Zinc and Copper Reduce Conjugative Transfer of Resistance Plasmids from Extended-Spectrum Beta-Lactamase-Producing *Escherichia coli*

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The present work addresses the effect of excess levels of ZnCl_2 and CuSO_4 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 *Escherichia coli*. Norwegian poultry are not treated prophylactically with antibiotics, but still, ESBL-producing *E. coli* are found in the chicken populations. Chickens 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 ZnCl_2 and CuSO_4 . Manual counting of trans-conjugants showed that ZnCl_2 and CuSO_4 reduced the conjugation frequency between *E. coli* strains in a concentration-dependent manner. Quantitative real-time PCR analyses showed that the presence of ZnCl_2 and CuSO_4 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.

Keywords: conjugation, CMY-2, antimicrobial resistance, poultry, zinc, copper

Introduction

SPREAD OF ANTIMICROBIAL RESISTANCE (AMR) has become a significant threat against human and animal health.¹ Parts 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 by which consumers can be exposed to AMR bacteria.^{2–6} In 2012, the NORM-VET monitoring program for AMR in the veterinary and food production sectors detected cephalosporin-resistant *Escherichia coli* in 43% of the Norwegian broiler flocks.⁷ In addition, 32% of *E. coli* from retail chicken meat were categorized as cephalosporin resistant, but even higher numbers were reported from Denmark and Sweden.⁸ 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.^{7,9} In Norway, conjugative extended-spectrum beta-lactamase (ESBL)-encoding plasmids have frequently been discovered in bacteria isolated from broiler chicken, implicating that these animals are a potential reservoir for cephalosporin-resistant *E. coli*.¹⁰

Third and fourth generation cephalosporins have been defined as critically important antimicrobials by the World Health Organization.¹¹ However, ESBL-producing bacteria have been isolated from a variety of animal species in different European countries, which could represent a major threat to public health.^{12–16} In 2015, the Norwegian Scientific Committee for Food and Environment concluded that “The probability of transfer of ESBL/AmpC-producing Enterobacteriaceae, quinolone-resistant *E. coli*, and their respective corresponding genes from live poultry and poultry meat is considered as non-negligible.”¹⁷ Exposure to ESBL-producing Enterobacteriaceae may result in consumers becoming carriers of resistant bacteria, if these bacteria establish themselves as part of the human gut microbiota.^{3,18–20} In situations where these bacteria cause disease, or spread their resistance genes to other pathogenic bacteria, their resistance characteristics may lead to treatment failure and increased mortality.^{21,22}

Conjugation allows bacteria to spread genetic information across diverse bacterial phyla by the use of mobile genetic elements.²³ Inter- and intraspecies dissemination of resistance plasmids is the main mechanism of horizontal gene

transfer of AMR between bacteria and is mediated by the type IV secretion system (T4SS).²⁴ The plasmid-located *tra* operon encodes the genes important for transport of the plasmid from the donor to the recipient cell. The TraB protein exhibit ATPase activity thought to provide energy for the assembly of the T4SS machinery and is known to play a major role in the conjugative transfer of plasmid DNA.^{25,26} Transfer of plasmid DNA is initiated and terminated at the origin of transfer, *oriT*. *NikB* encodes a relaxase, responsible for site- and strand-specific cleaving and re-joining of *oriT* at the nick site of the plasmid.²⁷ Hansen *et al.*²⁸ showed that horizontal transfer of plasmids is more important than clonal dissemination for transmission of *bla*_{CMY-2} mediated cephalosporin resistance between animals and humans.

Mo *et al.*²⁹ described two *bla*_{CMY-2} encoding plasmids (pNVI1292/IncK and pNVI2798/IncI1), which were found in *E. coli* strains isolated from retail chicken meat in Norway. They further showed that the plasmids could spread to a variety of Enterobacteriaceae species by conjugation. These plasmids encode two plasmid stability systems, namely *relBE/stbDE* and *pndAC*, which presumably facilitate dissemination and stability of the *bla*_{CMY-2} encoding plasmids. However, the importance of this stability system is not well studied.

Metals like Zn and Cu have antimicrobial effects; the bacterial toxicity of Zn may be due to the chemical affinity for thiol groups of biomolecules and Cu toxicity is based on production of hyperoxide radicals and interactions with cell membranes.³⁰ Bacteria acquire resistance genes against antimicrobials and metals with antibacterial properties on mobile genetic elements.³¹ When two or more resistance genes are present on the same genetic element, or the same mechanism provides resistance against several substances, it may result in co-selection of genes conferring metal and antibiotic resistance. By these mechanisms, selection for resistance to zinc (Zn), copper (Cu), and other potentially toxic metals may act as drivers for spread of AMR.^{32,33} However, data on the required concentration and time exposure for this effect to occur is still lacking.³⁴

Zn and Cu are important elements in the cellular metabolism; they allow many critical enzymes to function properly and are also essential for wound healing, protein synthesis, and maintaining the strength of the skin, blood vessels, and various tissues in the organism.³⁵ Copper and zinc are routinely used as additives in animal feed in livestock farming, however, when animals receive feed containing larger amounts of Zn and Cu than what they biologically require the excess of metals are thereby released into the environment.³⁶ Zn and Cu are therefore commonly found in soil, water, plants, and in manure from various farm animals, including chicken.³⁷ The occurrence of Zn and Cu may have beneficial fertilizing properties, as these are important trace elements for plants, but may also be of environmental concern when present in large quantities, affecting groundwater, surface water, and aquatic animals.^{38–41}

We hypothesize that excess levels of Zn and Cu promote transfer of resistance plasmids from ESBL-producing *E. coli*. The aim of our study was to assess the effect of Zn and Cu on conjugation of *bla*_{CMY-2} carrying plasmids from ESBL-producing *E. coli* collected from retail chicken meat.

Materials and Methods

Bacterial strains and growth media

Two *E. coli* strains isolated from retail chicken meat were used as plasmid donors in the conjugation experiments; *E. coli* 2012-01-1292 (pNVI1292/IncK) and *E. coli* 2012-01-2798 (pNVI2798/IncI1), hereafter named *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1), respectively. The strains were collected through NORM-VET in 2012, and harbored the *bla*_{CMY-2} gene,⁴² the most common plasmid-mediated AmpC-beta-lactamase in *E. coli*.⁴³ Both strains have recently been whole genome sequenced (Mo *et al.*).⁴² The *E. coli* DH5 α strain, resistant to nalidixic acid (Nal^R), was used as recipient. Both donors ferment lactose, while the recipient does not. The bacteria were cultured in Luria-Bertani (LB) broth (Sigma-Aldrich, Germany) or Brain Heart Infusion broth (Sigma-Aldrich) throughout the whole study unless otherwise stated. ZnCl₂ (Sigma-Aldrich) and CuSO₄ (Merck, Germany) were used as sources of Zn and Cu throughout the whole study.

In vivo and experimental concentrations of Zn and Cu

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

Experimental concentrations for ZnCl₂ and CuSO₄ to be used in the conjugation assays were chosen according to the two following criteria: (1) without exceeding the respective minimum inhibitory concentrations (MICs) and (2) reflect the range of concentrations found *in vivo*.

Minimum inhibitory concentrations

MICs for ZnCl₂ and CuSO₄ were determined for all strains by serial dilutions (Supplementary Table S1) in LB-broth.⁴⁴ The concentrations tested ranged from 0.03 μ g/mL to 3 mg/mL for ZnCl₂ and 0.01 μ g/mL to 1 mg/mL for CuSO₄.

Bacterial growth of donor and recipient strains

Overnight cultures of each strain were diluted 1:1,000 in fresh LB-broth containing the selected experimental concentrations of ZnCl₂ and CuSO₄. A control without supplemented metals was included in each experiment. A volume of 200 μ L of each sample was transferred to a 96-well microtiter plate (Greiner; Sigma-Aldrich) and incubated at 37°C in Tecan platereader. The optical density (OD₆₀₀) was measured in the cultures every 10 min for 24 hr. Each experiment was performed in three biological replicates, with three replicates of each sample.

Conjugation study

Conjugation experiments were conducted in LB-broth according to Sunde and Sorum,⁴⁵ with minor modifications. Briefly, the donor and recipient strains were grown overnight in LB-broth at 37°C and subsequently diluted to an OD₆₀₀ equivalent to a McFarland standard no. 1 ($\sim 3 \times 10^8$

bacteria/mL).⁴⁶ A volume of 500 μ L of the recipient strain culture and 10 μ L of the donor strain culture was mixed in 4 mL LB-broth containing the selected experimental concentrations of ZnCl₂ or CuSO₄, respectively, including a control without supplements. All cultures were incubated for 4 hr at 37°C. LB-broth supplemented with Zn or Cu were prepared 1 day before the experiment and incubated at 37°C overnight under agitation to prevent precipitation of the added metal. Dilutions of each mating culture were plated on Mueller-Hinton agar plates (Sigma-Aldrich) supplemented with 20 mg/L nalidixic acid and/or 0.5 mg/L cefotaxime, and incubated for 24 and 48 hr at 37°C. To quantify the conjugation and to test for toxic effects of the metals on the donor and recipient strains individually, we plated the mating cultures on donor-, recipient-, and transconjugant selective plates. The conjugation frequency was determined by manual counting of colony forming units (CFU) and dividing the number of transconjugants with the number of recipients.

Representative colonies from each transconjugant-selective plate was plated on bromothymol lactose blue agar (Sigma-Aldrich) to distinguish transconjugants from spontaneously mutated donors. In addition to different abilities to ferment lactose, the transconjugants and mutated donors are distinguishable by colony morphology. PCR analysis of bacterial colonies was conducted to confirm that the transconjugants harbored the *bla*_{CMY-2} gene.⁴⁷

Plasmid stability

The transconjugants from the conjugation assay (DH5 α with pNV11292/IncK or pNV12798/IncI1) were propagated by serial transfers as previously described.^{48,49} with minor modifications. Briefly, 10 μ L of stationary phase culture was transferred into 990 μ L of fresh LB-broth supplemented with 0.05 mg/mL ZnCl₂ or 0.01 mg/mL CuSO₄ every 12 hr for 5 days, corresponding to $\sim$ 300 generations. Cultures in LB-broth without metal supplements were used as controls. The bacteria were grown at 37°C under agitation (180 rpm). To confirm the presence or absence of plasmids pNV11292/IncK and pNV12798/IncI1, serial diluted samples from each transfer were plated on Mueller-Hinton agar plates with or

without antibiotics (20 mg/mL nalidixic acid and 0.5 mg/mL cefotaxime). Plates without antibiotics were incubated at 37°C for 24 hr and plates containing antibiotics were incubated for 48 hr. The number of CFU was counted manually. The presence of the *bla*_{CMY-2} plasmid was confirmed by colony PCR.⁴⁷

Sample preparation, RNA isolation, and quantitative PCR

The transcriptional analysis of genes involved in conjugation, *nikB* and *traB*, was performed in *E. coli* 1292 (IncK). An overnight culture was inoculated in fresh LB-broth with ZnCl₂ or CuSO₄. Bacteria cultured in plain LB media were used for comparison. The samples were incubated for 4 hr at 37°C. Total RNA was isolated from harvested *E. coli* using PureLink RNA Mini Kit (Life technologies, Carlsbad, CA) according to the manufacturer's protocol. On-column PureLink DNase (Life technologies) treatment was performed according to the protocol. An amount of 100 ng of total RNA was used to synthesize cDNA using High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, California, CA) according to the manufacturer's protocol. Primers were designed by Primer Express software, and the sequences are listed in Table 1. A standard curve using serial dilution of DNA from *E. coli* 1292 was made to calculate PCR efficiency for each primer pair. Real time reactions were performed using Power SYBR Green PCR Master Mix (Life Technologies) and real time amplification was carried out using Step One Real Time PCR system (Applied Biosystems). The data were collected and analyzed by normalization against the endogenous control gene *rpoA* using StepOne Software v2.3.

Statistical analysis

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 ZnCl₂ and CuSO₄ levels on the experiments. We adjusted for the impact of strain and biological replicate. Results were

TABLE 1. PRIMERS USED IN THIS STUDY FOR QUANTITATIVE POLYMERASE CHAIN REACTION FOR EVALUATION OF EXPRESSION OF GENES INVOLVED IN CONJUGATION

Gene	Primer sequences		Slope ^a	%Eff ^b	Gene description ^c
	Forward (5'-3')	Reverse (5'-3')			
<i>nikB</i>	CGCCTGATAATGGCTGCTTT	CGCTGTTTTGCGCACATA	-3.44	95.05	Conjugal transfer relaxase protein NikB
<i>rpoA</i>	GGCACAATCGATCCTGAAGAG	TTCCAGTTGTTTCAGCCAGAATG	-3.37	97.85	DNA-directed RNA polymerase, alpha subunit
<i>traB</i>	GGCAAAAACCGCGAACAT	TCCAGGGAAGGACGTGTTG	-3.4	96.75	Type IV secretion/conjugal transfer ATPase, VirB4 family

^aThe slope was calculated from the regression line in the standard curve.

^bThe efficiency was calculated using the slope of the regression line in the standard curve.

^cAccording to UniProt Database.

reported as coefficients with corresponding p -values when 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

Selected Zn and Cu concentrations

The poultry feed contained 100 mg/kg Zn and 15 mg/kg Cu, and the results from the poultry cecum-content ranged from 8.52 to 83.5 mg/kg Cu and 71.9 to 225 mg/kg Zn. MIC data for *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) is shown in Table 2. Thus, the experimental concentrations were selected as follows: 0.05, 0.125, and 0.2 mg/mL for ZnCl₂, and 0.01, 0.255 and 0.5 mg/mL for CuSO₄. This is equivalent to 0.024, 0.06, and 0.096 mg/mL elemental Zn and 0.0039, 0.101, and 0.199 mg/mL elemental Cu.

Bacterial growth

Growth curves for the donor and recipient strains are shown in Fig. 1. The lowest concentration of ZnCl₂ did not appear to have any effect on planktonic growth, while the two highest concentrations displayed a delayed growth rate (0.125 and 0.2 mg/mL). None of the concentrations of CuSO₄ appear to have any effect on the planktonic growth of the strains. The two donor strains *E. coli* 1292 (IncK) and *E. coli* 2798 (IncI1) showed higher growth rates compared to the recipient *E. coli* DH5 α strain. Growth rates are provided in the supplementary material (Supplementary Table S2).

Conjugation study

The conjugation frequency was first determined in a pilot study where samples were taken after 4 and 24 hr of mating. No difference in the conjugation frequency was observed between the two time points (data not shown). Therefore, 4 hr was chosen for further conjugation and transcriptional studies. The mating cultures were plated on donor-, recipient-, and transconjugant selective plates to calculate the conjugation frequency (Table 3) and to evaluate any inhibiting effect of the metals in the mating cultures. Neither the donors nor the recipient showed any reduction in CFU compared to the control (data not shown).

As shown in Fig. 2 the effect of increasing levels of ZnCl₂ and CuSO₄ on conjugation were clear. These findings were supported by the nonparametric regression analysis, where strong effects of Zn and Cu levels were found ($p < 0.001$). There was no effect of the donor strains on the number of recipients in the conjugation experiments in ZnCl₂ ($p =$

FIG. 1. Growth curves. (A) *Escherichia coli* 1292 (IncK), (B) *E. coli* 2798 (IncI1), (C) *E. coli* DH5 α (Δ) 0.05 mg/mL ZnCl₂, ($\blacktriangle$) 0.125 mg/mL ZnCl₂, ($\blacktriangle$) 0.2 mg/mL ZnCl₂, ($\square$) 0.01 mg/mL CuSO₄, ($\blacksquare$) 0.255 mg/mL CuSO₄, ($\blacksquare$) 0.5 mg/mL CuSO₄, ($\circ$) Control. The data are based on three biological replicates with three technical replicates each.

0.31) and CuSO₄ ($p = 0.55$), which confirms that the recipients are not outcompeted by the donors (Supplementary Fig. S1). No statistical effect of replicate could be observed in the data. Our results show that conjugation of the IncK plasmid was reduced by more than 98% at all concentrations of Zn tested compared to the control. This was also observed for the two highest concentrations of CuSO₄ while 0.01 mg/mL CuSO₄ gave a 90% reduction of conjugation for the IncK plasmid. At the two highest concentrations of Zn and Cu there was a more than 90% reduced conjugation of the IncI1 plasmid, while the lowest concentrations of Zn and Cu gave

TABLE 2. DETERMINED MINIMUM INHIBITORY CONCENTRATIONS OF ZnCl₂ AND CuSO₄ FOR DONOR AND RECIPIENT STRAINS

Metal/ strain	<i>E. coli</i> 1292 (IncK), mg/mL	<i>E. coli</i> 2798 (IncI1), mg/mL	<i>E. coli</i> DH5 α , mg/mL
ZnCl ₂	0.4	0.4	0.3
CuSO ₄	0.9	1	0.75

TABLE 3. CONJUGATION FREQUENCIES IN RESPONSE TO DIFFERENT CONCENTRATIONS OF ZnCl_2 AND CuSO_4

Strain	Additive	mg/mL	Conjugation frequency ^a	SD+/-
<i>E. coli</i> 2798 (IncI1)	CuSO_4	0.01	1.26E-04	1.03E-04
		0.255	3.95E-06	9.12E-06
		0.5	NTD	0.00E+00
	ZnCl_2	0.05	9.31E-05	6.00E-05
		0.125	1.69E-05	6.85E-06
		0.2	NTD	0.00E+00
<i>E. coli</i> 1292 (IncK)	Control	0	2.04E-04	1.31E-04
		0.01	3.37E-05	4.57E-05
	CuSO_4	0.255	2.37E-06	2.28E-06
		0.5	NTD	0.00E+00
	ZnCl_2	0.05	1.04E-06	1.35E-06
		0.125	NTD	0.00E+00
		0.2	NTD	0.00E+00
	Control	0	9.21E-05	5.31E-05

^aConjugation frequencies were calculated as the mean number of transconjugants divided by the mean number of recipients from all replicates, for each combination.
NTD, no transfer detected (no colonies detected) on transconjugant selective plate.

a reduction of 58% and 41%, respectively. A representative selection of colonies was picked for further confirmation and all of them were confirmed PCR-positive for the *bla*_{CMY-2} gene. Furthermore, all tested transconjugants gave a negative result on the bromomethyl lactose agar, confirming the recipient phenotype.

Plasmid stability

Both plasmids carry genes encoding stability systems (*relBE/stbDE* and *pndAC*). We wanted to investigate whether these stability systems promote plasmid maintenance, and if Zn and Cu had any impact on the stability of the plasmids. By propagating the transconjugants in monocultures for ~300 generations and calculate CFU after plating on transconjugant selective plates, we could show that both plasmids were maintained within the transconjugants (Supplementary Fig. S2). Furthermore, the results showed that there was no difference in plasmid stability between the IncK- and the IncI1 plasmid and that subinhibitory concentrations of ZnCl_2 and CuSO_4 did not have any influence on the maintenance of the plasmids ($p < 0.05$).

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

Qualitative PCR analysis showed that genes involved in transfer of the IncK plasmid had a significantly reduced expression following exposure to 0.05 mg/mL ZnCl_2 or 0.01 mg/mL CuSO_4 compared to the control ($p < 0.05$) (Fig. 3). Specifically, the expression of *traB* was reduced by 87% and 92% in the samples with ZnCl_2 and CuSO_4 compared to the control without ZnCl_2 or CuSO_4 . The expression of *nikB* was reduced by 97% and 96% in response to ZnCl_2 and CuSO_4 compared to the control.

Discussion

Conjugation is a complex mechanism that allows bacteria to spread genes encoding beneficial traits that will increase

FIG. 2. Distribution of transconjugants demonstrating a dose-dependent reduction of transconjugants in the presence of ZnCl_2 (A) and CuSO_4 (B). Concentrations are provided in mg/mL for ZnCl_2 and CuSO_4 . The horizontal line within the box represents the median. Boxes represent 50% of the data and the whiskers, highest and lowest values, while dots represent outliers. The data are based on four biological replicates with three technical replicates each.

bacterial survival. A previous study has shown that the two plasmids included in this study are inter- and intra-species transferable at different conditions, indicating that they may contribute to the maintenance of antibiotic-resistant genes in the environment.²⁹ In contrast to earlier studies, which show

FIG. 3. Expression of genes involved in conjugation in *Escherichia coli* 1292 (IncK) in response to ZnCl_2 and CuSO_4 . The data are presented as mean values $\pm$ SD ($n = 6$).

that Zn and Cu are associated with increased conjugation,^{50–53} we found that Zn and Cu reduced the conjugation frequency between *E. coli* strains in a concentration-dependent manner. There was also no difference in the number of colonies on the donor- and recipient-selective plates from the mating cultures, which confirms that Zn and Cu does not affect growth of donor- or recipient strains. Toxic effects of the metals can therefore not explain the observed reduction in conjugation frequencies. A pilot study was performed at both 4 and 24 hr of mating (data not shown). No difference in conjugation rate was observed between the two time points, which is consistent with a previous report by Mo *et al.*²⁹ Altogether, this justifies our choice of using the 4 hr time point in the mating experiments. The growth curves showed that the recipient strain grew slightly slower than the donor strain, however, this was compensated for by using larger amounts of recipient cells in the conjugation experiments.

The plasmids used in this study did not contain any known Zn or Cu resistance genes,⁴² which rules out co-selective mechanisms. Our results are consistent with a recent study, which showed that metal stress (Zn and Cu included) decreased plasmid transfer frequencies to bacterial communities independent of metal-resistance.⁵⁴ Suzuki *et al.*⁵⁵ also showed a reduction in horizontal transfer of the tetracycline resistance gene *tet*(M) in response to Zn and Cu exposure. Reduced plasmid transfer in response to metal stress could be a consequence of changes in metabolic status, decrease in plasmid replication, activation of the SOS-response, or a combination of different mechanisms.^{56–58} This study also showed that both the IncK and IncII plasmids remained stable in their host throughout several generations, independent of presence of Zn or Cu.

The molecular mechanisms of heavy metals on conjugative transfer of resistance genes, with exception of co-selective mechanisms, have rarely been investigated.⁵⁹ To understand the effect of Zn and Cu on conjugation in our study, we performed a real-time transcriptional analysis that showed a reduction in expression of conjugation-associated genes in response to subinhibitory concentrations of Zn and Cu. However, our findings contrast the report by Zhang *et al.*,⁵² who found that different concentrations of heavy metals increased conjugation and upregulated the expression of genes involved in conjugation of plasmids. Strain background, concentrations of the metals, and experimental conditions might explain the contradictory results.

Conjugative transfer of plasmids are controlled by a wide range of genes.⁶⁰ A failure to form a functional relaxosome, mediated by the NikB protein, can result in an incapability of the plasmid to mobilize.⁶¹ Our results from the transcriptional analysis could therefore indicate that the reduced expression of *nikB* in response to Zn and Cu could lead to a dysfunctional relaxosome, and the incapability of the plasmid to transfer from the donor to the recipient strain. The expression of the *traB* gene involved in plasmid transfer was also significantly reduced, which indicates that metals disturb the function of the conjugation machinery.

Our goal was to use concentrations of Zn and Cu that mimic the conditions found in the chicken intestines as closely as possible. We focused on the effect of heavy metals on the plasmid-encoded genes by using an experimental setup that excluded the effect of co-selection.

However, we cannot exclude that Zn and Cu interfere with expression of chromosomal genes or genes located in the recipient strain. The effect of Zn and Cu on the SOS-response may be of future interest, in addition to investigate the combined effect of Zn and Cu on conjugation. The expression of other conjugational genes needs to be studied to learn more about how metals interfere with conjugation at a transcriptional level. It would also be beneficial to study horizontal gene transfer in more complex models than the ones used in the present study; preferable models that to a larger degree resembles the “real life” conditions in the chicken intestinal environment. Nevertheless, our results indicate altogether that Zn and Cu interfere with genes involved in conjugation, and thereby decrease the frequency of conjugational transfer of plasmids between *E. coli*.

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Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2

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