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Mechanisms of oxidative stress caused by CuO nanoparticles to membranes of the bacterium *Streptomyces coelicolor* M145

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ABSTRACT

Toxic effects of widely used CuO nanoparticles (NPs) on the genus *Streptomyces* has been seldom studied. This work investigated toxicities of several sizes of CuO nanoparticles (NPs) to *Streptomyces coelicolor* M145 (*S. coelicolor* M145). Compared with NPs, toxicity of micrometer-sized CuO on M145 was trivial. In 0.9% NaCl, when the concentration of CuO NPs was 100 mg/L, survival of bacteria increased from 18.3% in 20 nm particles to 31.1% in 100 nm particles. With increasing concentrations of CuO, the level of ROS gradually increased and there were significant differences ($p < 0.05$) in ROS exposed to 20, 40 and 100 nm (80 nm) CuO NPs. In TSBY medium, toxicity of CuO NPs was less and mainly attributed to release of Cu^{2+}, analysis by confocal laser scanning microscope (CLSM) showed that size of the mycelium did not change although some individual bacteria died. This was likely due to Cu^{2+} released from NPs entering cells through the membrane, while in 0.9% NaCl, lesions on membranes was caused by NPs outside the bacteria. This research indicated that toxicity of CuO NPs to *S. coelicolor,* is related to both size of NPs and is dependent on characteristics of the medium.

Capsule: This is the first time to measure the toxicity of nano materials to *Streptomyces*, and toxic CuO NPs to *Streptomyces* have been shown to differ depending on medium.

1. Introduction

NPs are materials ranging in size from 1 nm to 100 nm in at least one dimension (Dinesh et al., 2012). Due to their unique antimicrobial, electronic, optical, and structural strength enhancement properties, today NPs are used in virtually all fields (Balazs et al., 2006; Lee et al., 2010). An inventory of NP-enabled products, which mainly includes metals and metal oxides, applications indicates that more than 1814 products are being manufactured and the number is projected to triple by 2020 (Robichaud et al., 2009; Vance et al., 2015; Woodrow Wilson Datatbase). Among them, CuO NPs is one of the most common NPs used in industry. Compared with bulk particle CuO (CuO BP), CuO NPs exhibit unique physical and chemical properties, so that it has been successfully used in many fields, such as electronic equipment, sensors, superconducting materials and thermal conductivity materials (Aprile et al., 2010; Ben-Moshe et al., 2009). In addition, because of its antibacterial ability, it can be used as a novel plastic antimicrobial agent and is widely used in breeding of livestock and poultry (Delgado et al., 2011).

The increasing usage of CuO NPs in industry, agricultural applications, consumer products and a variety of medical applications, inevitably leads to releases of CuO NPs into the environment. Currently, due to the potential risks to ecological systems, these releases have become an emerging issue (Eduok et al., 2013; Schaumann et al., 2015). As an important part of ecosystems, toxic effects of NPs on bacteria had aroused extensive attention. It had been confirmed that CuO NPs can inhibit many kinds of bacteria , such as *Vibrio fischeri* (Heinlaan et al., 2008), *Escherichia coli* (*E. coli*), *Bacillus subtilis*, *Streptococcus aureus* (Baek and An, 2011). However, studies of a unique bacterium-*Streptomyces* have been rare. *Streptomyces* is a high genome G + C, gram-positive, filamentous bacterium that can generate various industrially important secondary metabolites (Borodina et al., 2008), two of which are pigmented: a diffusible blue pigmented actinorhodin and undecylprodigiosin (cell wall associated, red pigment) (Kim et al., 2016). Actinorhodin is a weak antibiotic, while undecylprodigiosin on the other hand, is known to have antimicrobial activities,

Abbreviations: NPs, nanoparticles; *S. coelicolor*, *Streptomyces coelicolor*; CLSM, confocal laser scanning microscope; BP, bulk particle; h, hours; *E. coli*, *Escherichia coli*; DLS, Dynamic Light Scattering; MS, mannitol soy; PI, propidium iodide; SEM, scanning electron microscoph

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immunosuppressive and anticancer properties (Williamson et al., 2006). *Streptomyces* have complex life cycles, undergoing differentiation from spore to substrate hyphae, aerial hyphae, spore chains, and mature spore (Bentley et al., 2002). Meanwhile, morphological differentiation of *Streptomyces* is generally sensitive to environmental stresses, such as heat, osmotic pressure and nutrients (Bibb, 2005). Therefore, *Streptomyces* can be an ideal model to study responses of microbes to NPs. However, the toxic effects of NPs to *Streptomyces* had been little studied and described.

Toxicity of CuO NPs has been studied and much debate still exists about the mechanisms of toxicity of CuO NPs. Results of recent studies indicated that oxidative stress and dissolved ions played important roles in toxicities of NPs (Jin et al., 2010; Horie et al., 2012; Napierska et al., 2012). Integrities of membranes was a primary indicator of effects of NPs on bacteria under adverse stress (Kang et al., 2008). Results of some studies suggested that CuO NPs functioned via the specific particle effects. After 2 h (hours) exposure, inhibition of 45.4% was observed on *E. coli* at 10 mg/L of CuO NPs, but the dissolved Cu^{2+} ions at this concentration had no obvious toxicity (0.83% inhibition) (Zhao et al., 2013). In another study using different bacteria, it appeared that particles played principal role in toxicity while that of copper ions was negligible (Baek and An, 2011). However, some researchers insisted that from a toxicology perspective, dissolved copper was more harmful to organisms than solid copper particles (Aruoja et al., 2009). Therefore, when studying toxicity of CuO NPs to microbes determining whether particles or released ions are responsible for toxicity. This is important for determining doses and affects of accessory factors on toxic potencies of CuO NPs. In addition, media used by various researchers were not the same. Some media were inorganic (Jin et al., 2010) while others contained mixtures of organic substances (Napierska et al., 2012) and some used water collected from natural environments (Tong et al., 2013). Results of several studies have shown that solubility of CuO NPs in solution was affected by organic matters (Gunawan et al., 2011; Zhao et al., 2013). Therefore, in order to verify whether the medium could affect the toxicity of CuO to *Streptomyces*, in this experiment, two typical medium, 0.9% NaCl and an organic rich medium- TSBY were used.

Toxicity to *S. coelicolor* M145 was determined and the mechanism was explored during exposure to CuO either in the form of NPs or BPs in each of two culture media, 0.9% NaCl or organic rich TSBY. To our knowledge, it was the first holistic investigation on the toxicity of CuO NPs to *Streptomyces*.

2. Materials and methods

2.1. Characterization of CuO NPs

CuO NPs of 20, 40, 80 or 100 nm as well as BPs (1 μm) were purchased from Shanghai Macklin Biochemical company (China). Sizes and morphologies of particles were evaluated by use of a JSM-7800 scanning electron microscoph (SEM, JEOL, Japan) (Rajesh et al., 2012). CuO NPs and BPs suspensions (10, 20, 40, 100 mg/L) were prepared by adding dry particles into 0.9% NaCl or TSBY media (Trypticase Soy Broth: 30 g/L, Sucrose: 340 g/L, Yeast extract: 5 g/L) (Yang et al., 2012), then sonicated (100 W, 40 kHz) for 30 min and shaken for 2 h to facilitate dispersion. Sizes of particles and agglomerates in solution were measured by Dynamic Light Scattering (DLS) with a Zetasizer nano ZS (Malvern, UK). The pH and EC of the particle suspensions were then measured (Wang et al., 2009). Data were collected in triplicate at 25 °C.

2.2. Cultivation of bacterial

The organism used in this study was a unique, Gram-positive bacterium, *S. coelicolor* M145, purchased from China General Microbiological Culture Collection Center (Beijing, China). For spore production, *S. coelicolor* was cultivated on mannitol soy (MS) agar plates for 7 days at 30 °C, and harvested by scraping and suspending in 20% (v/v) glycerol and stored at − 80 °C (Sigle et al., 2016). Seed culture of *S. coelicolor* was prepared by inoculating spores into a special shaking flask with 100 mL of TSBY liquid medium, and incubated at 30 °C with shaking at 150 rpm for 48 h. Germinated spores were harvested by centrifugation at 6000 × *g* for 10 min (Bhatia et al., 2016).

To measure growth of *S. coelicolor* M145, triplicate samples of mycelium were washed three times with 0.9% NaCl and collected on a preweighed filter by vacuum. Filters with associated mycelium were freeze-dried and mass determined (Hesketh et al., 2007; Huang et al., 2015). The difference between the latter and the initial values was defined as the dry mass of bacteria.

2.3. Viability staining and confocal laser scanning microscope analysis

Cytotoxicity to *S. coelicolor* M145 was assessed by measuring changes of relative abundance of viable cells with the LIVE/DEAD Bac-Light bacterial viability kit (L-13152; Invitrogen). Cells were stained with two stains, propidium iodide (PI), a red fluorescent nucleic acid stain in order to detect dead cells and SYTO 9, a green fluorescent nucleic acid stain to detect the viable cells. SYTO 9 green fluorescent stain labels all of the cells no matter the membranes intact or damaged. In contrast, PI as a non-cell-permeating stain, labels only bacteria with damaged membranes. Thus, in the presence of both stains, bacteria with intact cell membranes appear fluorescent green whereas bacteria with damaged membranes appear red (Binh et al., 2014).

S. coelicolor M145 was cultured for 48 h in TSBY medium before measuring toxicity of CuO, and then the solution was centrifuged and washed with 0.9% NaCl three times. Series concentration of CuO NPs were first prepared with two different solutions − 0.9% NaCl and TSBY medium. Suspensions of 50 μL were added to 96 well microplates with 3 replicates per treatment. Plates were incubated at 30 °C with shaking at 150 rpm on an orbital shaker (HNY-2102C, Honour, China). After 4 h of incubation, 50 μL Bac-Light solution (SYTO 9: PI = 1:1) was added to each well and plates were incubated with shaking for 15 min in the dark. The green (excitation 485 nm and emission 530 nm) and red (excitation 485 nm and emission 630 nm) fluorescence of each well were measured by Microplate reader (synergy h4, BioTek). Relative abundances of viable bacterial cells in each well were expressed as a ratio of green to red fluorescence signals (live dead ratio). A calibration curve was obtained by using cultures of *S. coelicolor* M145 mixtures with known percentages of live and dead cells. The percentage of viable bacteria for different treatments was normalized by the calibration curve (Tong et al., 2013). After staining, samples were examined under a confocal laser scanning microscope (LSM880 with Airyscan, Zeiss, Germany) with the same wavelengths as the microplate reader.

2.4. Intracellular reactive oxygen species (ROS)

Concentrations of intracellular ROS in *S. coelicolor* M145 were determined by ROS Assay Kit (Beyotime, China). In the kit, a cell permeable 2′,7′-dichlorofluorescein diacetate (DCFH-DA) is used as a oxidation-sensitive dye, which can be deacetylated by esterases into dichlorofluorescein (DCFH), and DCFH can't cross cell membranes freely. Finally DCFH is oxidized into fluorescent 2′,7′-dichlorofluorescein (DCF) by intracellular ROS to indicate concentrations of intracellular ROS. Briefly, after 4 h exposure, the pellets were centrifuged and washed three times with 0.9% NaCl. Then suspended with 10 μmol/L DCFH-DA and incubated in the dark at 30 °C for 30 min, followed by washing three times with 0.9% NaCl. Then 100 μL solution was added to 96 well microplates. The fluorescence intensity was measured by microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The relative ROS level was represented as the fluorescence intensity ratio of the exposure group to the control group with the same dry mass (Li et al., 2016).