of plasmids between *E. coli*.

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365  proofreading.

366

**Author Disclosure statements**

368  The authors have no conflict of interest to declare.

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**Tables:**

**Table 1**: Primers used in this study for qPCR for evaluation of expression of genes

involved in conjugation.

| | Primer sequences | | | | |
|---|---|---|---|---|---|
| Gene | Forward (5`- 3`) | Reverse (5`- 3`) | Slope[a] | %Eff[b] | Gene description[c] |
| *nikB* | CGCCTGATAATGGCTGCTTT | CGCTGTTTTGCGCACAATA | -3.44 | 95.05 | Conjugal transfer relaxase protein NikB |
| *rpoA* | GGCACAATCGATCCTGAAGAG | TTCCAGTTGTTCAGCCAGAATG | -3.37 | 97.85 | DNA-directed RNA polymerase, alpha subunit |
| *traB* | GGCAAAAACCGCGAACAT | TCCAGGGAAGGACGTGTTG | -3.4 | 96.75 | Type IV secretion/conjugal transfer ATPase, VirB4 family |

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536   [a] The slope was calculated from the regression line in the standard curve.

537   [b] The efficiency was calculated using the slope of the regression line in the standard

538   curve.

539   [c] According to UniProt Database.

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559    **Table 2:** Determined minimum inhibitory concentrations of Zn and Cu for donor and

560    recipient strains.

| Metal/Strain | E. coli 1292 (IncK) | E. coli 2798 (IncI1) | E. coli DH5α |
|---|---|---|---|
| Zn (ZnCl$^-$) | 0.4 mg/mL | 0.4 mg/mL | 0.3 mg/mL |
| Cu (CuSO$^4$) | 0.9 mg/mL | 1 mg/mL | 0.75 mg/mL |

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579 **Table 3:** Conjugation frequencies in response to different concentrations of Zn and Cu.

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| Strain | Additive | mg/mL | Conjugation frequency[a] | SD +/- |
|---|---|---|---|---|
| *E.coli* 2798 (IncI1) | Cu | 0.01 | 1.26E-04 | 1.03E-04 |
| | | 0.255 | 3.95E-06 | 9.12E-06 |
| | | 0.5 | NTD[b] | 0.00E+00 |
| | Zn | 0.05 | 9.31E-05 | 6.00E-05 |
| | | 0.125 | 1.69E-05 | 6.85E-06 |
| | | 0.2 | NTD[b] | 0.00E+00 |
| | Control | 0 | 2.04E-04 | 1.31E-04 |
| *E.coli* 1292 (IncK) | Cu | 0.01 | 3.37E-05 | 4.57E-05 |
| | | 0.255 | 2.37E-06 | 2.28E-06 |
| | | 0.5 | NTD[b] | 0.00E+00 |
| | Zn | 0.05 | 1.04E-06 | 1.35E-06 |
| | | 0.125 | NTD[b] | 0.00E+00 |
| | | 0.2 | NTD[b] | 0.00E+00 |
| | Control | 0 | 9.21E-05 | 5.31E-05 |

581 [a]Conjugation frequencies were calculated as the mean number of transconjugants divided
582 by the mean number of recipients from all replicates, for each combination.
583 [b]NTD = No transfer detected (no colonies detected) on transconjugant selective plate.
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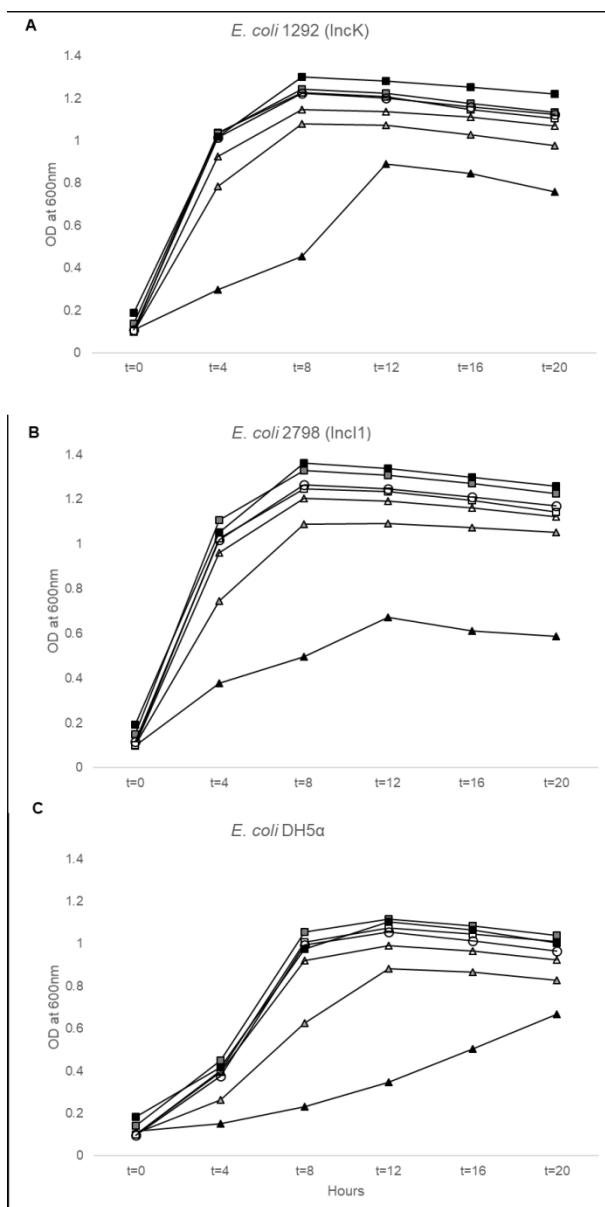51
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592 **Figures:**

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594 **Figure 1:** Growth curves. (A) *E. coli* 1292 (IncK), (B) *E. coli* 2798 (IncI1), (C) *E. coli* DH5α

595 (△) 0.05 mg/mL ZnCl,(⬤) 0.125 mg/mL ZnCl, (▲) 0.2 mg/mL ZnCl, (▫) 0.01 mg/mL

596 CuSO$_4$, (▫) 0.255 mg/mL CuSO$_4$, (■) 0.5 mg/mL Cu SO$_4$, (○) Control. The data is based

597 on three biological replicates with three technical replicates each.

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599 **Figure 2:** Distribution of transconjugants demonstrating a dose-dependent reduction of

600 transconjugants in the presence of Zn (A) and Cu (B). The horizontal line within the box

601 represents the median. Boxes represents 50% of the data and the whiskers the highest

602 and lowest values, while dots represent outliers. The data is based on four biological

603 replicates with three technical replicates each.

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605 **Figure 3**: Expression of genes involved in conjugation in *E. coli* 1292 (IncK) in response

606 to ZnCl and CuSO$_4$. The data is presented as mean values ± SD (n = 6).

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Figure 1: Growth curves. (A) E. coli 1292 (IncK), (B) E. coli 2798 (IncI1), (C) E. coli DH5α (▲) 0.05 mg/mL ZnCl,(▲) 0.125 mg/mL ZnCl, (▲) 0.2 mg/mL ZnCl, (□) 0.01 mg/mL CuSO4, (■) 0.255 mg/mL CuSO4, (■) 0.5 mg/mL Cu SO4, (○) Control. The data is based on three biological replicates with three technical replicates each.

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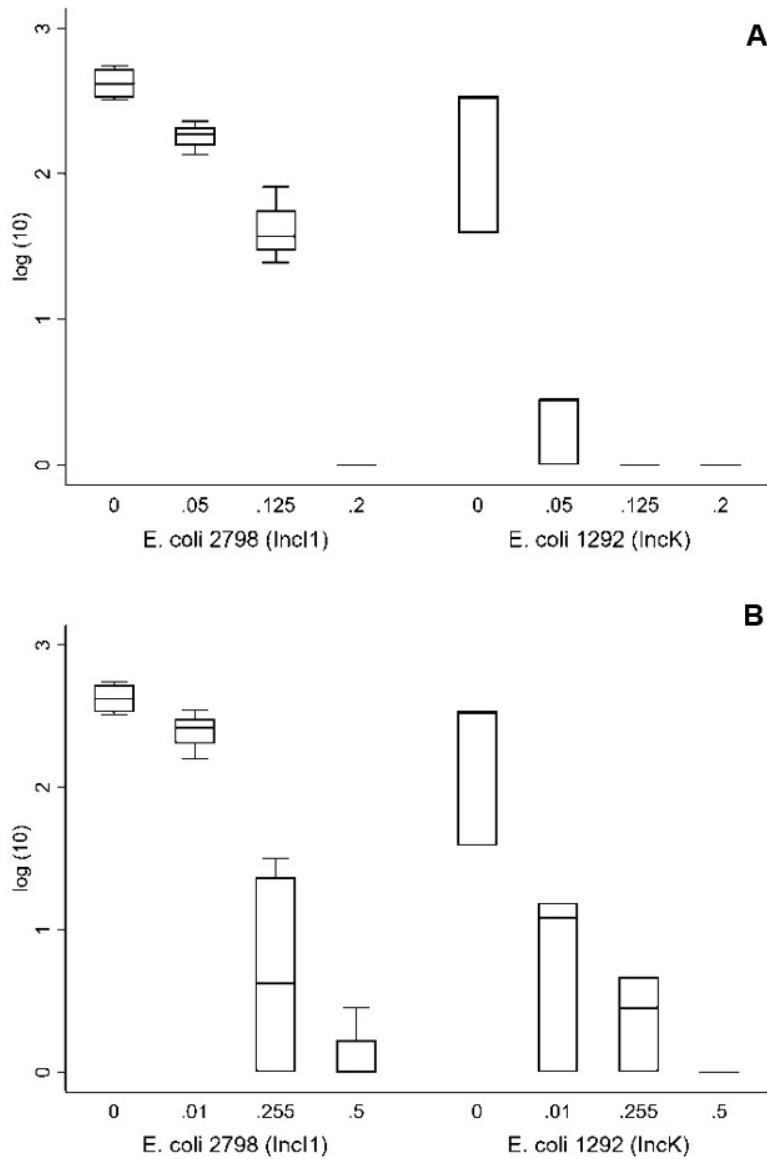
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Figure 2: Distribution of transconjugants demonstrating a dose-dependent reduction of transconjugants in the presence of Zn (A) and Cu (B). The horizontal line within the box represents the median. Boxes represents 50% of the data and the whiskers the highest and lowest values, while dots represent outliers. The data is based on four biological replicates with three technical replicates each.

Figure 3: Expression of genes involved in conjugation in E. coli 1292 (IncK) in response to ZnCl and CuSO4. The data is presented as mean values ± SD (n = 6).

124x76mm (150 x 150 DPI)

**Supplement**

**Table S1:** MIC determination performed by stepwise dilution. First tenfold, then twofold and finally with a 0.1 mg/mL interval.

| Metal | 1. Tenfold mg/mL | 2. Twofold mg/mL | 3. Serial mg/mL |
|-------|------------------|------------------|-----------------|
| Zn | 3 | 3 | 0.7 |
| | 0.3 | 1.5 | 0.6 |
| | 0.03 | 0.75 | 0.5 |
| | 0.003 | 0.375 | 0.4 |
| | 0.0003 | | 0.3 |
| | 0.00003 | | 0.2 |
| Cu | 1 | 1 | 0.5 |
| | 0.1 | 0.5 | 0.6 |
| | 0.01 | 0.25 | 0.7 |
| | 0.001 | 0.125 | 0.8 |
| | 0.0001 | | 0.9 |
| | 0.00001 | | |

Buberg

22  **Table S2:**
23  Growth rates from the growth curves.

| Strains | Concentration/Additive | Growth rate | Doubling time (min) |
|---|---|---|---|
| *E. coli* 2012-2798 (Inc I1) | 0.05 mg/mL Zn | 0.691039 | 86.82578 |
| | 0.125 mg/mL Zn | 0.638383 | 93.98744 |
| | 0.2 mg/mL Zn | 0.426022 | 140.8377 |
| | 0.01 mg/mL Cu | 0.708375 | 84.70087 |
| | 0.225 mg/mL Cu | 0.617336 | 97.19182 |
| | 0.5 mg/mL Cu | 0.540696 | 110.9681 |
| | Control | 0.662754 | 90.53137 |
| *E. coli* 2012-1298 (Inc K) | 0.05 mg/mL Zn | 0.676801 | 88.65231 |
| | 0.125 mg/mL Zn | 0.637863 | 94.06413 |
| | 0.2 mg/mL Zn | 0.343206 | 174.8221 |
| | 0.01 mg/mL Cu | 0.697226 | 86.05528 |
| | 0.225 mg/mL Cu | 0.612096 | 98.02391 |
| | 0.5 mg/mL Cu | 0.527978 | 113.6412 |
| | Control | 0.68022 | 88.20681 |
| *E. coli* - DH5α | 0.05 mg/mL Zn | 0.530787 | 113.0397 |
| | 0.125 mg/mL Zn | 0.344963 | 173.9315 |
| | 0.2 mg/mL Zn | 0.110234 | 544.2974 |
| | 0.01 mg/mL Cu | 0.544715 | 110.1494 |
| | 0.225 mg/mL Cu | 0.450441 | 133.2029 |
| | 0.5 mg/mL Cu | 0.334976 | 179.1174 |
| | Control | 0.527805 | 113.6783 |

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24    **Figures:**

25    **Figure S1**: Plasmid stability. Ten µL of stationary phase cultures of each transconjugants

26    (DH5α with pNVI1292/IncK and DH5α with pNVI2798/IncI1) was transferred into 990 µL

27    of fresh LB-broth supplemented with ZnCl or $CuSO_4$, 0.05 mg/mL and 0.01 mg/mL,

28    respectively. LB-broth without Zn or Cu was used for comparison. Transfers were done

29    every 12 h for 5 days, corresponding to approximately 300 generations. Samples from

30    the different transfers were plated on Mueller-Hinton agar plates with or without antibiotics

31    (20 mg/mL nalidixic acid and 0.5 mg/mL cefotaxime). The plates were incubated at 37°C

32    for 24 or 48 h, and CFU were calculated as explained above. (○) DH5α with

33    pNVI1292/IncK control, (■) DH5α with pNVI1292/IncK with Zn, (□) DH5α with

34    pNVI1292/IncK with Cu, (●) DH5α with pNVI2798/IncI1 control, (▲) DH5α with

35    pNVI2798/IncI1 with Zn, (◊) DH5α with pNVI2798/IncI1 with Cu.

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37    **Figure S2:** Distribution of recipients in the presence of Zn and Cu. No statistical effects

38    of strain was seen for the recipients for Zn (A) (p = 31) and Cu (B) (p = 0.55). The

39    horizontal line within the box represents the median. Boxes represents 50% of the data

40    and the whiskers the highest and lowest values, while dots represent outliers. The data

41    is based on four biological replicates with three technical replicates each.

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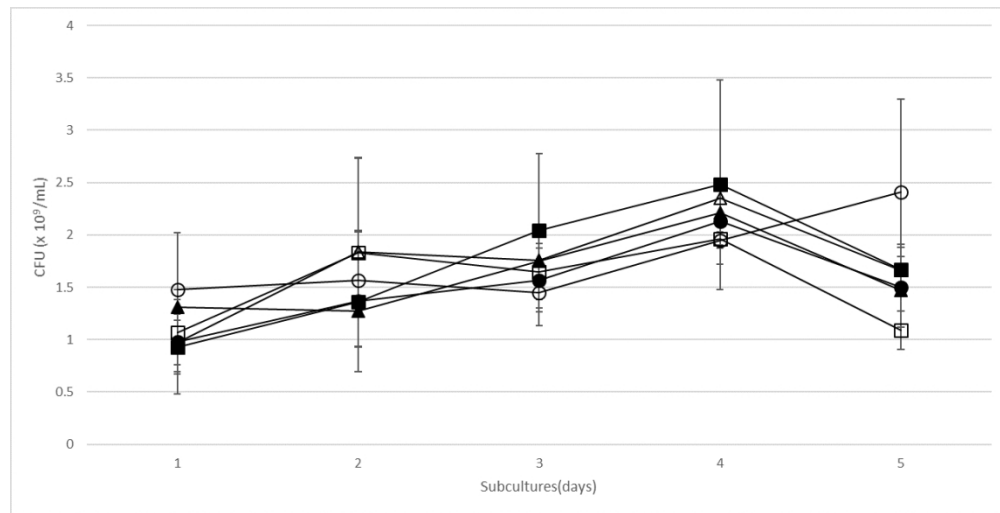
Figure S1: Plasmid stability. Ten μL of stationary phase cultures of each transconjugants (DH5α with pNVI1292/IncK and DH5α with pNVI2798/IncI1) was transferred into 990 μL of fresh LB-broth supplemented with ZnCl or CuSO4, 0.05 mg/mL and 0.01 mg/mL, respectively. LB-broth without Zn or Cu was used for comparison. Transfers were done every 12 h for 5 days, corresponding to approximately 300 generations. Samples from the different transfers were plated on Mueller-Hinton agar plates with or without antibiotics (20 mg/mL nalidixic acid and 0.5 mg/mL cefotaxime). The plates were incubated at 37oC for 24 or 48 h, and CFU were calculated as explained above. (○) DH5α with pNVI1292/IncK control, (■) DH5α with pNVI1292/IncK with Zn, (□) DH5α with pNVI1292/IncK with Cu, (●) DH5α with pNVI2798/IncI1 control, (▲) DH5α with pNVI2798/IncI1 with Zn, (◇) DH5α with pNVI2798/IncI1 with Cu.

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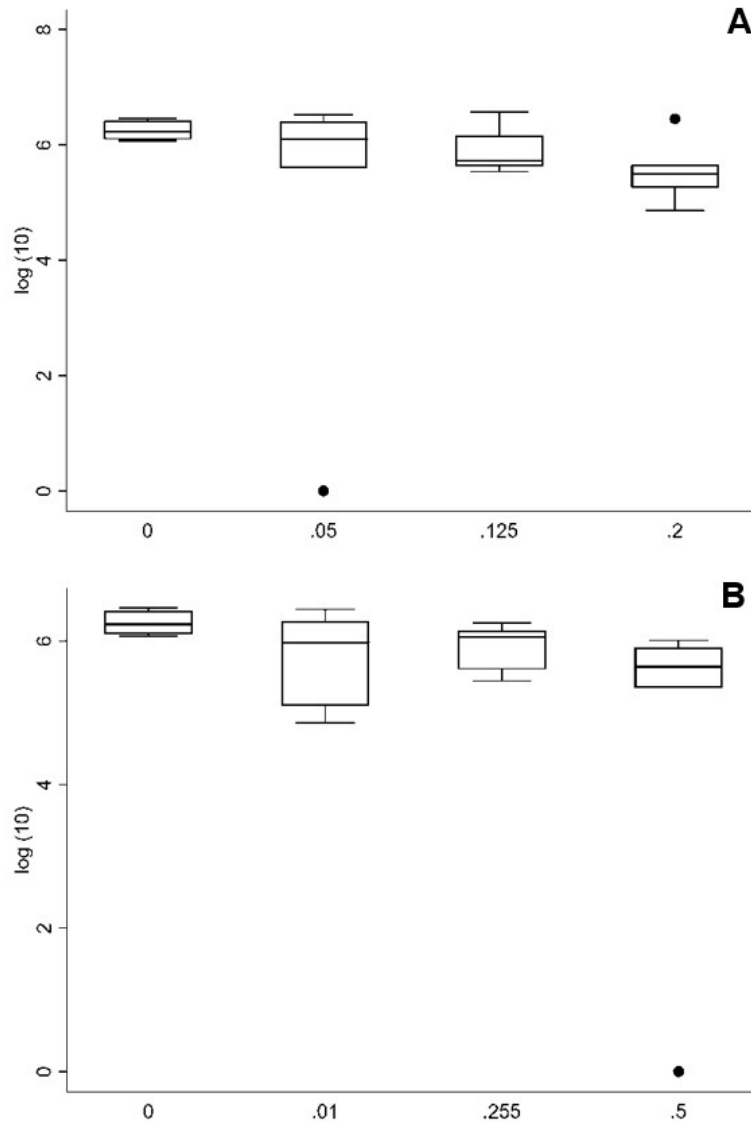41
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Figure S2: Distribution of recipients in the presence of Zn and Cu. No statistical effects of strain was seen for the recipients for Zn (A) (p = 31) and Cu (B) (p = 0.55). The horizontal line within the box represents the median. Boxes represents 50% of the data and the whiskers the highest and lowest values, while dots represent outliers. The data is based on four biological replicates with three technical replicates each.

165x235mm (96 x 96 DPI)