Legionella pneumophila Transcriptional Response following Exposure to CuO Nanoparticles

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Copper ions are an effective antimicrobial agent used to control Legionnaires’ disease and Pontiac fever arising from institutional drinking water systems. Here, we present data on an alternative bactericidal agent, copper oxide nanoparticles (CuO-NPs), and its efficacy on Legionella pneumophila. In broth cultures, the CuO-NPs caused growth inhibition, which appeared to be concentration and exposure time dependent. The transcriptomic response of L. pneumophila to CuO-NP exposure was investigated by using a whole-genome microarray. The expression of genes involved in metabolism, transcription, translation, DNA replication and repair, and unknown/hypothetical proteins was significantly affected by exposure to CuO-NPs. In addition, expression of 21 virulence genes was also affected by exposure to CuO-NP and further evaluated by quantitative reverse transcription-PCR (qRT-PCR). Some virulence gene responses occurred immediately and transiently after addition of CuO-NPs to the cells and faded rapidly (icmV, icmW, lepA), while expression of other genes increased within 6 h (ceg29, legLC8, legP, lem19, lem24, lpg1689, and rtxA), 12 h (cegC1, dotA, enhC, htpX, icmE, pvcA, and sidF), and 24 h (legP, lem19, and ceg19), but for most of the genes tested, expression was reduced after 24 h of exposure. Genes like ceg29 and rtxA appeared to be the most responsive to CuO-NP exposures and along with other genes identified in this study may prove useful to monitor and manage the impact of drinking water disinfection on L. pneumophila.

Legionella pneumophila is the causative agent of Legionnaires’ disease and can be isolated from natural water environments as well as from engineered systems worldwide. Different strategies have been used to control this environmental pathogen, such as the use of copper/silver ions as bactericidal agents (1) to manage their growth in premise plumbing or other devices where drinking water is used. Currently, little has been reported on the antibacterial properties of copper nanoparticles. Coiffi et al. (2) demonstrated the bactericidal effects of copper nanoparticles on Escherichia coli, while Yoon et al. (3) reported a greater antibacterial activity of copper nanoparticles on E. coli and Bacillus subtilis than of silver nanoparticles. Furthermore, the bactericidal effects of copper oxide nanoparticles (CuO-NPs) were evaluated with four strains of E. coli, B. subtilis, and three strains of Staphylococcus aureus, and it was shown that the sensitivity to CuO-NPs is species specific and that B. subtilis was the most sensitive of the three bacterial species studied (4). To our knowledge, there has been no report on the study of CuO-NPs against Legionella spp.

The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface-to-volume ratio, which allows them to interact closely with microbial surfaces. For CuO-NPs, their toxicity may be due in part to either the uptake of copper ions released from the CuO-NPs by the bacteria or the interactions of the nanoparticles with the microbial organics and free radical formation, or both (5). Previous studies have shown that copper ions may bind to DNA molecules, disrupting biochemical processes (6, 7), or cause damage to the helical structure by cross-linking within and between the nucleic acid strands (4). CuO-NPs can also cause cellular membrane damage via specific or nonspecific interactions or membrane wrapping of the NPs (8). For example, exposure of Campylobacter jejuni to ZnO-NPs resulted in the increased expression of two oxidative stress genes and a general stress response gene (9), which was most likely a response to the disruption of the cell membrane and oxidative stress in C. jejuni. In any case, copper NPs have been shown to have higher cytotoxicity than copper ions because they may penetrate the cell membrane more efficiently and release copper ions inside the cell (3, 10). However, the exact mechanism(s) behind the bactericidal effect of copper nanoparticles is not known and requires further study.

Reported effects of nanometals on legionellae appear limited to Au-NPs. Stojak et al. (11) reported that 4- and 18-nm Au-NPs caused morphological changes to L. pneumophila in biofilms, with cells forming chain-like assemblages and filamentous, elongated planktonic cells at stationary phase, as well as thickening of biofilms. It was assumed that Au-NPs might have decreased expression of the global regulator csrA, which might induce flaA and flaA transcripts, which are associated with filament or biofilm formation (12).

Pathogenicity or virulence of L. pneumophila is considered dependent upon the presence or absence of certain virulence genes (13). Of those genes, type IV secretion systems (T4SSs), which are related to bacterial conjugation systems and are classified into two subgroups, typeIVA (T4ASS) and type IVB (T4BSS), play a central role in the pathogenicity (14). The most-characterized virulence system of L. pneumophila is the Icm/Dot T4BSS (14) and its transported substrates (15). The most recently characterized T4BSSs were reported to include more than 300 effectors (16) and

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275 substrates (17). Some components (DotG/IcmE) might be pivotal (14), while others promote evasion of death pathways (sdhA, sidF, and plaA) (18). Recently, the T4ASS (termed lvh) has been reported to contain three core complex proteins, VirB7, VirB9, and VirB10 (14). Previously reported virulence genes included pore-forming toxin (rtxA), enhanced entry protein (patD) (20), an invasion factor (hipB) (21), and a developmental marker (magA) (21). Other well-documented virulence factors have been reviewed by Shevchuk et al. (22). They include trans-isomerase (mip), GTP-dependent Fe(II) transporter (fsbB), peptide transporter (iraA and iraB), putative adenylate cyclase (ladC), estimated entry protein (enhC) (19), various dehydrogenases (bdhA), phospholipase A and lyso-phospholipase A activities (patD) (20), an invasion factor (hipB) (21), and a developmental marker (magA) (21). Other well-documented virulence factors have been reviewed by Shevchuk et al. (22).

The objectives of this study were to test the efficacy of CuO-NP disinfection on L. pneumophila viability and to identify genes differentially expressed following exposure that may prove useful to understanding the control of this environmental pathogen, along with similar pathogens that respond by expressing virulence factors to environmental stressors (23).

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**MATERIALS AND METHODS**

Preparation and characterization of CuO-NPs. CuO-NPs were prepared at the laboratories of the U.S. Environmental Protection Agency (EPA), Cincinnati, OH, using quality-approved protocols in a single batch. In brief, 40 ml of 0.5 M NaOH solution was combined with 20 ml of 0.5 M CuCl2 solution at room temperature, and then 50 ml of 0.1 M ascorbic acid solution was slowly added (over 30 min) with vigorous stirring, which resulted in Cu2O production. The yellow Cu 2O nanoparticles were harvested by centrifugation at 2,500 rpm and washed with deionized water three times. CuO-NPs were obtained by heating the Cu2O at 300°C for 4 h. The particles were then measured by transmission electron micrograph (TEM) (JEM-2100; JEOL, Japan) (Fig. 1A), and the majority of particles (79%) ranged between approximately 40 and 80 nm in diameter (Fig. 1B).

Exposure of L. pneumophila to CuO-NPs. L. pneumophila strain Lp02 was used in this study as previously described (24). Bacteria were grown on buffered charcoal yeast extract (BCYE) agar plates (BD Diagnostic Systems, Franklin Lakes, NJ) for 2 to 4 days at 37°C. A number of single colonies were transferred to a flask of BYE broth (N-[2-acetamido]-2-aminoethanesulfonic acid-buffed yeast extract broth supplemented with 0.4 mg ml−1 l-cysteine, 0.1 mg ml−1 thymidine, and 0.135 mg ml−1 ferric nitrate) and then incubated for 15 to 24 h at 37°C with constant shaking. The bacterial culture density was determined by measuring the optical density at 600 nm (OD600) calibrated to a predetermined standard curve of CFU using a Hach DR 2800 spectrophotometer (Loveland, CO). A broth culture starting with an OD600 of 0.3 to 0.6 was used for disinfection experiments. Two pilot growth inhibitory experiments were undertaken to determine a suitable range of CuO-NP concentrations (20, 40, and 80 μg ml−1) and 80, 160, and 240 μg ml−1 plus untreated control (denoted as “control” in the following text) based on previous studies (6 to 50 μg ml−1 for human cells [10]; 20 to 300 μg ml−1 for bacterial pathogens [4]). Given the significant inhibition of growth (OD600) observed from the pilot experiment at the highest three concentrations (see Fig. S1 in the supplemental material), the CuO-NP experimental concentrations were set as 80, 160, and 240 μg ml−1 (made from 100-μg ml−1 stock suspension) and compared to a control group (no treatment) all in a total of 30 ml BYE broth. The first batch of subsamples was taken immediately after CuO-NPs were added to each flask, and then the flasks were incubated with shaking (150 rpm) at 37°C. Over a 24-h incubation period, five subsamples were taken (3, 6, 9, 12, and 24 h). At each time point, duplicates of 5-μl, 200-μl, and 1-ml samples were taken from each flask for quantitative reverse transcription-PCR (qRT-PCR), plate counts, and total RNA isolation, respectively. All samples were immediately placed on ice and processed within 3 h.

**Cell lysis and total RNA extraction.** Each 5-μl sample was lysed with 25 μl of lysis buffer (SYBR green cell-to-CT kit; Life Technologies, Foster City, CA). DNA was subsequently digested with Turbo DNA-free DNase (Life Technologies, Foster City, CA) for 1 h at 37°C as described by the manufacturer. RNA from each 1-ml sample was isolated using TRIzol reagents as described by the manufacturer (Life Technologies, Foster City, CA), first treated with Turbo DNA-free DNase according to the manufacturer’s instruction to remove contaminating DNA. The purity and integrity of the resulting RNA dissolved in 20 μl nuclease free water was determined with a Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA), and the concentration was measured by Nanodrop (Nanodrop Products, Wilmington, DE).

To confirm complete removal of DNA contamination, 2 μl of the treated total RNA and a 10-fold dilution of the original RNA were added to the qPCRs with rpsL. qPCR assay as described below. The quality controls of qPCRs showed no DNA contamination found during the entire experiment, indicating the reagent and procedure were robust.

**Microarray procedure.** L. pneumophila exposed to 160 μg ml−1 for 3 h was compared with controls sampled at the same time point using a whole-genome microarray as previously described (25). Fifteen micrograms of RNA from each replicate/condition was labeled independently by reverse transcription using amino-allyl dUTP and random hexamers (25). Bacterial gDNA was used as the reference channel on each slide to allow comparison of the treated samples (26). Five micrograms of gDNA was labeled with amino-allyl dUTP using Klenow fragment and random primers (Invitrogen) at 37°C for 18 h (27). DNA was subsequently coupled to the succinimimidyl ester fluorescent dye Alexa Fluor 546 (for cDNA) or Alexa Fluor 647 (for gDNA) (Invitrogen) by following the manufac-
turer’s protocols. Hybridization to the microarrays and data acquisition was performed as previously described (25). Local background was removed from spot signal intensities, and normalization was carried out by calculating the fraction over the total signal intensity in both channels as previously described (27). Signal levels that were lower than background levels in experiments and controls were filtered out. A total of six cDNA-to-reference ratios were recorded for each condition, since the probes on the microarray are spotted in duplicate. Statistical analysis between test and control conditions was performed using an unpaired one-tailed Student t test. Genes were considered differentially expressed if they demonstrated a ratio-to-control value of ±2-fold with a P value of <0.005.

qRT-PCR. For new qRT-PCR assays, gene sequences were referenced from Legionella pneumophila subsp. pneumophila, strain Philadelphia 1 (http://biocyc.org/LPNE27624/organism-summary), and 21 new primers (see Table S1 in the supplemental material) used in this study were designed using the same IDT software (http://www.idtdna.com Primerquest/Home/Index) with the same parameters for all primers. To test primer melting temperature (Tm) (°C) and performance of PCR assays, gradient PCR for each assay was conducted against genomic DNA of strain Lp02. Furthermore, using the optimal Tm (~62 to 64°C), PCR assays were run against a panel of 20 various Legionella strains (see Table S2 in the supplemental material). In this study, only those assays showing strong PCR signals specific to strain Lp02 and of the expected amplification size and Tm (~1.5°C) were used. Total RNA samples from direct cell lysis were subjected to reverse transcription using the SYBR green cells-to-CT kit by following the manufacturer’s instructions (Life Technologies) on an MJ Research PTC-200 Peltier thermal cycler (Bio-Rad, Hercules, CA). Quantitative PCR was performed with primers listed in Table S1 in the supplemental material using the Applied Biosystems StepOne Plus 96-well real-time PCR system with Power SYBR green PCR master mix from SYBR green cells-to-CT kit (Life Technologies) by following the manufacturer’s instructions. All qPCR assays were run under the following conditions: initial incubation for 3 min at 95°C, followed by 40 cycles of 10 s at 95°C, 30 s at 63°C, and 20 s at 72°C. Melt curve analysis was also performed to evaluate PCR specificity and resulted in single 40 cycles of 10 s at 95°C, 30 s at 63°C, and 20 s at 72°C. Melt curve analysis was also performed to evaluate PCR specificity and resulted in single primer-specific melting temperatures. For each qRT-PCR run, the calculated threshold cycle (Ct) was normalized to the Cq of the internal control rpsL, amplified from the corresponding samples (denoted ΔCt-rpsL) and the fold change (2ΔΔCt) compared to the control was calculated (denoted ΔΔCt) as previously described (28). In order to test whether rpsL was a stably expressed gene, 1 μg RNA was taken from the samples with triplicate RNA samples in control and treatments (80 and 160 μg ml⁻¹) at five time points (0, 3, 6, 9, and 12 h), and the qRT-PCR targeting rpsL was assayed. Adjustments were undertaken when different amplification efficiencies were seen between internal controls and other target genes as specified by the manufacturer (ABI protocol, version 3.1).

Data analysis for the efficacy of CuO-NPs on L. pneumophila and qRT-PCR. The experiments were designed to compare one control against three CuO-NP treatments over five time points in pairwise comparisons for each sample with three replicates and two repeat samplings for each replicate. A general linear model (GLM) was used as follows: response variable = treatment + time + treatment × time. The GLM was run with SAS Systems version 9.2 (SAS, Cary, NC) to display the effect of CuO-NP concentrations, exposure time, and interactions between treatment and time. The response variable in the analysis was the concentration of RNA or the relative changes (fold changes) in gene expression from the qRT-PCR assay.

Microarray accession number. The microarray data can be downloaded from the GEO database, accession number GSE41966 (http://www.ncbi.nlm.nih.gov/geo/).

RESULTS
CuO-NP toxicity to L. pneumophila. Pilot experiments demonstrated significant reduction in L. pneumophila growth after exposure to 80, 160, and 240 μg ml⁻¹ with 3-h delay responses in OD₅₆₂ readings (see Fig. S1 in the supplemental material) but not at lower concentrations for <12 h of exposure. Thus, further analyses of L. pneumophila disinfection effects were performed based on the measurements of CFU (Fig. 2) and total RNA concentrations under CuO-NPs at 80, 160, and 240 μg ml⁻¹ (Fig. 3) compared to controls, which showed significant exponential increase in the total RNA quantity (Fig. 3; R² = 0.9624). Overall, CuO-NPs significantly (P value for F test [P_F-test] < 0.0001) inhibited Lp02’s growth (both CFU and total RNA yield). Based on CFU, the control Lp02 continued to grow, whereas cells exposed to 80 and 160 μg ml⁻¹ CuO-NPs showed no significant increase, and a significant decrease at the highest dose (240 μg ml⁻¹) was observed, which resulted in a linear decrease (R² = 0.7627) until no colonies were observed by 24 h after CuO-NP addition (Fig. 2). Compared to CFU, the total RNA measurement was more accurate in identifying cell inhibition (Fig. 3).

GLM analysis for total RNA showed that CuO-NP dose, time, and time-dose interactions were factors or cofactors for L. pneumophila growth inhibition. Specifically, from one dose to another dose, all pairwise comparisons except 160 versus 240 μg ml⁻¹ (P value for t test [P_t-test] = 0.111) showed significant differences (P_t-test ≤ 0.0003). From one time point to another time point, there was also a highly significant difference (P_t-test < 0.0001), except for 0 h versus 3 h, 6 h versus 9 h, and 12 h versus 24 h. Growth inhibition was apparent at 12 h for 80 and 160 μg ml⁻¹ of CuO-NPs and at 6 h for 240 μg ml⁻¹ (Fig. 2). Moreover, a concentration of 240 μg ml⁻¹ seemed to be lethal for L. pneumophila Lp02, and this effect was apparent as early as 9 h posttreatment (Fig. 2). At 24 h posttreatment at 240 μg ml⁻¹, no viable L. pneumophila was recovered (Fig. 2). Inhibition of transcription, measured by the amount of RNA recovered from the cells, was detected as early as 3 h posttreatment and for each of the concentrations tested (Fig. 3). Thus, it seems that L. pneumophila cells were sublethally impacted at the two lower concentrations (80 and 160 μg ml⁻¹) and lethally impacted at the higher concentration (240 μg ml⁻¹) after an exposure time of 24 h.

L. pneumophila transcriptome after exposure to CuO-NPs. The transcriptomic response of L. pneumophila Lp02 to 160 μg ml⁻¹ CuO-NPs after 3 h of exposure was analyzed using microarrays. Compared to untreated controls, microarray analysis showed that the exposure to CuO-NPs affected 966 genes (−1 > log₂ > 1; P < 0.005), 595 positively and 371 negatively. The analysis through the database of Clusters of Orthologous Groups of proteins (COG) revealed that the categories “Nucleotide metabolism,” “Replication and repair,” “Transcription,” “Translation,” and “Unknown/hypothetical function” were highly induced. The genes in the last three categories were upregulated by CuO-NP exposures (Fig. 4). In contrast, the gene categories “Chromatixis/motility/cell division,” “Protein fate/hydrolase/secretion,” and “Signal transduction/other regulatory function” were repressed by CuO-NP exposure (Fig. 4). Surprisingly, genes associated with the cellular response to copper showed no significant changes in expression (copA, copper efflux ATPase; copF, copper oxidase) or decreased expression (copper efflux ATPase, heavy metal efflux, heavy metal efflux pump CzaA, and metal ion transporter). There were more (n = 41) repressed genes involved in inner membrane transport and binding than induced ones (n = 34), but of the latter genes, most were virulence genes (type IV secretion system containing). Interestingly, four icm dot system genes (icmQ, icmR, icmW, and dotA) and 27 icm dot effectors/substrates were induced

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following exposure to CuO-NPs, including lem (3, 4, 19, 22, and 24), lepA, leg (A2, A5, L3, K2, LC8, and P), lpg (1689 and 2575), ceg (14 and 17), vpdA, and sdhA, and 28 effectors/substrates were repressed (Fig. 5).

The expression of 29 genes was tested by qRT-PCR (see Table S1 in the supplemental material), including virulence genes (n/H11005/H11022), cell replication-associated genes (ftsY, fusA, and gyrA), copper efflux ATPase (copA), biosynthesis-associated gene (pvcA), and sigma factor (rpoD). The ribosomal gene (rpsL) was used as the internal control. The validation experiment for internal control (rpsL) using qRT-PCR against normalized RNA among various treatments and time points showed that there was no significant difference among treatments ($P_{F_{tot}} = 0.277$), indicating that rpsL was a valid internal control. Further validation for amplification efficiencies between rpsL and the 12 important assays (icm, sidF, lem19, rpoD, ceg29, dotA, cegC1, lemA, rtxA, icmV, pvcA, and lem22) showed no significant differences ($n = 12$, at 0.05 level).

For qRT-PCR gene expression comparisons (all pairwise combinations of time points, treatments, and interaction), the fold changes ($2^{\Delta \Delta C_{T}}$) over 24 h were grouped according to their patterns. The level of differential gene expression ranged from 0.004- to 38.8-fold against a baseline of 1. There were 21 genes that demonstrated significant induction (>4-fold; $P < 0.0001$ for control compared to three treatments and zero time point compared to other time points), while the other genes showed weak expression (rpoD and lem22), no significant change in expression (ceg17, csrA, lpg2145, and icmQ), or reduced expression (copA). For those genes showing more than a 4-fold increase in expression, most showed similar trends for all three CuO-NP doses. However, four patterns of dose-time response were seen: (i) early induction that peaked at 3 h (Fig. 5A, icmV; see also Table S1 in the supplemental material, icmW, lepA, and umuD); (ii) midtime induction of replication-related genes (Fig. 5B, fusA; see also Table S1, fusY and gyrA); (iii) extended midtime induced genes (Fig. 5C and D, ceg29 and rtxA;
see also Table S1, legLC8, legP, lem19, lem24, and lpg1689; and (iv)
induction at all time points assayed (Fig. 5E and F, cegC1 and dotA;
see also Table S1, enhC, htpX, icmE, pvcA, and sidF). Further anal-
ysis identified two genes (ceg29 and rtxA) that were not only dose
dependent, i.e., increasing with CuO-NP concentrations (ceg29,
\(R^2 = 0.569\), \(n = 138\); rtxA, \(R^2 = 0.479\), \(n = 138\)) but also time
dependent, increasing from 0 h to 9 h (ceg29, \(R^2 = 0.516\), \(n = 92\);
rtxA, \(R^2 = 0.351\), \(n = 92\)) but decreasing from 9 h to 24 h (ceg29,
\(R^2 = 0.389\), \(n = 69\); rtxA, \(R^2 = 0.617\), \(n = 69\)). Copper-associated
gen (copA) appeared unchanged over the 24-h time course (see
Table S1).

**DISCUSSION**

The goal of this study was to investigate the concentration of CuO-
NPs required to inactivate *L. pneumophila* and to investigate tran-
scriptional effects, with a view to identifying useful genes to target
in situ. Toxicity and differential gene expression in *L. pneumophila*
were demonstrated for cells exposed to CuO-NPs, based on CFU
counts, mRNA yield, whole-genome microarray expression, and
qRT-PCR targeting 21 genes. Overall, CuO-NP-exposed *L. pneumo-
phila* Lp02 cells displayed change in gene expression involving
metabolism, nucleotide replication, transcription, DNA repair-
ing, and complete lethality following exposure to 240 \(\mu\)g ml\(^{-1}\) after 24 h. For laboratory testing, total RNA was the preferred
growth indicator, consistent with Kemp et al. (29), who
demonstrated a strong correlation in RNA concentration and
growth rate. The concentration of CuO-NPs resulting in Lp02
toxicity was consistent with previous reports for other bacteria;
pecifically, the MICs of CuO-NPs (7- to 17-nm diameter) were
between 140 to 280 \(\mu\)g ml\(^{-1}\) for four *Escherichia coli* strains, 20 \(\mu\)g
ml\(^{-1}\) for *Bacillus subtilis*, and 140 \(\mu\)g ml\(^{-1}\) for three *Staphylococcus
aureus* strains (4). For an accurate estimate of the Cu-NP MIC, the
agar plate method may need to be used, considering static prop-
erty. Given the likely role of free-living protozoa in supporting
growth of *L. pneumophila* reviewed in Lau and Ashbolt (30), it is
also interesting that exposure to 80 \(\mu\)g ml\(^{-1}\) CuO-NPs (30-nm
diameter) induced significant changes in the composition of *Tet-
rahymena thermophila* membrane fatty acids (5). On a more cau-
tionary note, cell lines exposed to 40 and 80 \(\mu\)g ml\(^{-1}\) of CuO-NPs
(20 to 40 nm) also resulted in cell damage or loss of viability and
oxidative activity (10, 31), indicating that these NPs may be un-
suitable for the treatment of water that comes in direct contact
with humans. While CuO-NPs as engineered nanomaterials
showed potential for disinfection of legionellae, like all technolog-
ical developments, unintended risks need to be fully examined,
such as described in EPA’s New Chemical Consent Orders and
Significant New Use Rules (SNURs) (http://www.epa.gov/oppt/
newchems/pubs/cnosnurs.htm).

In this study, it was shown by microarray data that many genes
involved in nucleotide metabolism were induced, some of which
were confirmed by qRT-PCR to exhibit long-lived induction (fisY,
fusA, and gyrA). However, a previously well-documented gene,
recQ, was repressed and encodes a protein belonging to the RecQ
family of DNA helicases and plays a critical role in maintaining
genomic stability (32). All those responses suggested high likeli-
hood of disruption of DNA by CuO-NPs. When *Staphylococcus
aureus* was exposed to peracetic acid, similar gene expressions
were observed, which were considered indicators of DNA disrup-

**FIG 4** Cluster of orthologous group analysis of the microarray data. The fraction of genes in orthologous groups induced or repressed after 3 h of exposure to 160 \(\mu\)g ml\(^{-1}\) of CuO-NPs compared to the untreated control are shown.

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L. pneumophila Response to CuO-NP Exposures

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Another significant response observed in this study was the induction of oxidative stress genes, *dnaK* and *katG*, suggesting the toxicity effects of CuO-NPs to *L. pneumophila* were similar to those observed in *C. jejuni* in response to ZnO-NPs and arsenic (9). Another possible consequence might have occurred in this study: when *L. pneumophila* Lp02 was exposed to 160 μg ml⁻¹ CuO-NPs for 3 h, the inner membrane transport and binding genes associated with transporters of amino acids, peptides, amines, cations, iron-carrying compounds, and carbohydrates through membranes were significantly repressed (*n* = 41), which may indicate cell membrane damage. Previous studies (9) on nanometal antibacterials describe direct damage to membrane and DNA and indirect impact, such as deleterious modification of protein function and increase in oxidative lesions (9). Cho et al. (34) reported cell wall rupturing in *E. coli* treated with silver nanoparticles. In addition to direct effects of nanoparticles to bacterial membranes, bacterial cells were also impacted by the release of metal ions in solution (4), which led to protein denaturation and cell death (35).

Virulence genes in *Staphylococcus aureus* following exposure to hydrogen peroxide, peracetic acid, and sodium hypochlorite disinfectants were induced (including exotoxins, coagulases, and intercellular surface adhesion proteins, respectively) (33, 36). The virulence enzyme-related responses in those studies were thought to be microbial defense systems against oxidants by damaging phagocytes and/or impairing oxidants (33). In the current study, a number of virulence genes were highly induced when *L. pneumophila* Lp02 was exposed to 160 μg ml⁻¹ CuO-NPs for 3 h, which...
was confirmed by qRT-PCR (Fig. 5). These virulence gene responses may also represent certain defense mechanisms to mitigate the toxic effect of CuO-NPs. For some genes, this response occurred immediately after addition of CuO-NPs and decreased to a lower level within 3 h (icmV, icmW, lepA), while others increased by 9 h (cag29, legL28, legP, lem19, lem24, lpgl1689, and rtxA), increased by 12 h (cagC1, dotA, cuhC, htpX, icmE, pveA, and sidF), and even kept high expression levels for up to 24 h (lepP, lem19, and ceg19). Possible due to metabolically stopping, the expression of most of the genes tested returned to negligible levels by 24 h. In a previous study, Staphylococcus aureus response to chlorination resulted in the induction of several virulence factors, and it was suggested that this may increase phagocyte invasion (36). In this study, a number of induced Icm/Dot effectors or substrates, which play a central role for Legionella phagocyte invasion, might suggest a similar upregulation of virulence defense response as seen with Staphylococcus aureus responding to chlorination (36).

In drinking water, the main environmental stresses faced by environmental bacteria are disinfectants and competition. This study developed a model to reveal some highly expressed legionellae genes for cells under disinfection stress, with the ultimate goal to use these activity assays for in situ detection of disinfection efficacy. Since DNA-based PCR methods do not differentiate DNA from live and dead bacteria, they may provide an overestimation of pathogen, unless targeting RNA via qRT-PCR assays. For example, comparable results between qRT-PCR and Legionella culture were recently reported and promoted as a rapid and accurate technique for improved surveillance of Legionella in drinking water (37). In our study, ceg29 and rtxA could provide qRT-PCR targets, which were significantly induced for 9 h in a dose- and time-dependent manner. Interestingly, rtxA is more frequently found in L. pneumophila serogroup 1 strains associated with human disease (13). Arushothy and Ahmad (38) isolated a total of 33 L. pneumophila serogroup 1 strains from 70 cooling tower water samples and reported that more than 82% (27 out of 33) had the rtxA gene. In Australia, Huang et al. (39) noted that 58% of their water isolates carried the rtxA gene. Therefore, further investigation of rtxA and similarly induced genes with more than 4-fold changes could serve as biomarkers associated with disinfection stressors. Regarding the internal control, a previously used 16S rRNA assay (27) was nonspecific to L. pneumophila, while the rpsL used in this study was specific and relatively stable for mRNA in L. pneumophila strain LP02.

In summary, CuO-NPs were shown to exhibit antibacterial activity and were lethal to L. pneumophila strain LP02 at a concentration of 240 μg ml⁻¹. CuO-NP exposure resulted in significant increases in the expression of genes involved in virulence and metabolism, as well as many unknown genes. To our knowledge, this is the first study to evaluate toxicity of CuO-NPs to Legionella pneumophila.

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