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Original Article

Copper affects virulence and diverse phenotypes of uropathogenic *Proteus mirabilis*

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KEYWORDS
Copper;
Virulence;
Uropathogenic *P. mirabilis*

Abstract *Background:* Copper plays a role in urinary tract infection (UTI) and urinary copper content is increased during *Proteus mirabilis* UTI. We therefore investigated the effect of copper on uropathogenic *P. mirabilis* and the underlying mechanisms, focusing on the virulence associated aspects.
Methods: Mouse colonization, swarming/swimming assays, measurement of cell length, flagellin level and urease activity, adhesion/invasion assay, biofilm formation, killing by macrophages, oxidative stress susceptibility, OMPs analysis, determination of MICs and persister cell formation, RT-PCR and transcriptional reporter assay were performed.
Results: We found that copper-supplemented mice were more resistant to be colonized in the urinary tract, together with decreased swarming/swimming, ureases activity, expression of type VI secretion system and adhesion/invasion to urothelial cells and increased killing by macrophages of *P. mirabilis* at a sublethal copper level. However, bacterial biofilm formation and resistance to oxidative stress were enhanced under the same copper level. Of note, the presence of copper led to increased ciprofloxacin MIC and more persister cell formation against ampicillin. In addition, the presence of copper altered the outer membrane protein profile and triggered expression of RcsB response regulator. For the first time, we unveiled the

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pleiotropic effects of copper on uropathogenic *P. mirabilis*, especially for induction of bacterial two-component signaling system regulating fitness and virulence.
Conclusion: The finding of copper-mediated virulence and fitness reinforced the importance of copper for prevention and therapeutic interventions against *P. mirabilis* infections. As such, this study could facilitate the copper-based strategies against UTI by *P. mirabilis*.

Introduction

Copper is an essential trace element with a crucial role in several bacterial biological processes as a cofactor of cuproenzymes[1]; however, high level of copper is toxic.[2,3] The toxicity can occur through copper-mediated generation of highly toxic reactive oxygen species (ROS) during redox cycling between Cu(I) and Cu(II), targeting bacterial membranes, DNA, etc. to cause cell damage and death, displacing other metal from mental-binding enzymes to inactivate enzymes or interacting with thiol groups to affect protein folding and function.[2–4] Therefore, intracellular copper concentrations need to be tightly regulated to maintain copper homeostasis. Bacteria have developed a number of mechanisms directed against excess copper. These mechanisms include induction of direct copper homeostasis such as the efflux and sequestration of copper and increased adaptive stress responses against ROS or misfolded proteins.[2,5,6] The multifaceted responses of pathogenic bacteria to copper include both common and species-specific adaptations.[7] The mechanisms used and the coordination of regulatory responses can vary significantly between bacterial species.

It has long been known that copper participates in the function of the immune system.[8] Copper accumulates at sites of infection,[9,10] including in urine and blood, and its increased influx into macrophages leads to bacterial clearance.[11] In this regard, copper-deficient animals are more prone to infection, whereas copper-rich diets increase their ability to resist invading pathogens.[12,13] Hence copper resistance is important for virulence of bacterial pathogens, enabling them to fight for the copper insult while keeping enough copper for essential cuproenzymes. Numerous studies have shown the linkage of copper and pathogenesis[14] and copper resistance has been shown to be a critical determinant of bacterial fitness at the host-pathogen interface in a variety of pathogens.[15–17]

Urinary tract infection (UTI) is a major global infectious disease affecting millions of people annually. *Proteus mirabilis* is an important pathogen of the urinary tract, especially in patients with indwelling urinary catheters.[18] It has been shown that human urinary copper content is elevated during UTI caused by the major uropathogens *Escherichia coli* (UPEC), *P. mirabilis* and *Klebsiella pneumoniae*.[12] In addition, it was found copper-deficient mice are highly colonized by UPEC, indicating that copper is involved in the limiting of bacterial growth within the urinary tract. Namely, copper is a host effector that is involved in protection against pathogen colonization of the urinary tract.

P. mirabilis exhibits a form of multicellular behavior, termed swarming,[19] which vegetative bacteria differentiate into elongated and hyperflagellated swarmer cells capable of rapid population migration until they slow down into consolidation phase. Swarming motility is associated with ascending UTI and the result of complex signal transduction and gene regulation[19,20] involving the flagellar master regulator FlhDC, chemotaxis proteins and specific environmental cues such as glutamine.[19] It is generally believed that signals could be sensed and transmitted by two-component systems (TCS) and then cytosolic regulators, leading to a complex regulatory network. Other strategies of pathogenesis employed by *P. mirabilis* include adherence against urine flow via fimbriae,[21] biofilm formation within catheters, and urease production.[22] It is worth noting that the ability of *P. mirabilis* to express virulence factors is coupled to swarming differentiation.

Knowing copper plays a role in UTI, urinary copper content is increased during *P. mirabilis* UTI and copper can generate diverse effects in different microbial cells,[2,5,6] we therefore investigated the effect of copper on uropathogenic *P. mirabilis*, especially focusing on the fitness and virulence associated aspects. Looking forward, we can capitalize on the knowledge gained and embark on a way towards identifying novel strategies that target virulence traits or copper-mediated regulatory pathways against UTI caused by *P. mirabilis*, for example to augment the copper-based host defense.

Methods

Mouse colonization

Five-week-old female ICR mice were provided drinking water alone ($n = 5$) or water supplemented with $CuCl_2$ (1 mg/ml) ($n = 5$) for 8 days. Mice were assessed for signs of dehydration and diarrhea every day. After 8 d, mice were transurethrally inoculated with 1.5×10^8 CFU of wild-type *P. mirabilis*. Three days later, mice were sacrificed and bacterial loads (CFU counts) of bladders and kidneys were determined as described.[13] All mice were purchased from the National Laboratory Animal Center, Taiwan, and housed at the Laboratory Animal Center of National Taiwan University College of Medicine. The Institutional Animal Care and Use Committee (IACUC) of the National Taiwan University College of Medicine approved the mouse care and handling procedures.

Bacterial strain, reagents and growth conditions

A bacterial strain used in this study is *P. mirabilis* N2, containing no native plasmid and being isolated from a patient of urinary tract infection involving renal stone formation. All chemicals were obtained from the Sigma—Aldrich, and primer sequences are given in Table 1. Bacteria were stored at −80 °C and routinely cultured in Luria—Bertani (LB) broth at 37 °C. The LSW agar was used to prevent swarming motility for colony counting.

Swarming/swimming assays, measurement of cell length, flagellin level and urease activity

The swarming and swimming assays were performed as described previously.[23] The overnight bacterial cultures (5 μl) were inoculated onto the center of LB swarming plates containing 1.5% agar, followed by incubation at 37 °C and the swarming distance was measured at 1-h intervals. The swimming migration was determined in a similar way except for incubation for 18 h on the 0.3% LB agar plate. Flagellin level examined by Western blot and cell length were determined as described.[23] Urease activity was measured by the phenol red colorimetric method.[24]

Adhesion and invasion to urothelial cells, biofilm formation and eDNA analysis

Adhesion and invasion assays were performed as described previously.[21,25] Biofilm-forming ability was assessed by measuring the ability of cells to adhere to the 96-well microtitre plate as described.[26] The eDNA amount was determined using overnight bacterial cultures as described previously.[26]

Bacterial killing by macrophages

Overnight bacterial cultures treated with $CuCl_2$ (1 mM) or not were washed and applied to THP-1 cells[21] grown to 90% confluence (about 5×10^5 cells) in the 12-well plates with multiplicity of infection (MOI) of 10 to evaluate killing of bacteria by macrophages. After co-incubation for 2 h in RPMI-1640, macrophages were lysed without affecting viability of bacteria to enumerate the viable bacterial count. The bacteria only controls incubated in RPMI with indicated concentrations of copper or not were used in parallel. The percent *P. mirabilis* survived macrophage killing in the presence and absence of copper was calculated by dividing CFU of macrophage-containing sample by CFU of control.

Menadione and H_2O_2 susceptibility

Overnight cultures were diluted, grown to OD_{600} of 0.6 with copper (1 mM) or not, and adjusted to 10^8 cells/ml in LB broth. For menadione susceptibility, cells were 10-fold serially diluted and a 5-μl aliquot was applied onto LSW agar plates containing menadione (40 μg/ml) or not for each dilution to determine the numbers of bacteria surviving the stress. For H_2O_2 susceptibility, cells were exposed to 30 mM H_2O_2 for 20 min at 37 °C and subject to serial dilution and determination of the bacterial numbers as for menadione. The percent survival in the presence and absence of copper was calculated by dividing CFU of menadione or H_2O_2 treated sample by CFU of the untreated control.

Determination of MICs and persister cell formation

MICs of antibiotics were determined by the broth microdilution method according to the guidelines proposed by the Clinical and Laboratory Standards Institute. Persister cell formation was performed as described.[27] Cells were grown in LB medium to a turbidity of 0.5 at 600 nm. The cell concentration was adjusted to obtain 10^8 CFU/ml and these cells were exposed to a lethal dose of ampicillin (100 μg/ml) for 4 h before 10-fold serial dilution and determination of persister cell CFU by applying a 5-μl aliquot for each

Table 1 Primers used in this study.

Primers	Sequence (5′ to 3′)	Description
gyrB-RT-F	GACCCGTACGCTAAACAAC	For *gyrB* real-time PCR. Paired with "*gyrB*-RT-R".
gyrB-RT-R	AGAAATAACCGCAATCAGG	
flhDC-RT-F	CACGAGCATGGACATTAG	For flhDC real-time RT-PCR. Paired with "flhDC RT R".
flhDC-RT-R	GCAGGATTGGCGGAAAGTT	
realtime-*sodA*-F	GTT TTG GTT CAG GTT GGG	For *sodA* real-time RT-PCR. Paired with "realtime-*sodA*-R".
realtime-*sodA*-R	ATG GGC TAT CTT GGT TTG	
rcsB new RT-F	AGGTTTCCTTGTCACTGAGATTGC	For *rcsB* real-time RT-PCR.
rcsB new RT-R	ATCATTGTCGACACCGAGCTT	
0749*vipA* promoter F	CTTAAGGGGCTTGAAAACCTATTATTCCC	For *vipA* reporter assay. Paired with "0749*vipA* promoter R".
0749*vipA* promoter R	CTGCAGGGCAACACTACCGAGATTTTTC	
pmfA RT F	GCCTGCACCAAAAGTAAGTTCA	For pmfA real-time RT-PCR. Paired with "pmfA RT R".
pmfA RT R	TGGCAGTTTTCGCTACAGTTGT	
scsA promoter-F	ATCGACCTGAGGCTCAAAGTCAAATGTACCAT	For scsA reporter assay. Paired with "scsA promoter-R".
scsA promoter-R	CTGTAGGATCCAGATTTCCTCATTTGTTGAC	

dilution onto LSW agar plates. Persistence frequency was calculated by dividing the number of CFU/ml in the culture after incubation with antibiotic by that before adding antibiotic.

Analysis of outer membrane proteins (OMPs)

Overnight bacterial cells were broken by a sonicator and the membrane fraction was prepared as described previously.[25] The inner membrane was solubilized by adding Sarkosyl NL-97 and the outer membrane fraction was pelleted by centrifugation and assayed by SDS-PAGE.

Real-time reverse transcription-PCR (RT-PCR)

The overnight bacterial cultures of the wild type were diluted 100-fold and incubated for 5 h at 37 °C. Total RNA was extracted and RT-PCR was carried out to determine the mRNA level of relevant genes normalized against the *gyrB* mRNA as described previously.[23]

Transcriptional reporter assay

For transcriptional reporter assay, the transcriptional reporter plasmids of relevant genes were constructed and the XylE activity was measured as described previously.[23]

Results

Copper supplementation mitigated mouse colonization by *P. mirabilis* in the urinary tract

Multiple host-pathogen interaction studies have garnered evidence that copper is weaponized by the host immune system.[11,28] Copper is a protective innate immune effector mobilized to urine as a host response to UTI caused by Gram-negative bacterial pathogens.[3,12] In addition, it has been shown that copper homeostasis is a key attribute for fitness and virulence during UTI.[13,29] Accordingly, it is not surprising that copper has appeared as a new antibacterial material. Hence it would be of great interest to assess the role of copper in the pathogenesis of UTI caused by *P. mirabilis*. We firstly tested if copper plays a role in mouse colonization using ICR female mice by providing drinking water with $CuCl_2$ or not. After transurethral inoculation with *P. mirabilis*, bacterial loads of bladders and kidneys were determined at day 3 as described in Methods. We found mice in the $CuCl_2$-supplemented group were colonized by lower levels of *P. mirabilis* both in the bladder and the kidneys than the control (Fig. 1), indicating that $CuCl_2$ supplementation could mitigate *P. mirabilis* colonization in the urinary tract of mice. Based on the involvement of copper in mouse colonization (in vivo), we therefore investigated the effect of copper on *P. mirabilis*, especially for attributes related to virulence and fitness, in vitro. We first examined the growth of *P. mirabilis* in the presence of different concentrations of $CuCl_2$ in LB broth. The LB broth contains undetected copper by using graphite furnace atomic absorption spectrophotometry. An overnight culture of *P. mirabilis* N2 was inoculated into LB broth containing 0, 0.5, 1, 1.5, 2 and 3 mM of $CuCl_2$ and the bacterial growth was monitored by measuring the OD_{600nm} during the 12-h period. As shown in Fig. 2, *P. mirabilis* N2 grew to a similar density in the presence of 0—2 mM $CuCl_2$ and $CuCl_2$ at 3 mM obviously inhibited the growth. We chose the sub-inhibitory $CuCl_2$ concentration of 1 mM not affecting bacterial growth to explore the copper effect.

Copper inhibited swarming and swimming

P. mirabilis swarming motility contributes to establishing a UTI by migrating along the catheters or the ureters.[19] In addition, virulence factors are coordinately expressed during swarming differentiation. Hence we tested the effect of copper on swarming motility and related phenotypes. In the presence of $CuCl_2$ at 1, 1.5, 2 and 3 mM, swarming was inhibited in a dose-dependent manner (Fig. 3A). Swarming cell length and swimming motility was also reduced by 1 mM $CuCl_2$ (Fig. 3B and C). Accordingly, *flhDC* expression and flagellin level revealed by RT-PCR and Western blot, respectively, were significantly reduced in the presence of 1 mM $CuCl_2$ (Fig. 3D and E).

Copper decreased urease activity but increased biofilm formation

P. mirabilis produces urease, an important virulent factor which hydrolyzes urea to provide nitrogen source and also contributes to the formation of urine stones and crystalline biofilms that block the catheter. We found urease activity was reduced in the presence of 1 mM $CuCl_2$ but biofilm formation was increased at the same concentration of copper (Fig. 4A and B). In this regard, eDNA level and

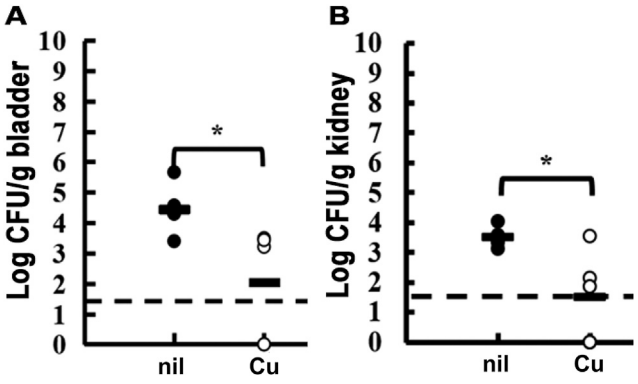

Figure 1. Copper supplementation mitigates wild-type *P. mirabilis* colonization in the urinary tract. ICR mice were provided water alone (n = 5) or water supplemented with $CuCl_2$ (1 mg/ml) (n = 5) for 8 days, followed by transurethral inoculation with wild-type bacteria and bacterial load was determined as described in the Methods. Each symbol corresponds to the data from a mouse, bars indicate median bacterial load and dotted line indicates limit of detection. The data are the averages and standard deviations of three independent experiments. Bacterial burden in control (nil) and copper-supplemented group (Cu) were compared by Mann—Whitney test. Significant difference is indicated with an asterisk (*$P < 0.05$).

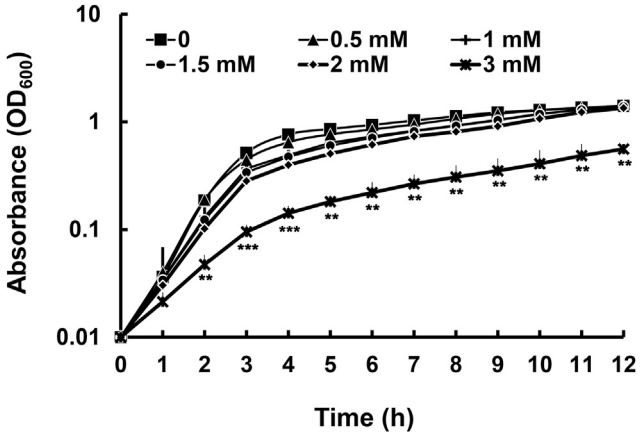

Figure 2. The effect of copper on the growth of wild-type *P. mirabilis*. We monitored the cell growth by measuring the optical density at 600 nm (OD_{600}) at 1 h-interval in LB broth in the presence of $CuCl_2$ at 0, 0.5, 1, 1.5, 2 and 3 mM. The data are the averages and standard deviations of three independent experiments. Significant differences between 0 and 3 mM of $CuCl_2$ were determined by using the Student's *t* test (**P < 0.01; ***P < 0.001).

expression of *pmfA* fimbrial gene were significantly increased by 1 mM $CuCl_2$ (Fig. 4C). The increased fimbrial *pmfA* and the decreased *flhDC* expression (Fig. 3D) correspond to being capable of reciprocal modulation of adherence and motility in *P. mirabilis*.

Copper facilitated macrophage-mediated killing of *P. mirabilis*

Copper is involved in innate protection against a broad range of pathogens. In this aspect, macrophages take advantage of the antibacterial properties of cupric ions mobilized into the sites of infection in the killing of bacterial intruders.[3] Consistently, data accumulated so far suggest that copper tolerance is a general mechanism of virulence in bacteria during infection.[15—17] In general, *P. mirabilis* is assumed not a professional intracellular pathogen; however, in the macrophage niche, survival within a short time represents an important fitness index for better initiating an infection.[25] In view that copper contributes to effective host defense against pathogens within macrophages, we wondered if copper affects the ability of macrophages to eliminate *P. mirabilis*. We challenged macrophages (THP-1 cells) with *P. mirabilis* cells in the presence and absence of $CuCl_2$ and determined the colony count after lysing THP-1 cells to assess the survival of *P. mirabilis*. The results showed the presence of $CuCl_2$ reduced significantly the survival of *P. mirabilis* (Fig. 5).

Copper compromised the ability of adhesion and invasion into the urothelial cells

To evade the host defense mechanism, uropathogens adhere and invade into the epithelium.[30] Given copper is employed by the body's innate immunity in a toxic attack against pathogenic bacteria, we then assessed the ability of *P.*

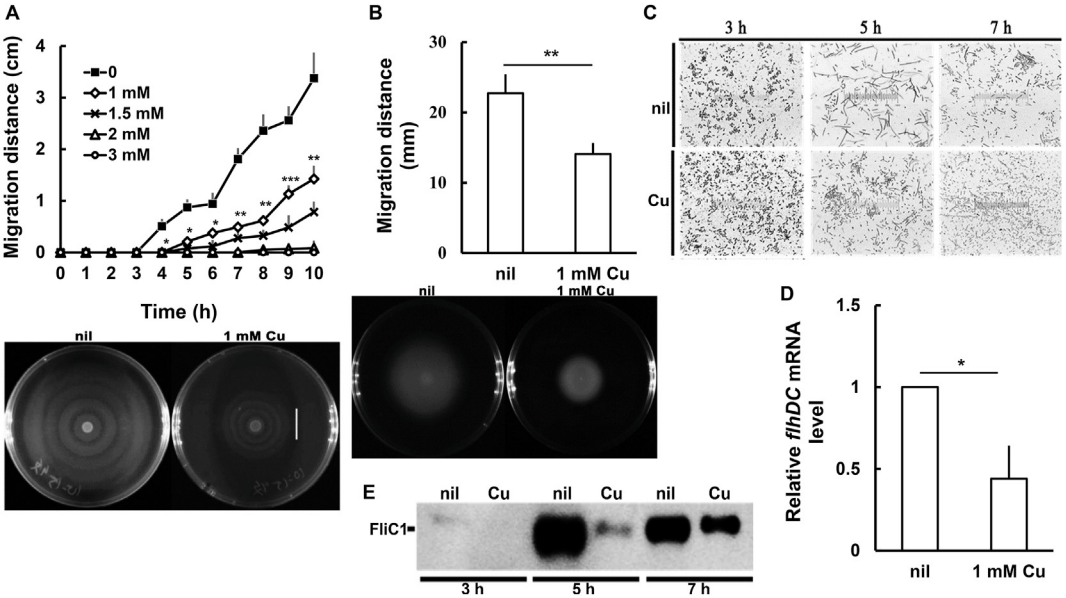

Figure 3. The effect of copper on swarming (A), swimming (B), cell length (C), *flhDC* expression (D) and flagellin level (E) of wild-type *P. mirabilis*. All assays were conducted as described in Methods with copper or not. Swarming motility was determined at 1-h interval on LB agar plate with copper indicated. Swimming motility was determined at 18 h after inoculation. Lower panels in **A** and **B** show the representative picture for swarming and swimming, respectively. Cell length and flagellin level were monitored at 3, 5 and 7 h after inoculation on LB agar plate. Western blot and RT-PCR were used to assess flagellin level and *flhDC* mRNA amount, respectively. In **D**, the value for the absence of copper was set at 1. The data are the averages and standard deviations of three independent experiments. Significant differences between the presence and the absence of copper were determined by using the Student's *t* test (*P < 0.05; **P < 0.01; ***P < 0.001). nil, no copper; Cu, 1 mM $CuCl_2$.

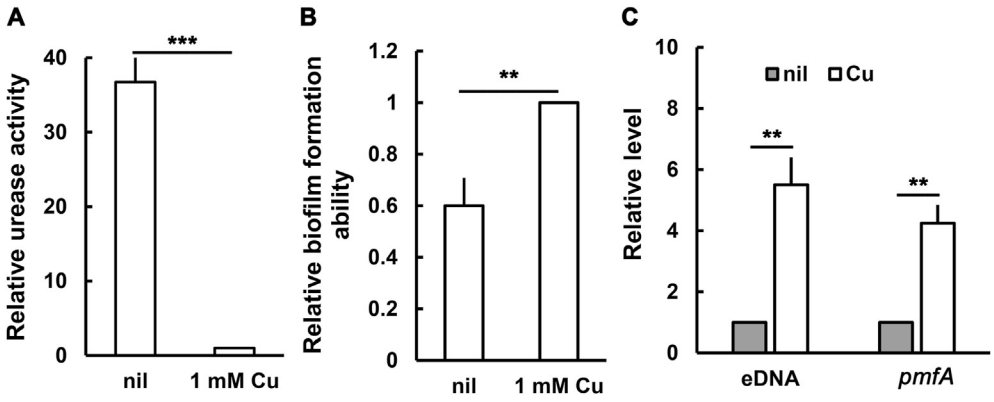

Figure 4. The effect of copper on urease activity (A), biofilm formation (B) and biofilm-associated eDNA production and *pmfA* expression (C) of wild-type *P. mirabilis*. Overnight cultures were subject to assess urease activity, biofilm and eDNA level as described in the Methods in the presence and the absence of copper. For urease activity, a mixture of phenol-red and urea in potassium-phosphate buffer was added to the bacterial pellet (1 ml, $OD_{600} = 0.5$), followed by incubation at 42°Cfor 2 h and the optical density at 560 nm of the supernatant was read. The mRNA amount of *pmfA* was measured from bacterial cultures grown for 5 h in LB broth with $CuCl_2$ or not. The data are the averages and standard deviations of three independent experiments. In **A** and **B**, the value for the presence of copper was set at 1. In **C**, the value for the absence of copper was set at 1. Significant differences between the presence and the absence of copper were determined by using the Student's *t* test (**, $P < 0.01$; ***, $P < 0.001$). nil, no copper; Cu, 1 mM $CuCl_2$.

mirabilis to adhere and invade into NTUB1 urothelial cells. Obviously, pretreatment with $CuCl_2$ impaired the ability of *P. mirabilis* to adhere and invade into urothelial cells (Fig. 6).

Copper could increase survival under H_2O_2 or menadione stress

Copper has been shown to catalyze redox cycling reactions with oxygen or nitrogen species, promoting the formation of reactive radicals.[3] We monitored the effect of copper on *P. mirabilis* survival upon exposure to H_2O_2 or a superoxide generator, menadione (MD). To our surprise, $CuCl_2$ at 1 mM increased survival of *P. mirabilis* from 15 to 43% and 10—55% for MD and H_2O_2 exposure, respectively (Fig. 7A). This indicates copper, as a stressor, can activate certain adaptive or protective responses to alleviate the menadione or H_2O_2-dependent stress in wild-type *P. mirabilis*. By the way, we

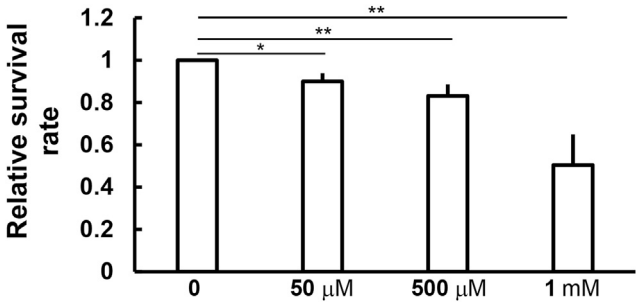

Figure 5. The effect of copper on killing of wild-type *P. mirabilis* by macrophages. Following overnight treatment of $CuCl_2$ (50 μM, 500 μM and 1 mM) or not, macrophage killing assay was performed as described in Methods. The value for the absence of copper was set at 1. The data are the averages and standard deviations of three independent experiments. Significant differences from the wild-type were determined by using the Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

examined the expression of *sodA* and *scsA* genes, responsible for defense against superoxide and H_2O_2, respectively,[31,32] in response to the presence of $CuCl_2$. The result showed $CuCl_2$ treatment led to increased expression of both *sodA* and *scsA* genes (Fig. 7B and C), corresponding to the increased survival of MD or H_2O_2 exposure after $CuCl_2$ treatment in Fig. 7A.

Copper affected expression of type VI secretion system

The type VI secretion system (T6SS), a molecular device for the delivery of proteins from one cell into another, is one of the weapons for different bacteria to compete with each other and gain predominance in their niches.[33] The T6SS

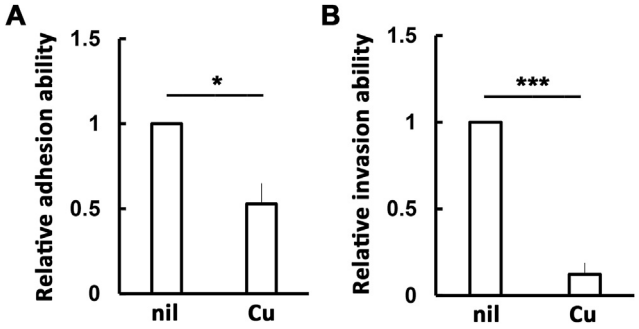

Figure 6. The effect of copper on adhesion (A) and invasion (B) abilities of wild-type *P. mirabilis*. Following overnight treatment of 1 mM $CuCl_2$ or not (nil), adhesion and invasion assays were performed as described in Methods. The data are the averages and standard deviations of three independent experiments. The value for the absence of copper was set at 1. Significant differences between the presence and the absence of copper were determined by using the Student's *t* test (*, $P < 0.05$; ***, $P < 0.001$).

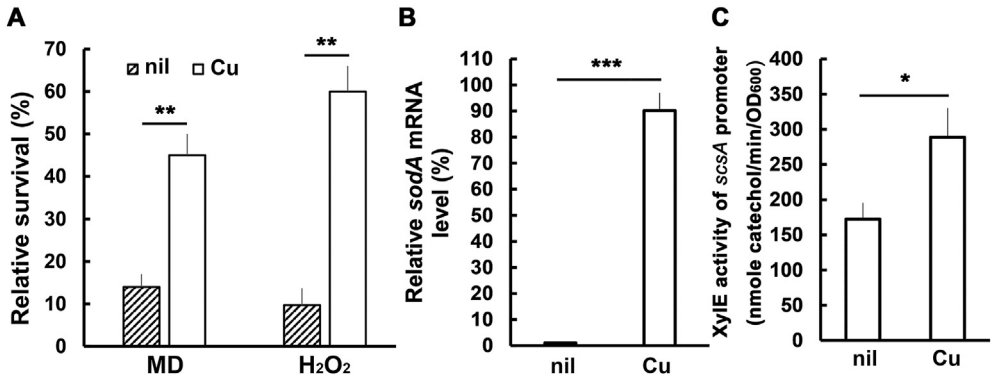

Figure 7. The effect of copper on tolerance of *P. mirabilis* to menadione or H_2O_2 (A), along with expression of *sodA* (B) and *scsA* (C). Following by treatment of 1 mM $CuCl_2$ for 5 h or not, the assessment of menadione (MD) and H_2O_2 susceptibility was performed as described in Methods. The numbers of bacteria surviving the stress were determined and relative bacterial cell survival (%) was obtained relative to the no stress control. The *sodA* mRNA level and *scsA* promoter activity were determined by RT-PCR and transcriptional reporter assay as described in Methods in the presence of copper or not. In **B**, the value for the absence of copper was set at 1. The data are the averages and standard deviations of three independent experiments. Significant differences between the presence and the absence of copper were determined by using the Student's *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). nil, no copper; Cu, 1 mM $CuCl_2$.

function depends on the contractile sheath comprised of VipA and VipB proteins that form a membrane-spanning complex to deliver the cell-puncturing proteins, Hcp and VgrG, and effector proteins.[34] In *P. mirabilis*, the 16 T6SS-associated genes constitute an operon starting with *vipA* (our unpublished data). We found $CuCl_2$treatment inhibited *vipA* promoter activity (Fig. 8), implying copper could modulate the T6SS function in *P. mirabilis*.

Copper brought about ciprofloxacin resistance

It was shown that metals such as copper could affect antibiotic activity.[35] For example, copper has been used as a carbapenem adjuvant to potentiate the antibacterial activity of carbapenems against NDM-positive *E. coli*.[36] However, there is a growing body of evidence for copper-driving antibiotic resistance in metal-treated bacteria, due to selection of genetic elements carrying both copper and antibiotic resistance genes or activation of antibiotic resistance pathways.[35] In addition, many classes of antibiotics can form complexes with copper to hinder (or enhance) antibiotic activity.[35] To test the effect of copper on drug susceptibility, we determined the MICs of ampicillin, gentamicin and ciprofloxacin, representing 3 major antibiotics classes and found that $CuCl_2$-treated *P. mirabilis* was more resistant to ciprofloxacin than the untreated control with an increase in MIC from 0.0125 to 0.2 (Fig. 9 A).

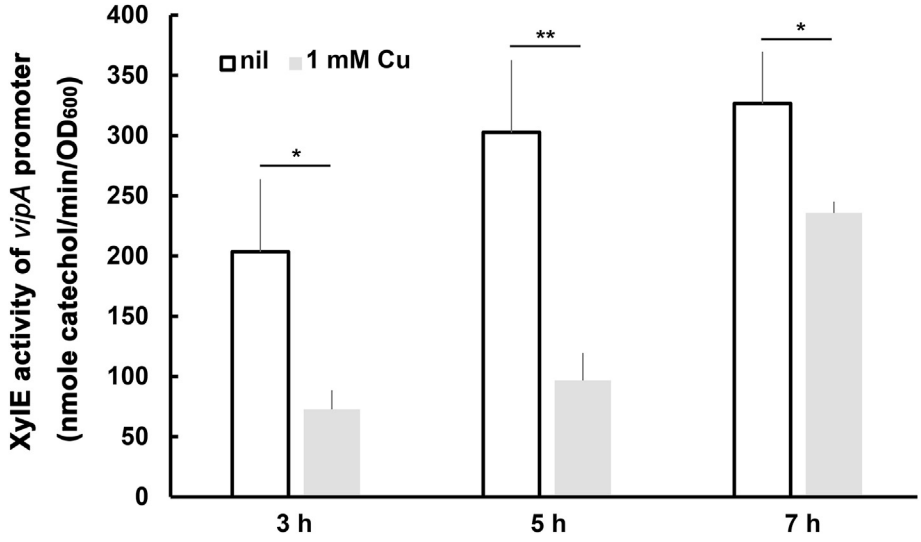

Figure 8. The effect of copper on the *vipA* promoter activity of wild-type *P. mirabilis*. The activity of promoter was determined in the presence of $CuCl_2$ or not (nil) by the transcriptional reporter assay in the *vipA-xylE* reporter plasmid-transformed wild-type after incubation in LB broth for 3, 5 and 7 h. The data are the averages and standard deviations of three independent experiments. Significant differences between the presence and the absence of Cu were determined by using the Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

A

The effect of copper on MICs of ampicillin, gentamicin and ciprofloxacin.

	MIC (μg/ml)		
	Ampicillin	Gentamicin	Ciprofloxacin
nil	16	4	0.0125
Cu	16	4	0.2

B

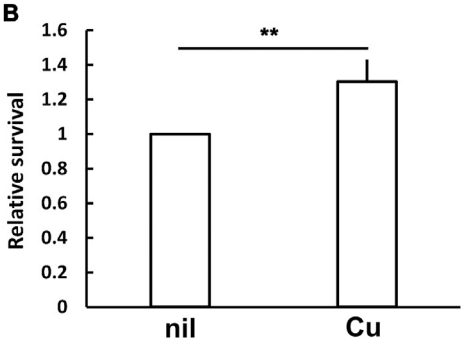

Figure 9. The effect of copper on MICs of ampicillin, gentamicin and ciprofloxacin (A) and persister cell formation against ampicillin (B) in *P. mirabilis*. MICs were determined according to the protocol of CLSI. In **B**, cells were challenged with lethal dose ampicillin to isolate persister cells, followed by calculating the relative survival as described in Methods. The value for the absence of copper was set at 1. The data are the averages and standard deviations of three independent experiments. Significant difference between the presence and the absence of copper was determined by using the Student's *t* test (**, $P < 0.01$). nil, no copper; Cu, 1 mM $CuCl_2$.

In addition, we disclosed $CuCl_2$ treatment induce persister formation against ampicillin (Fig. 9 B).

Copper altered the outer membrane protein (OMP) profile

Cu-provoked protein misfolding, disruption of OM lipoprotein trafficking and peptidoglycan crosslinking compromise envelope integrity and lead to envelope malfunction.[4,37,38] Thus the adaptation to copper stress requires the activation of the envelope stress response. The cell envelope of Gram-negative bacteria, where most cuproproteins are localized, is particularly prone to copper toxicity. Bacterial envelope stress responses induced by cell envelope damages or defects could alter the transcriptome to relieve destructive stresses, which is particularly important for survival and virulence modulation. Knowing OMPs ComC, lowering the OM permeability to copper, and OmpC, an OM porin, were induced upon copper stress,[5,39] we then examined the OMP profile in the presence of copper or not. Fig. 10A indicates a different OMP profile upon exposure to 1 mM $CuCl_2$. Two obvious bands indicated (*), one almost invisible and the other increased significantly upon $CuCl_2$ exposure, were subjected to analysis of matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). The copper-dependent activation of TCSs, Cpx and Rcs, has been shown to affect bacterial fitness and virulence.[31,40,41] In this study we disclosed $CuCl_2$ at 1 mM increased the level of *rcsB* transcript, an indicator of the RcsB pathway activation since Rcs system possesses a mechanism of autoregulation[42] (Fig. 10B). This suggests Cu-responsive regulons are a part of Rcs signaling pathway in *P. mirabilis*.

Discussion

Studies from various groups have revealed a role for copper during host-pathogen interaction.[3] In this regard, ceruloplasmin, the primary carrier of circulating copper, found at higher levels in urine from UTI patients is positively correlated with urinary copper content[12] and copper supplementation results in decreased UPEC colonization during UTI.[12,13] For the first time, we identified the diverse effects of $CuCl_2$ at a sublethal concentration in vitro on uropathogenic *P. mirabilis* including attributes of virulence and fitness in this study. Our study indicated copper was a critical determinant for *P. mirabilis* to establish a UTI, also supporting that copper could be a protective effector in bacterial UTI and play an antimicrobial role. We noted that $CuCl_2$-supplemented mice were more resistant to *P. mirabilis* colonization in the bladder and kidneys (Fig. 1),

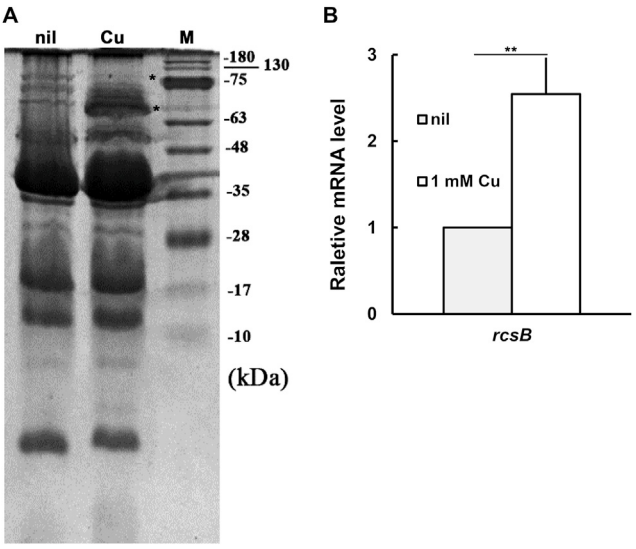

Figure 10. The effect of copper on the OMP profile (A) and the *rcsB* mRNA level (B) of wild-type *P. mirabilis*. The SDS-PAGE profile of OMPs was obtained from overnight cultures of wild-type *P. mirabilis* in the presence and absence of copper. The representative result from three independent experiments is shown. The *rcsB* mRNA level was determined by the RT-PCR assay as described in Methods. The value for the absence of copper was set at 1. The data are the averages and standard deviations of three independent experiments. Significant difference between the presence and the absence of copper was determined by using the Student's *t* test (**, $P < 0.01$). M, marker; nil, no copper; Cu, 1 mM $CuCl_2$.

together with in vitro results of decreased motility (swarming and swimming), ureases activity, T6SS expression and adhesion/invasion ability to urothelial cells and increased killing by macrophages of *P. mirabilis* in the presence of a sublethal $CuCl_2$ level (Figs. 3, 4A, 5, 6 and 8). However, biofilm formation and resistance to oxidative stress were enhanced under this $CuCl_2$ level (1 mM) (Figs. 4B and 7). Notably, the presence of $CuCl_2$ led to increased ciprofloxacin MIC and more persister cells against ampicillin (Fig. 9).

Researchers have garnered several lines of evidence for copper-dependent killing of pathogens in host macrophages.[3,11] First, macrophages from copper-deficiency rats display poor killing of pathogens compared to the adequate copper control. Second, the copper efflux pumps provide protection from macrophage killing in *Salmonella enterica*. Third, copper deficiency inhibits the inflammasome activation in macrophages. As such, copper-resistance should contribute to virulence during UTI. We have isolated *P. mirabilis* copper-susceptible mutants of *copA*, *cueO* and *cueR*, encoding efflux pump, copper oxidase and copper sensing regulator, respectively.[43] The study to investigate the role of *P. mirabilis* copper-resistance in mouse colonization is underway.

As for increased ciprofloxacin resistance, antagonistic effects of copper on the interaction between quinolones and DNA as well as DNA gyrase were reported.[44] Based on the recent study showing development of antibiotic resistance due to oxidative stress under sublethal copper exposure,[45] the role of oxidative stress in copper-mediated ciprofloxacin resistance will be explored. Care must be taken using copper as a drug adjuvant because of the potential for copper to induce drug resistance or persister cell formation as was reminded by previous studies.[6,35] Biofilms, in which bacteria are embedded in a polymeric matrix, are highly antibiotic-resistant than their free planktonic counterparts. As a corollary, our finding of copper-induced biofilm production in *P. mirabilis* implicates the possibility for copper-mediated antimicrobial resistance.

We have demonstrated that cupric ions could act as a signal for CpxR to increase biofilm formation[26] and $CuCl_2$ induced Rcs activation (Fig. 10B), suggesting copper responsive regulons are parts of Rcs and Cpx signaling systems. Both Cpx and Rcs signaling systems are known to sense and respond to cell envelope disturbance.[40,41] The Cpx TCS is induced by defects in membrane protein secretion or by misfolding proteins, which may occur due to changes in pH or osmolarity, peptidoglycan defects, and copper exposure. Activation of Cpx TCS was shown to participate in relieving envelope stress by regulating protein folding and degrading factors,[41] together with regulation of the virulence potential of a number of pathogens.[41] The Rcs TCS is activated by outer membrane defects, peptidoglycan perturbation, and lipoprotein mislocalization, which then changes expression of genes involved in motility, biofilm formation, and virulence.[40] It is noteworthy, copper tolerance in bacteria requires activation of multiple accessory pathways not only to control intracellular copper homeostasis, but also to protect the function of the cell envelope and repair the copper-causing damages.[6] In view of multiple layers of gene regulation required for an adaptation to copper stress, the pleotropic effects of copper we observed is reasonable. Based on the complex copper-mediated phenotypes observed, transcriptome analysis is on the way to elucidate the Rcs and Cpx TCS-regulated regulons in response to copper. The preliminary data of OMPs (Fig. 10) analyzes by MALDI-TOF MS revealed siderophore TonB-dependent receptor and Pta autotransporter proteins were increased and decreased, respectively, in response to $CuCl_2$. Further studies to clarify the copper-associated proteins is underway.

In this study, we used $CuCl_2$ at 1 mM in rich LB medium, which contains undetectable copper, to investigate the effect of copper on *P. mirabilis*. Our unpublished data also showed $CuCl_2$, at lower effective doses 50—500 μM in glucose minimum medium, led to similar trends of urease activity, biofilm formation, H_2O_2 sensitivity and adhesion ability to urothelial cells. It has been shown that copper level increases with aging in mouse tissues.[46] Our observations of fitness or virulence-associated attributes after high $CuCl_2$ (1 mM) exposure may have clinical relevance since the increased copper microenvironments may be attainable in people of aging or abnormal copper homeostasis.[10]

There is a dire need for antibacterial drug design for the rise of highly resistant pathogenic bacterial superbugs. It has been known for long that copper was employed by host's immune system to attack against pathogenic bacteria. Copper-centric strategies for antibacterial preventative and therapeutic applications were developed. Promising results were obtained for the use of copper-containing materials in the hospital to minimize bacterial infections[47] and copper-based small molecules demonstrate potential synergism with classical drugs.[48] For the first time, we unveiled the pleiotropic effects of cupric ions on uropathogenic *P. mirabilis*, including swarming, biofilm-forming, urease activity, adhesion/invasion ability, killing by macrophages, MD/H_2O_2 susceptibility, drug resistance, T6SS expression, OMP profile and mouse colonization, together with the induction of TCS in this study. This implies the bacterial copper regulatory pathways could be new drug targets. We will explore the copper effect on other *P. mirabilis* clinical isolates. Though preliminary, our study of copper-mediated effect on virulence and fitness highlighted the involvement of copper in prevention and therapeutic interventions against *P. mirabilis* infections. These results will facilitate further studies to develop innovative strategies against UTI caused by *P. mirabilis*.

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Declaration of competing interest

We declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.02.007.