Journal of Bioscience and Bioengineering
VOL. 112 No. 6, 570–576, 2011

www.elsevier.com/locate/jbiosc

Copper as an antibacterial agent for human pathogenic multidrug resistant *Burkholderia cepacia* complex bacteria

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Received 5 July 2011; accepted 19 August 2011
Available online 16 September 2011

The *Burkholderia cepacia* complex (Bcc) consists of 17 closely related multidrug resistant bacterial species that are difficult to eradicate. Copper has recently gained attention as an antimicrobial agent because of its inhibitory effects on bacteria, yeast, and viruses. The objective of this study was to evaluate the antibacterial activity of copper surfaces and copper powder against members of the *B. cepacia* complex. The antibacterial activity of different copper surfaces was evaluated by incubating them with Bcc strain suspensions (5×10^7 cfu/ml). The bacterial survival counts were calculated and the data for various copper surfaces were compared to the data for stainless steel and polyvinylchloride, which were used as control surfaces. The antibacterial activity of copper powder was determined with the diffusimetrical technique and the zone of inhibition was evaluated with paper disks. A single cell gel electrophoresis assay, staining assays, and inductively coupled plasma mass spectroscopy were performed to determine the mechanism responsible for the bactericidal activity. The results showed a significant decrease in the viable bacterial count after exposure to copper surfaces. Moreover, the copper powder produced a large zone of inhibition and there was a significantly higher influx of copper ions into the bacterial cells that were exposed to copper surfaces compared to the controls. The present study demonstrates that metallic copper has an antibacterial effect against Bcc bacteria and that copper adversely affects the bacterial cellular structure, thus resulting in cell death. These findings suggest that copper could be utilized in health care facilities to reduce the bioburden of Bcc species, which may protect susceptible members of the community from bacterial infection.

[**Key words:** *Burkholderia cepacia* complex; Opportunistic pathogen; Multidrug resistant; Copper; Antibacterial actvity]

The *Burkholderia cepacia* complex (Bcc) is a group of genetically distinct, phenotypically similar gram-negative bacteria. This group of bacteria emerged in the 1980s as an important causative agent of opportunistic and nosocomial infections. Currently, 17 species have been formally designated as members of the complex, including *Burkholderia cepacia*, *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Burkholderia stabilis*, *Burkholderia vietnamiensis*, *Burkholderia dolosa*, *Burkholderia ambifaria*, *Burkholderia anthina*, *Burkholderia pyrrocinia*, *Burkholderia ubonensis*, *Burkholderia latens*, *Burkholderia diffusa*, *Burkholderia arboris*, *Burkholderia seminalis*, *Burkholderia metallica*, *Burkholderia lata*, and *Burkholderia contaminans*. These species have been extensively reviewed by Vandamme and Dawyndt (1). Almost all of the Bcc species have been diagnosed in cystic fibrosis patients (CF patients) with a low or high prevalence (2,3) and the most commonly reported causative species are *B. cepacia*, *B. cenocepacia*, and *B. multivorans* (4). The Bcc species are also known to infect immunocompromised individuals and can cause diseases such as meningitis and septicemia (5,6).

There are several lines of evidence that suggest that the Bcc species may be transmitted between CF patients, both within and outside the hospital (7,8). These outbreaks are contingent upon a number of risk factors, including the bacterial strain type (9–12), the use of contaminated medical devices (13), and inappropriate treatment practices in infection control centers (14). Moreover, there are several reports of Bcc infections in non-CF patients, where these patients were likely exposed through the environment. Many of these bacterial strains are multi-drug resistant, which makes them difficult to treat with clinical antimicrobials (15–17). These multi-drug resistant strains pose a particularly dangerous threat to susceptible members of the community. Doorknobs and other tactile surfaces in hospitals are notorious primary sources of nosocomial infections because these surfaces are often contaminated by opportunistic pathogens that can cause fatalities in intensive care units or nurseries (18). As more and more of these outbreaks are caused by Bcc bacteria, particularly in hospitals, there is an urgent medical need to identify novel methods to inhibit surface contamination by this pathogenic family of microorganisms.

Copper is an essential element that functions as a cofactor during aerobic metabolism; however, in low or excessive amounts copper can have deleterious effects (19–22). The toxicity of copper is largely due to

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doi:10.1016/j.jbiosc.2011.08.017

generation of reactive oxygen species (ROS), which is probably mediated by redox cycling between the different copper species, Cu (0), Cu(I), and Cu(II) (23). Currently, the antimicrobial properties of metallic copper surfaces are being tested against numerous pathogens. Humans have used copper as far back as the 5th and 6th millennia (24); however, it was not until 2008 that its ability to inactivate microbes upon contact was identified (25–28). Subsequently, rigorous testing by the US Environmental Protection Agency (EPA) led to the registration of copper alloys as antimicrobial agents. EPA support has resulted in increased interest in the use of copper as an antimicrobial agent. The human pathogens that have been the most widely tested against copper include Methicillin-resistant *Staphylococcus aureus* (MRSA), *Clostridium difficile*, *Escherichia coli*, *Listeria monocytogenes*, *Influenza A* (H1N1), *Aspergillus niger*, and *Pseudomonas aeuroginosa*, as reviewed by Grass et al. (29). These tests indicate that copper can have antibacterial effects in applications where control of these germs would be beneficial. An Irish hospital (St. Francis Private Hospital) was recently the first hospital in the world to embrace this innovation by incorporating hygienic copper door handles. Additionally, the use of copper vessels for drinking water has been incorporated in developing countries as a low-cost alternative in the prevention of bacterial infections (30).

The purpose of this study was to determine whether copper has antimicrobial effects toward the Bcc species and determine which Bcc species is the most susceptible to copper. Finally, we also sought to identify the molecular mechanisms of copper contact-induced bacterial death.

MATERIALS AND METHODS

Bacterial strains and culture conditions The antibacterial activity of copper contact was evaluated with suspensions of various Bcc species (*B. cepacia* Y3; *B. multivorans* PE26 and PW99; *B. cenocepacia* Y5 and Y10; *B. vietnamiensis* S13; *B. arboris* HT1; *B. contaminans* Y4 and Y1, and *B. seminalis* S9 and 0901) that were indentified in our previous studies (31–34), as well as reference strains that were acquired from the Prof Swing BCCM/LMG Bacteria Collection at the University of Ghent, Belgium. All of the strains that were tested are listed in Table 1. All of the Bcc strains were stored in 20–30% glycerol (Shanglin Industries Hangzhou China) at −80°C and were cultured for 24–48 h in Brain Heart Infusion (BHI) agar at 37°C. Single colonies were inoculated into 20 ml of BHI broth and incubated at 37°C overnight; on the following day, the cultures were diluted 1:100 with 10 ml of fresh BHI in 50 ml flasks to an approximate optical density (OD) of 3.0. The bacterial OD was determined with a spectrophotometer (Perkin Elmer Lambda35 UV/VIS) by reading the absorbance at 600 nm.

Copper metallic surfaces and copper powder The metallic copper coupons (1.5 cm × 1.5 cm × 0.5 mm) and copper powder (≥99.9% purity) were obtained from Teachn Industrial Technology Development Co., Limited, Hunan, China. The stainless steel sheets (0.5 mm) and polyvinylchloride (PVC) were obtained locally and were used as control surfaces.

Viability assays on copper and control surfaces The viability assays were performed as described by Mehtar et al. (35) with some modifications. The bacterial culture was serially diluted in phosphate buffered saline (PBS) and 20 μl of a Bcc suspension (5×10^7 cfu/ml) was overlaid on either a sterile copper surface, PVC or stainless steel surface and the overlays were stored at room temperature. This technique is called the wet inoculation technique or moist copper exposure. The viable bacterial counts were measured at 0, 2, 4, 8, and 12 h to determine the effect of the surface on bacterial growth with time. The copper, stainless steel, and PVC coupons were kept in Petri dishes that were maintained in a closed plastic box. Whatman filter paper was placed inside the wall of the box and was kept wet with sterile distilled water until the experiment was complete. After the samples were collected at the various time points, the coupons were placed in 10 ml of PBS with 2 mm glass beads (PBSG), centrifuged for 30 s at 300 × *g*, and then serially diluted to assess the bacterial counts. The assays were performed in duplicate and were repeated three times.

Antibacterial activity of copper powder The antibacterial activity of the copper powder was determined according to the diffusimetrical technique as described by Fit et al. (36). This technique determines the sensitivity of the microbes to antibiotics and chemotherapeutic agents in vitro. Müeller–Hinton (MH) agar (Qingdao Hope Biotechnology, China) plates were made according to the manufacturer's instructions by dissolving 42 g of MH agar in 1 l of distilled water. Approximately 10 ml of autoclaved MH agar was poured into sterilized Petri plates and dried at 37°C prior to inoculation. The paper disks were prepared by cutting Whatman No. 1 filter paper with a hole punch to produce uniformly sized circular disks. An overnight bacterial culture was diluted to 0.5 OD on the McFarland scale and the plates were flooded with the Bcc culture and dried. The copper powder (0.25 mg/disk) was placed on the paper disks (4 disks/plate) that were soaked with distilled water to determine the zone of inhibition. Paper disks loaded with sodium chloride (NaCl, 0.25 mg/disk) were used as a control. The plates were incubated for 24–48 h at 37 ± 1°C and the diameter of the zone of inhibition was measured.

TABLE 1. Copper antimicrobial activity against Bcc species tested using diffusimethrical method.

Bcc species and strains	Inhibition area diameter (mm)	Origin, characteristics and restrictions
B. cepacia		
LMG1222 (AF143786)	15.3 ± 0.14	Onion, human opportunistic pathogen
Y3	15.4 ± 0.5	CF, human opportunistic pathogen
B. multivorans		
PE26 (GQ306216)	15.8 ± 0.5	Water, human opportunistic pathogen
PW99 (QQ306219)	15.9 ± 0.5	Water, human opportunistic pathogen
B. cenocepacia		
Y5 (EU500765)	15.9 ± 0.04	CF, human opportunistic pathogen
Y10 (EF426457)	16.2 ± 0.3	CF, human opportunistic pathogen
B. vietnamiensis		
LMG10929 (AF097534)	12.4 ± 0.09	Rice, human opportunistic pathogen
S13 (GQ306221)	12.3 ± 0.8	Water, human opportunistic pathogen
B. arboris		
LMG24066 (AM747630)	14.3 ± 1.9	CF, human opportunistic pathogen
HT1 (FJ897564)	13.2 ± 1.4	Bamboo rhizosp., human opportunistic pathogen
B. contaminans		
Y1 (EU521726)	12.1 ± 0.1	CF, human opportunistic pathogen
Y4 (EU500764)	12.3 ± 0.16	CF, human opportunistic pathogen
B. seminalis		
S9 (QQ306220)	9.6 ± 0.08	Water, human opportunistic pathogen
0901 (GU188877)	9.4 ± 0.12	Soil, human opportunistic pathogen
B. dolosa		
LMG18943 (AF323971)	13.3 ± 0.04	CF, human opportunistic pathogen
B. lata		
LMG22485 (CP000150)	13.3 ± 0.04	Forest soil, human opportunistic pathogen

Single cell gel electrophoresis (SCGE) A comet assay, or SCGE was performed as described by Sing et al. (37) with some modifications. *B. cenocepacia* suspensions (5×10^7 cfu/ml) were streaked on to dry copper surfaces using sterile cotton swabs. The cells were removed with 10 ml of PBSG containing 20 μM EDTA, treated with lysozyme (20 mg/ml), and incubated at 37°C for 20 min. The cells were subsequently mixed with 0.8% agarose and applied to glass slides that were pre-coated with 1.5% agarose. After the agarose cell suspensions polymerized, the slides were immersed in lysis buffer (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris–HCl, pH 10, 10% Triton X-100, and 1% dimethyl sulfoxide [DMSO] to prevent oxidation during lysis) and were carefully agitated at 25 rpm at 4°C for 5 min. After washing with deionized water, the slides were treated with denaturation buffer (300 mM NaOH and 1 mM EDTA, pH >13), incubated briefly with excess Tris–borate–EDTA (TBE) buffer, and subjected to electrophoresis at 25 mV and 15 mA for 3 min. The slides were removed, washed with ice-cold deionized water, immersed in absolute ethanol, and air-dried overnight. Finally, the slides were stained with ethidium bromide (Sangon Biotech China) in TBE and incubated for 1 min in the dark. The fluorescence was observed with an inverted confocal fluorescence microscope (Olympus DP50 BX 51) at an excitation wavelength [λ_{Ex}] of ~490 nm and an emission wavelength [λ_{Em}] of ~520 nm.

ICP-MS analysis The influx of copper ions into 9 different Bcc species was analyzed with inductively coupled plasma mass spectroscopy (ICP-MS) (Agilent ICP-

MS model 7500a). The Bcc cells were exposed to copper and PVC surfaces for 6 h as described for the viability assays. The surface challenged cells on the moist copper and PVC surfaces were removed and washed with ice-cold PBSG buffer containing 20 μM EDTA and the viable bacterial counts were determined by plating as described above. In parallel, samples were mineralized with concentrated 70% (v/v) nitric acid for 2 h at 70°C and diluted to a final concentration of 5% (v/v) nitric acid. Germanium [$Ge(NO_3)_3$] was added at a final concentration of 50 ppb as an internal standard. The samples were loaded with an auto sampler and subsequently analyzed. Each sample was performed in triplicate.

Testing cell disintegration using general staining methods To examine changes in the bacterial cells after exposure to various surfaces, the *B. cenocepacia* suspensions were exposed to dry metallic copper surfaces and PVC as described above. The cells were resuspended in 100 μl of PBS buffer on the coupons, transferred onto a glass slide, and air-dried. The cells were subsequently stained with safranin O and the glass slides were examined under oil immersion at 40× with light microscopy (Axioskop 2 Plus *Zeiss* fluorescence microscope).

Statistical analyses The data were analyzed with an analysis of variance test and the mean values were compared with the least significant differences test.

RESULTS

Antibacterial efficiency of metallic copper surfaces toward Bcc To determine the antibacterial effects of copper toward the Bcc, bacterial suspensions (approximately 5×10^7 cfu/ml) were overlaid on copper coupons for 0, 2, 4, 8, or 12 h and the viable bacterial count was determined. The results indicate that there was a decrease in the viable bacterial count over time on the copper surfaces compared to PVC or stainless steel. The *B. cepacia* strains LMG1222 and Y3, *B. multivorans* PW99 and PE26, *B. cenocepacia* Y5 and Y10, *B. arboris* LMG24066 and HT1, *B. dolosa* LMG18943, and *B. lata* LMG22485 showed significantly lower counts on the copper surfaces at 2–4 h at room temperature compared to the PVC or stainless steel surfaces. These differences were significantly more pronounced at 6–8 h ($P<0.05$), as is shown in Fig. 1 (plots A, B, C, E, and H). At 8 h, the antibacterial activity of copper was

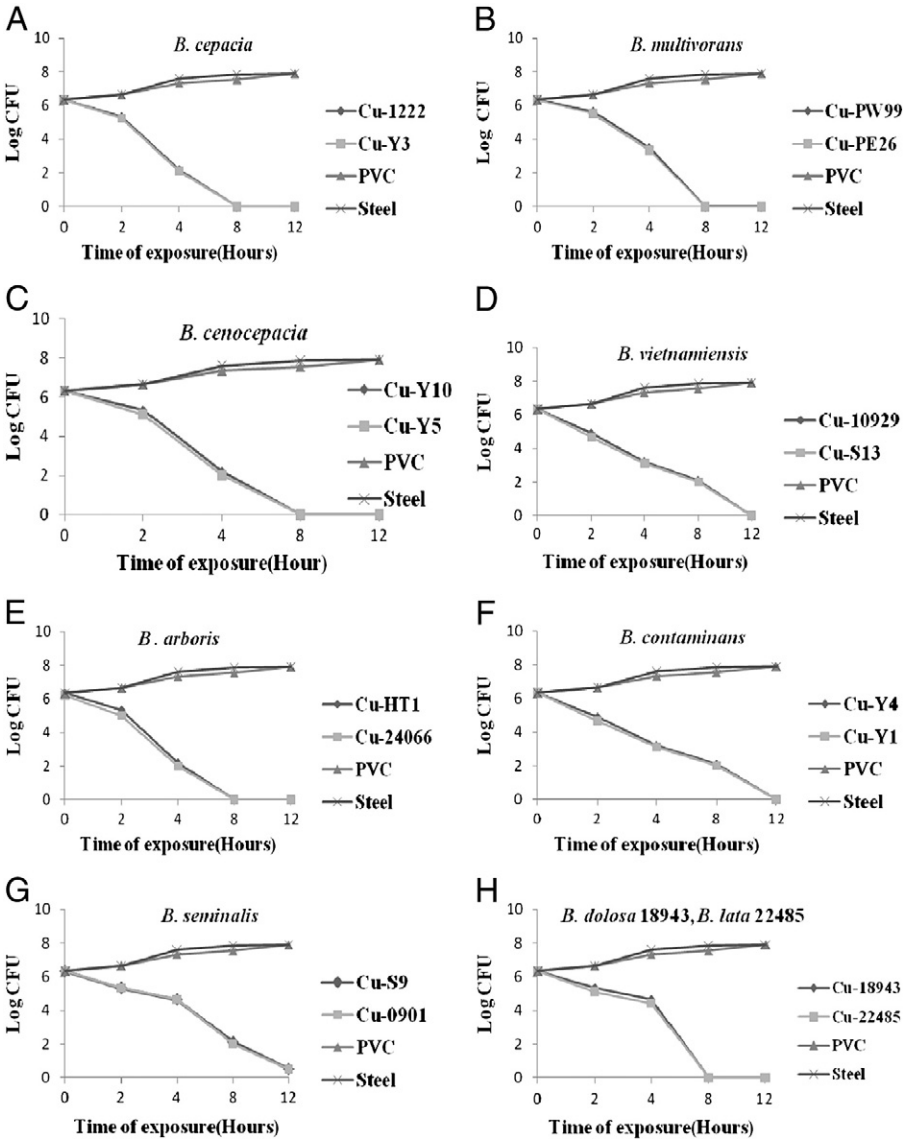

FIG. 1. Survival of the Bcc bacterial suspensions on copper, PVC, and steel surfaces. Bcc cells suspensions (5×10^7 cfu/ml) were overlaid on copper, PVC, or stainless steel surfaces, and samples were obtained at 0, 2, 4, 8, and 12 h. The plots compare the logarithms of the mean of the surviving bacterial counts (cfu/ml) for the copper and control surfaces. Plots A, B, C, E, and H show the significant differences between exposure to copper, PVC, and stainless steel surfaces after 2 h; these differences were highly significant after 4, 8, and 12 h ($P<0.05$). In plots D and F, there is a significant difference between the surviving bacterial counts (cfu/ml) at each time interval, although the difference is the most significant at 12 h. Plot G clearly indicates that copper has a lower antibacterial activity toward *B. seminalis* compared to the other 8 strains, although the antibacterial activity of copper toward *B. seminalis* is still higher compared to PVC or stainless steel.

maximal, while the PVC and steel surfaces exerted no lethal effects (Fig. 1, plots A, B, C, E, and H). According to Fig. 1, there is no significant difference in the death rate between strains of the same species.

Fig. 1 plots D and F show the results for the *B. vietnamiensis* strains LMG10929 and S13 and the *B. contaminans* strains Y1 and Y4 after exposure to the different surfaces. The plate counts for the *B. vietnamiensis* and *B. contaminans* cultures that were maintained on the copper surfaces are lower compared to the control surfaces. The differences between the copper and control surface viable bacterial counts were statistically significant at 8–12 h ($P<0.05$), as is shown in Fig. 1 (plots D and F). The antibacterial activity of the copper was maximal at 12 h. Neither the stainless steel nor the PVC demonstrated lethal effects on *B. vietnamiensis* or *B. contaminans*, as is shown in Fig. 1 (plots D and F).

The viable bacterial count for *B. seminalis* decreased over time compared to the PVC and stainless steel, although the difference between the counts for these surfaces did not become statistically significant until hour 12 ($P<0.05$), as is shown in Fig. 1 (plot G). Full inhibition of bacterial growth was achieved at 14 h (data not shown). The antibacterial effect of copper on Bcc species was significantly increased ($P<0.05$) compared to the control surfaces, with the exception of some variability in *B. seminalis*, which required 14 h for full inhibition, compared to 8 or 12 h for the other species. The results also indicate that there was no difference in the copper antibacterial effect among Bcc species that were derived from environmental or clinical sources.

Antibacterial efficiency of metallic copper powder toward Bcc Previous in vitro studies have demonstrated that copper powder has antimicrobial activity when it is mixed with different metal alloys (36). To determine if pure copper powder possesses antibacterial effects, growth of all of the Bcc strains was tested with copper powder according to the diffusimetrical technique. The diameter of the mean zone of inhibition (mm) was measured for all of the Bcc species. The largest zone of inhibition measured was 16.2 mm for *B. cenocepacia* Y10, as is shown in Fig. 2A and listed in Table 1. The saline control produced no zone of inhibition, as is shown in Fig. 2B. *B. cenocepacia* Y5 was also sensitive to copper and had a 15.9 mm zone of inhibition, as is listed in Table 1. The mean diameter for the zone of inhibition for the other strains tested was 15.8 mm and 15.9 mm for *B. multivorans* PE26 and PW99 respectively and 15.3 mm and 15.4 mm for *B. cepacia* LMG1222 and Y3 respectively. These values are significantly higher compared to *B. seminalis* S9 and 0901 ($P<0.05$), where the zone of inhibition was 9.6 mm and 9.4 mm respectively ($P<0.05$). These results are listed in Table 1. The mean diameter of the zones of inhibition for *B. arboris* LMG24066 and HT1, *B. dolosa* LMG18943, *B. lata* LMG22485, *B. vietnamiensis* LMG10929 and S13, and *B. contaminans* Y1 and Y4 are significantly larger, although they are smaller than the values for *B. cepacia*, *B. multivorans*, and *B. cenocepacia*. These values are also larger than the mean diameter of the zone of inhibition for *B. seminalis*, as is shown in Table 1.

B. seminalis showed the minimum inhibitory zone with the copper powder compared to other strains in this experimental panel, which supports the results from the copper coupon surface studies.

Acute copper toxicity and DNA damage Espirito Santo et al. (23) demonstrated that the acute bacterial toxicity (long exposure of bacterial cells) of metallic copper surfaces is caused by copper-mediated DNA damage in *E. coli*. We used the SCGE test to study the Bcc bacterial DNA after dry metallic copper exposure. This assay was performed after a 15 s or 1 h exposure to dry copper metallic surfaces, and the comet tails or DNA breaks were visualized with gel electrophoresis. The cells that were exposed for 1 h are shown in Fig. 3B; in contrast, no comet tails or DNA fragmentation was observed in the cells that were exposed to the PVC control surface (Fig. 3A) or in the cells that were exposed to the dry metallic copper surfaces for 15 s (data not shown). These results indicate that the bacterial DNA is lethally damaged by the dry metallic copper surfaces after 1 h of exposure but not after 15 s of copper exposure.

Cell disintegration by copper We performed a general bacterial staining method after exposure to the dry metallic copper surface to determine if exposure physically damaged the cells. After 15 s of exposure, some of the cells disintegrated, resulting in visible cellular debris (Fig. 4B). In contrast, the control cells that were exposed to PVC remained unaffected (Fig. 4A). These results indicate that bacterial exposure to copper led to severe structural damage and cell lysis.

Accumulation of copper ions by bacterial cells Current studies have suggested that copper toxicity is mediated by the influx of copper ions into bacterial cells (23). ICP-MS was performed to determine the intra-cellular copper ion accumulation of each Bcc species after copper exposure. The cellular copper ion concentration was increased in the cells that were exposed to moist copper surfaces compared to PVC, as is shown in Fig. 5. *B. cepacia*, *B. cenocepacia*, *B. arboris*, and *B. multivorans* accumulated significantly higher amounts of copper compared to *B. seminalis* ($P<0.05$), as shown in Fig. 5. *B. seminalis*, however, accumulated 6.5 ± 1.2 μg/ml copper ions, which is significantly higher than the PVC control that remained constant at 0.82 ± 0.21 μg/ml ($P<0.05$). *B. seminalis* growth was completely inhibited after 14 h on copper surfaces (data not shown) and this species took longer than the other Bcc species to show copper toxicity, as shown in Fig. 1 (plots A, B, C, D, E, F, and H); accordingly, this species had a lower copper ion concentration. These results indicate that the Bcc strains that accumulate more copper have a shorter survival time after exposure to copper surfaces. These data indicate that copper influx into the Bcc bacterial cells is associated with cell death and may be the mechanism responsible for copper antibacterial activity.

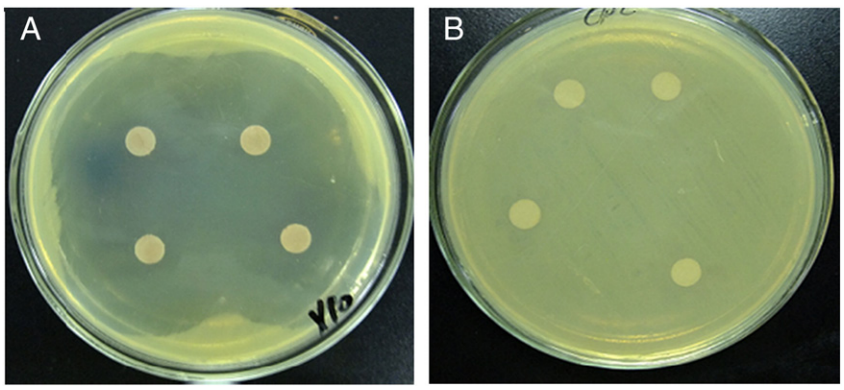

FIG. 2. *B. cenocepacia* sensitivity to copper powder versus the control (NaCl). (A) The inhibition zone obtained with copper powder. (B) No inhibition zone was obtained with the control (NaCl).

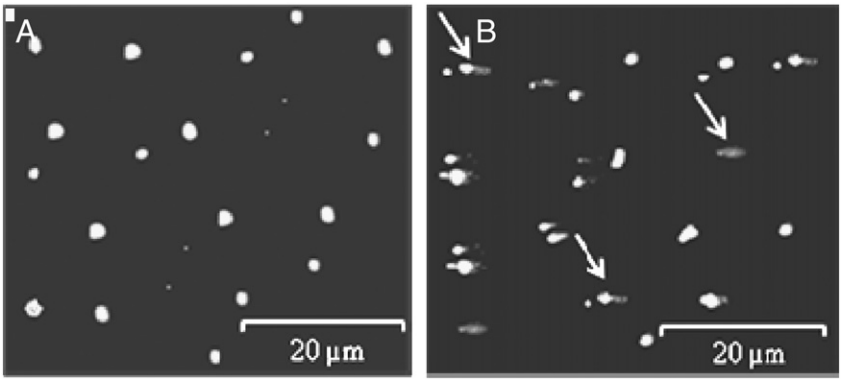

FIG. 3. Exposure of *B. cenocepacia* to copper surfaces causes DNA damage. (A) Exposure of *B. cenocepacia* to PVC control surfaces. (B) Exposure of Bcc to dry metallic copper surfaces. Bar = 20 um.

Taken together, these findings suggest that copper has a strong antibacterial effect against Bcc. Copper accumulation within the cell, cell death, and DNA damage assays all indicate that copper has lethal effects toward this group of bacteria. Among the Bcc species that were tested, *B. cepacia*, *B. multivorans*, *B. cenocepacia*, and *B. arboris* were more susceptible to copper compared to *B. vietnamiensis*, *B. contaminans*, and *B. seminalis*.

DISCUSSION

The goal of this study was to test the antibacterial activity of copper on emerging, highly infectious, and multidrug-resistant Bcc bacterial strains. Collectively, our results indicate that copper has a strong antibacterial effect toward Bcc bacteria. The copper surface tests were conducted at room temperature to mimic environmental conditions of surface contamination in hospitals.

Faundez et al. (25) reported that copper antibacterial effects against *Salmonella enterica* and *Campylobacter jejuni* bacterial suspensions were dependent upon the time of exposure. In this study, the antibacterial activity of copper toward *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. arboris*, *B. dolosa*, and *B. lata* was apparent after 2 to 4 h and became more pronounced after 8 h. After 8 h, no viable bacterial counts were obtained (Fig. 1, plots A, B, C, E, and H). The antibacterial activity of copper toward *B. vietnamiensis* and *B. contaminans* at 2, 4, and 8 h was significantly higher compared to the PVC and stainless steel control surfaces; however, this difference became more significant at 12 h (Fig. 1, plots D and F). *B. seminalis* was the only exception, where some bacterial counts were recorded at 12 h after copper exposure (Fig. 1, plot G); however, the growth of this bacterium was completely inhibited after 14 h of exposure (data not shown). Grass et al. (29) have indicated that exposure time is an important factor for bacterial death. We observed that the time required for the full antibacterial activity of copper varied for the different Bcc species tested. The minimal time recorded for complete *B. seminalis* death was 1 min on dry copper surfaces and 14 h on moist copper surfaces (data not shown). In contrast, *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. arboris*, *B. dolosa*, and *B. lata* required a minimum of 8 h of copper exposure (Fig. 1, plots A, B, C, E, and H). Thus, our results indicate that the copper surface exposure time is a crucial parameter for Bcc death, which agrees with the data published by Grass et al. (29) for other bacterial species. The physical appearance of the copper surfaces changed during the antibacterial copper sheet assays, where the copper sheets turned a dark brown color with time and the bacterial suspensions became a pale blue color, which Faundez et al. (25) have indicated is caused by Cu^{++} ion release. Over time, this pale blue color became deepened. Thus, our data indicate that the exposure time is a vital factor that influences bacterial death, and that *B. seminalis*, which requires a longer exposure time for complete bacterial death, is more resistant to copper exposure than the other species that we tested.

Our data also clearly show that pure copper powder has antibacterial activity toward the Bcc species. *B. seminalis* was the only species tested in this study that had a low sensitivity to copper powder (Table 1). These results agree with the data from the copper surface contact assay and demonstrate that copper powder has a strong antibacterial effect against *B. seminalis*, although this effect is lower compared to the other species tested. Previously, various granulations of copper in combination with different salts, Bismuth compounds, and Bismuth salicylate were reported

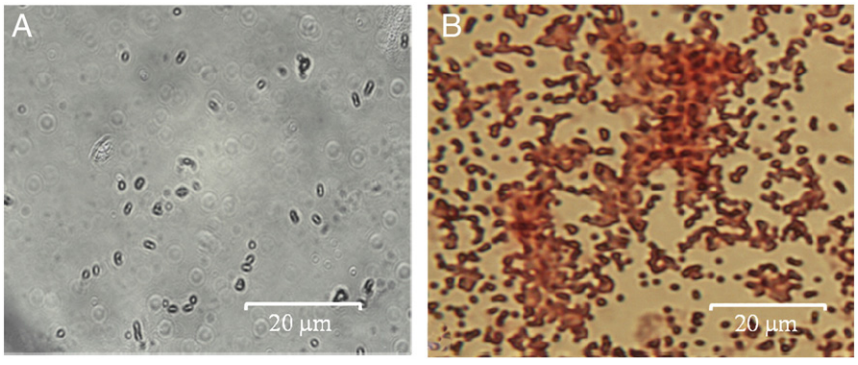

FIG. 4. Exposure of *B. cenocepacia* to dry metallic copper surfaces results in cellular disintegration. (A) *B. cenocepacia* cells exposed to PVC control surfaces for 15 s. (B) *B. cenocepacia* cells exposed to dry metallic copper surfaces for 15 s. Bar = 20 um.

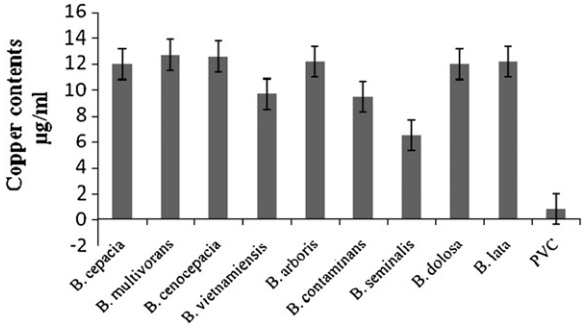

FIG. 5. The influx of copper ions into Bcc cells. Bcc cellular copper accumulation during a 6-hour exposure to copper surfaces compared to PVC.

(36,38,39) to have antimicrobial activity toward numerous gram-negative pathogenic bacteria. Therefore, copper/salt formulations have been traditionally used in medicinal preparations to prevent bacterial contamination. In this study, our data indicate that copper powder has antibacterial activity against the nine Bcc species tested (Table 1) and thus may be a useful antibacterial agent.

Our data, along with the existing literature, suggest that bacterial death via copper is preceded by cell membrane damage, copper influx into the cells, oxidative damage, and DNA damage (23). In the Bcc species tested here, we observed that a 15-second exposure resulted in no DNA fragmentation or comet formation, similar to the control cells (Fig. 3A). However, after 1 h of exposure, DNA fragmentation or tailed DNA was clearly visible in the copper treated cells (Fig. 3B). Our comet assay results indicate that Bcc bacteria exposure to dry copper surfaces for short or extended periods of time results in DNA damage that likely precedes cell death. These findings are consistent with the results previously reported by Espirito-Santo et al. (23), who demonstrated that *E. coli* exposure to dry metallic copper surfaces resulted in toxicity due to DNA damage following prolonged exposure.

Copper accumulation in bacterial cells is the key factor that mediates copper toxicity and subsequent cell death in *E. coli* (23). In our study, the ICP-MS data demonstrate that the differences in the cellular Cu^{++} influx concentration (Fig. 5) and the time until complete Bcc cell death (Fig. 1) are associated. This correlation indicates that the more rapid antibacterial activity of copper toward *B. cepacia*, *B. cenocepacia*, *B. multivorans*, and *B. arboris* compared to *B. seminalis* may be due to a faster intracellular accumulation of copper, which damages the bacterial DNA. The data indicate that Bcc cell death following copper contact was due to the intracellular accumulation of copper. Previous studies have shown that free copper ions may cause a selective change in the cell membrane (40), and that the cell membrane is directly targeted by copper exposure (23). In this study, copper exposure also led to other cellular changes, including disintegration of the Bcc bacterial cells (Fig. 3B); in contrast, no disintegration or cell debris were observed in the control cells that were exposed to PVC. This finding is consistent with previous studies that have shown that bacterial exposure to copper results in cellular disruptions (23,40).

Our data indicate that copper has antibacterial activity against several Bcc species. The Bcc is an emerging, infectious, multidrug-resistant group of human bacteria that are present in a variety of environmental niches, plant rhizospheres, soil, water, hospital patients, hospital equipment, and industrial contaminants, as reviewed by Pope et al. (17). However, our data indicate that Bcc bacterial contamination may be controlled with copper antibacterial applications. Currently, copper is used in numerous effective antimicrobial agents, including fungicides, antifouling paints, antimicrobial medicines, oral hygiene products, hygienic medical devices, and antiseptics (41). Experimental evidence has shown that copper-containing items significantly reduce the number of microorganisms in the clinical environment (42) and these applications are now being applied elsewhere to protect susceptible members of the community. We hypothesize that similar copper antibacterial applications can be expanded and applied to Bcc bacteria.

As reviewed by Grass et al. (27), a majority of the current studies indicate that copper has an antibacterial effect on a range of disease causing organisms. However, to the best of our knowledge, no information is available concerning the antibacterial activity of copper against Bcc species (clinical or environmental isolates). Thus, our current study investigated the antibacterial effect of copper toward Bcc. This study provides important information about copper applications that may eventually be applied to control Bcc environmental contamination. Currently, Bcc is a highly multidrug-resistant, nosocomial, and important opportunistic human pathogen in hospitalized and immunocompromised patients (15–17). However, our data indicate that copper applications may reduce the environmental bioburden of these bacteria, particularly in hospitals and CF centers.

Our data clearly indicate that copper exerts strong antibacterial effects toward the Bcc bacteria and mediates cell death upon contact due to a bacterial intra-cellular influx of copper ions. Future studies are needed to investigate the effects that copper resistance genes (43,44) have on the survival of these bacteria on copper surfaces.

ACKNOWLEDGMENTS

This project was supported by the Special Fund for Agro-scientific Research in the Public Interest (201003029, 201003066) and the National Natural Science Foundation of China (30871655).

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