CHARACTERIZATION OF ANTIBACTERIAL MECHANISMS OF ZINC OXIDE IN *STAPHYLOCOCCUS AUREUS*

By

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CHARACTERIZATION OF ANTIBACTERIAL MECHANISMS OF ZINC OXIDE

IN *STAPHYLOCOCCUS AUREUS*

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Abstract

Rising precedence of antibiotic resistance has increased interest in nontraditional antibacterial agents such as zinc oxide nanoparticles (ZnO NPs). Although the anti-microbial activity of ZnO NPs is well established, the mechanism of this activity is unknown. Current literature hypothesizes that ZnO NP cytotoxicity could be mediated through one or multiple proposed mechanisms including production of reactive oxygen species (ROS), release of toxic ZnO^{2+} ions, and charged interactions that disrupt the cell wall and cause osmotic stress. Literature also suggests bacteria may be unable to gain resistance to ZnO because antibacterial action occurs through multiple mechanisms. To illuminate the properties of ZnO and determine which of the proposed mechanisms occur, ZnO susceptibility was assessed in *Staphylococcus aureus*. To determine if bacteria gain resistance to ZnO, *S. aureus* was passed in ZnO at sublethal doses. We find that *S. aureus* swiftly gain antibiotic resistance, suggesting ZnO antibacterial activity may operate through a single mechanism. To determine the predominant mechanism, susceptibility assays were performed in *S. aureus* mutants with deletions in *katA*, a gene important to defense against H_2O_2, and *mprf*, a gene important to cell wall charge. We find that production of H_2O_2 and charged interactions with the cell wall are not significant in ZnO susceptibility. Lastly, we find that media conditioned with ZnO effectively inhibits bacterial growth in the absence of ZnO particles. We conclude that physical contact with *S. aureus* is not necessary for ZnO activity, although the precise mechanism by which bacterial growth is inhibited is not yet elucidated.

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Table of Contents

Introduction

The discovery and subsequent development of penicillin in the early 20th century led to an explosion in the discovery of antibiotics. This explosion of antibiotics has reduced the prevalence of many infectious diseases and established antibiotics as the gold standard of treatment for most bacterial infections. Use of antibiotics has not been limited to medical treatments in humans [1]. There has also been widespread use of antibiotics in agriculture, particularly in the chicken and cattle industry. This ubiquitous and often improper use of antibiotics has created conditions ideal for the selection of antibiotic resistance in bacteria. Antibiotic resistance arises when bacteria acquire new genes or mutations in existing genes that provide resistance to a given antibiotic. While antimicrobial agents have saved countless lives since their advent, the use of antibiotic drugs has directly led to the increase in antibiotic resistance in a wide range of bacteria strains through the selective survival of cells with resistance genes.

This rise in antibiotic resistant bacteria is of clinical significance as an increasing number of bacterial infections in human isolates display antibiotic resistance. As a result of this resistance, the gold standard antibiotics are no longer viable treatments and physicians must resort to increasingly toxic antibiotics. It is estimated that antibiotic resistant infection will cause 10 million deaths worldwide by year 2050 if the current trend of inappropriate and excessive use of antibiotics continues [2]. This medical crisis is approaching a breaking point as the development of novel antibiotics has plateaued. Methicillin-resistant *Staphylococcus aureus* (MRSA) embodies this crisis. *S. aureus* is a gram-positive cocci-shaped bacterium that is often found as part of the human skin microbiota. In some cases, *S. aureus* can also become an opportunistic pathogen that is associated with skin infections and food poisoning. Standard *S. aureus* strains are susceptible to a wide range of antibiotics; however, MRSA strains have become resistant to multiple antibiotics [3]. These multidrug-resistant strains have become extremely difficult to treat. As the incidence of multidrug-resistant “superbugs” rise, the need for novel antibiotics has become vital.

As such, there has been increased interest in unorthodox antibacterial agents. One such agent is metal oxide nanoparticles (NPs). Many metal oxide NPs such as zinc oxide (ZnO) have received increased attention due to the unique properties they exhibit when the size of the particle decreases into the nanometer range. Among these properties are, a high charge to surface ratio, high electron mobility, a wide band gap, and its ability to act as a semiconductor [4-6]. These properties have led to widespread applications from use in sunscreen to solar panels to light emitting diodes (LEDs). Another notable property that has received attention are the antibacterial properties of ZnO coupled with its relative low toxicity to human cells, that set it up to be a novel antibiotic [7-12]. ZnO NPs have possible applications in water purification [13], food canning [14], and sterilizing medical equipment and textiles [11]. ZnO NPs could be particularly effective in isolated areas of the world where food and waterborne illnesses are abundant due to their long-term stability.

Although there is evidence that ZnO NPs are efficacious against multidrug resistant strains of *S. aureus* and have broad spectrum cytotoxicity to both gram-positive and gram-negative strains of bacteria [15, 16], there is controversy over the mechanism of bacterial killing. Among the most often proposed mechanisms are production of reactive oxygen species (ROS), damage of cell membranes, electrostatic interactions, internalization that leads to disruption of cell metabolism or DNA replication, or generation of Zn^{2+} ions that can lead to mis-metalation of coenzymes and interference with enzyme function [7, 12, 17-21]. Other literature suggests that more than one of these mechanisms could be responsible for the antimicrobial activity of ZnO. [8, 22]

Production of ROS has had the most support in recent years because of multiple studies that directly or indirectly demonstrate that ZnO NPs are able to produce ROS. This ability to produce ROS, however, seems to be highly dependent on the context and conditions of the ZnO NPs. Some data indicates that light is required to activate the production of ROS, while others suggest that light is not necessary[19, 21]. Several studies have also studied the expression of oxidative stress genes in response to ZnO exposure with contradicting reports. Some suggest that oxidative stress genes are upregulated, while others suggest that

there is no increase or even a decrease in the expression of oxidative stress genes in response to exposure to ZnO [20, 23]. Further conflicting the ROS mechanism, some studies have shown that bacteria experience an increase in lipid peroxidation in response to exposure to ZnO NPs, while others do not [17, 20]. In addition, Kadiyala et al 2021 showed that application of antioxidants was not able to protect pathogens from ZnO mediated death which further calls into question the role of ROS is ZnO mediated death [20].

Dissolution of toxic Zn^{2+} ions has also been proposed [4, 8, 11, 12, 22] but has received significantly less experimental attention. While the role of Zn^{2+} in the ZnO NP cytotoxic mechanism is still unconfirmed, the ability of Zn^{2+} ions to induce bacteria death is established [24]. The majority of evidence supporting release of toxic Zn^{2+} as the mechanism come from studies that study the relationship between the ZnO NP surface and interactions with the media [25, 26]. Specifically, an increase in the number of oxygens deficiencies and abundance of surface trap states would suggest an excess of Zn^{2+} ions at the polar surfaces of ZnO MPs (Dustins unpublished paper). In opposition to this, other data has indicated that Zn2+ ions are not responsible for bacterial death because physical ZnO exposed supernatant was not able to inhibit growth and addition of soluble Zn^{2+} did not increase killing [19, 20].

In addition to this, the role of internalization, as well as physical contact has received significant attention in multiple studies [7, 9, 14]. Many of these studies rely on SEM or TEM microscopy to make claims that physical contact is inducing damage and morphological change to the cell envelope [7, 9, 14, 27]. Others identified specific functional groups that ZnO interacts with on the cell surface through FTIR and Raman microscopy [7, 9]. Many sources report both contact with and internalization of ZnO NPs occur, but few of them have evaluated ZnO cytotoxicity in the absence of physical contact. As a result, it is possible that damage to the cell membrane may occur through a mechanism not dependent on physical contact. While some sources found that internalization on NPs enhances killing [14], Reeks et al. 2021 found that the antibacterial activity was not dependent on internalization [28].

The purpose of this study is to characterize the antibacterial mechanisms of ZnO in *S. aureus*. Specifically, we will use wild-type and mutant S. aureus strains to investigate the importance of media type, hydrogen peroxide (H_2O_2), physical contact, and cell charge for the antimicrobial properties of zinc oxide against *S. aureus*. We will also look at the susceptibility of ZnO NPs to the development of resistance. Better insight into the antimicrobial mechanism of ZnO NP will allow researchers to develop morphologies that are optimized for microbial cytotoxicity. Development of more efficacious particles could lead to widespread implementation of ZnO as a sterilization tool in food handling/packaging and the medical field.

Procedures and Methods

Bacteria Strains, Culture Conditions, and Zinc Oxide

S. aureus strains (Newman and SA113) were grown in Mueller Hinton Broth (MHB; Hardy Diagnostics) medium at 37°C under aerobic conditions. *S. aureus* Newman with a deletion in *katA* (Δ*katA*) and *S. aureus* SA113 with a deletion in *mprF*, Δ*mprf*, were created as previously described by [29, 30]. ZnO resistant *S. aureus* were derived through *in vitro* passage as described in methods. Zinc oxide was either obtained commercially from Sigma-Aldrich, Alfa Aesar, or Zochem,

ZnO MIC Assays

Cultures were grown to early log phase to an optical density of 0.4 at 600nm then diluted 1:200 in microcentrifuge tubes with varying concentrations of ZnO particles in a final volume of 1 ml. The microcentrifuge tubes were inverted using an electronic inverter for 16-20 hours at 37° Celsius in order to maximize the interactions between the ZnO particles and *S. aureus*. Microcentrifuge tubes containing the same concentrations of ZnO particles in 1ml MHB without *S. aureus* were co-incubated at the same time. After incubation, the microcentrifuge tubes were centrifuged at 100 rpm for 30 sec to separate the ZnO particles from the *S. aureus*. 200µl of supernatant were then transferred into a 96-well plate and the OD600 values were measured using a Fluostar Omega plate reader (BMG Labtech). The OD600 readings for the ZnO control tubes were then subtracted from the OD600 readings from the tubes containing the same concentration of ZnO with *S. aureus* to determine the growth of the *S. aureus* independent of the turbidity caused by remaining ZnO particles.

Hydrogen Peroxide (H_2O_2) MIC Assays

Cultures were grown to early log phase to an optical density of 0.4 at 600nm and diluted 1:200 in 96-well plates with varying H_2O_2 (Sigma Aldrich) concentrations in a final volume of 200ul of MHB. The

microcentrifuge tubes were then incubated statically for 16-20 hours at 37° Celsius. After incubation, the OD600 values were measured to determine bacterial growth.

Generation of ZnO Resistance

S. aureus was grown in sublethal concentrations of ZnO (Sigma Aldrich) for 24-hrs and passed daily into fresh ZnO-containing media by centrifuging at 200 rcf for 30 seconds to pellet the ZnO and transferring 20 µl of supernatant into a new 1.75ml microcentrifuge tube containing 500 µl of 0.313 mg/ml ZnO. MICs were conducted on days 0,2,4,6,8,10, and 15 by inoculating 3 ml of MHB with 35 ul of the passaged *S. aureus*, growing to an OD600 of 0.4, and then further diluting to a final ratio 1:200 in MHB before conducting ZnO MIC assays as described above. Data are plotted as fold change from the original log phase MIC of 1.25 mg/ml.

Bacterial Survival

S. aureus were grown until early log phase (OD600 of 0.4) and then washed and resuspended in MHB, phosphate buffered saline (PBS), or saline as indicated before being diluted 1:100 in the respective medias. PBS was created by dissolving 4.0g NaCl, 0.1g KCl, 0.72g sodium phosphate-dibasic (anhydrous), and 0.12g KH_2PO_4, in 500ml of H_2O and adjusting the pH to 7.4 before autoclaving. Saline was created by dissolving 4.0g NaCl and 0.1g KCl in 500ml of H_2O and adjusting the pH to 7.4 before autoclaving. 750µl of the diluted log phase cultures were incubated inverting at 37° C with 750µl of 5 mg/ml ZnO suspended in the respective medias to create a final concentration of 2.5 mg/ml ZnO and 1:200 diluted log-phase *S. aureus*. At the indicated time points, 200 µl of culture from each tube was removed and centrifuged at 100 rcf for 2 minutes to pellet the ZnO particles. 10-fold dilutions of the supernatants were then plated to enumerate surviving CFU/ml.

Conditioned Media

Conditioned media was made by immersing Sigma Aldrich ZnO particles in MHB, PBS, or saline media, produced as previously described, to create 20 mg/ml ZnO stock solutions in clear conical tubes.

These solutions were incubated uncovered at room temperature for the indicated time. At the specified times, 1 ml of the media was removed and centrifuged at 16,000 rcf for 5 minutes to pellet any suspended ZnO. The supernatant was then removed and a 100 ul was added to a well of a 96-well plate. Log phase *S. aureus* were then diluted 1:100 in regular MHB and 100 ul was added to the same wells (100 µl conditioned media plus 100 µl diluted *S. aureus* culture) for a final concentration of log-phase *S. aureus* diluted 1:200 and conditioned media diluted 1:2. As a positive control, 200µl of log phase cultures diluted 1:200 in regular MHB (no conditioned media or ZnO) were grown in one well and as a negative control, 200µl of conditioned media (no *S. aureus*) was placed in another well. The plate was then incubated shaking at 37° for 16-20 hours. After incubation, the OD600 values were measured using a Fluostar Omega plate reader from BMG Labtech to measure bacterial growth.

Cytochrome C Assay

S. aureus cultures grown overnight in MHB were diluted 1:100 and grown for ~2 hours at 37° C until log phase with an OD600 of 0.4. These cultures were centrifuged, washed, and resuspended in 20 mM MOPS buffer. MOPS buffer was created by dissolving 0.419g of MOPS (Sigma Aldrich) in 100 ml of H_2O. *S. aureus* quantified by optical density using OD600 and volume of the culture needed to produce an OD600 of 1.0 in 250 ul was calculated. This volume of *S. aureus* was incubated at room temperature for 30 minutes with varying concentrations of cytochrome c from *Saccharomyces cerevisiae* (Sigma Aldrich) in 250ul of MOPS. This mix was then pelleted, 200ul of the supernatant was transferred into a 96 well-plate, and the amount of cytochrome c was quantified using OD530.

Daptomycin Assay

Strains were propagated overnight for approximately 16 hours and then diluted 1:100 and grown to early log phase at an optical density (OD) of 0.4 at 600 nm wavelength. Cultures were pelleted, washed, and re-suspended in an equivalent volume of Mueller-Hinton Broth (Hardy Diagnostics) supplemented with 50µg/ml calcium chloride to make CA-MHB. *S. aureus* were diluted to a final dilution

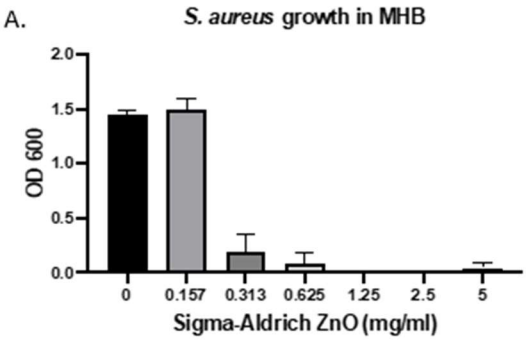
of 1:200 CA-MHB with 0.5 µg/ml daptomycin. Assays were performed in flat bottom 96-well plates, which were incubated statically overnight for approximately 16 hours at 37°C. Following incubation, the optical density of each well was measured at 600 nm wavelength.

Results

To characterize the antibacterial effect of ZnO NPs from various sources, MIC assays were conducted as described in the methods with each of the particle types indicated in Fig.1b. Results indicate ZnO particles from commercial sources (Sigma Aldrich, Alfa Aesar, and ZoChem) display antibacterial properties. However, not all NPs performed equally. Both Sigma-Aldrich and Alfa Aesar NPs displayed MICs of 1.25 mg/ml whereas ZoChem NPs had a slightly higher MIC of 2.5 mg/ml. We chose to use the Sigma particles as our primary ZnO particle as it was slightly more effective than the Alfa Aesar particle and was abundant in the lab.

Fig. 1

A.



B.

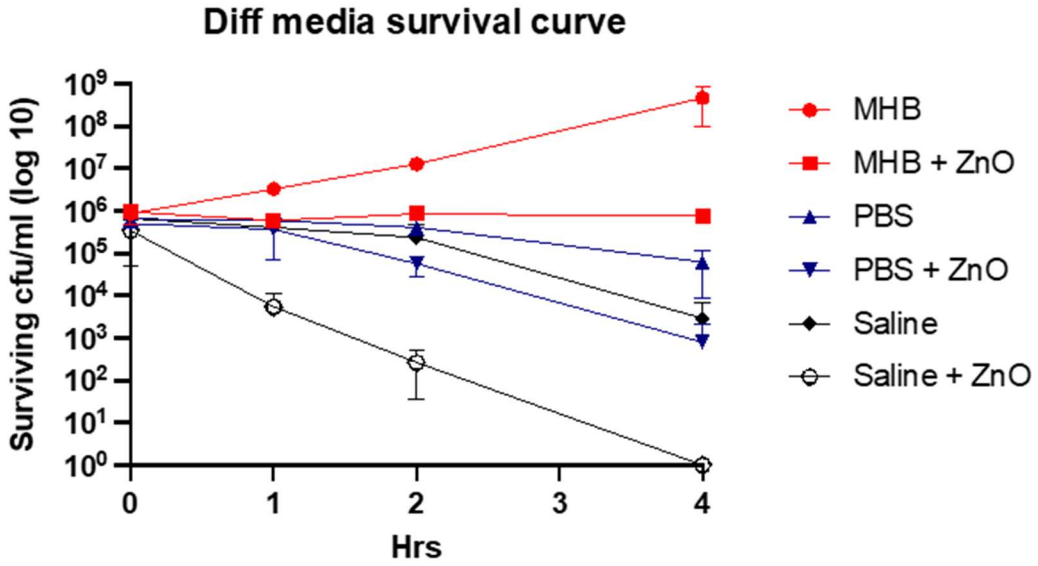
Particle Type	Size	Shape	MIC (mg/ml)
Sigma Aldrich	<100nm	Amorphous	1.25
Alfa Aesar	<100nm	Amorphous	1.25
ZoChem	<100nm	Amorphous	2.5

Collection of different ZnO NPs demonstrate conserved antibacterial action. a) *S. aureus* growth in Sigma-Aldrich NPs. b) Comparison of the size, shape, and MICs of indicated ZnO NPs. Data is presented as the mean of at least three independent trials, and SD is represented by the error bars.

We next assessed the role of different types of media on the effectiveness of ZnO activity as there have been contradicting reports of the activity in different medias [8, 16, 20]. To do this, survival of *S. aureus* was monitored over time using Sigma Aldrich ZnO NPs. MHB has traditionally been used in

antibiotic MIC testing, so we used this as our bacterial growth media. PBS was chosen due to data that indicated that phosphates in the media may increase the cytotoxic effect of ZnO NP [31]. Saline was later used to evaluate the role of phosphates in the media when decreased antibacterial activity of PBS was observed. The results indicate that media type significantly impacts the bacteriostatic verses bactericidal properties of ZnO NPs. In MHB, ZnO NPs act in a bacteriostatic manner, whereas ZnO NPs are bactericidal in saline. In PBS bactericidal activity of ZnO was inhibited presumably by the presence of phosphate ions. Literature supported this conclusion and indicated that phosphates in the PBS may react with zinc species to produce zinc phosphate crystals that ameliorate the antibacterial effect of ZnO NPs [26, 32].

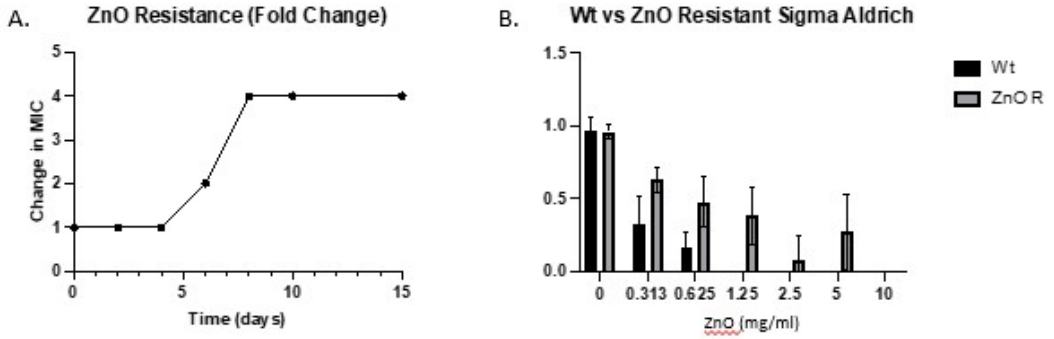
Fig. 2



Antibacterial action of ZnO is dependent on the type of growth media used. *S. aureus* survival over-time with Sigma-Aldrich ZnO NP in indicated medias.

We next wanted to investigate whether *S. aureus* can gain resistance to ZnO. Previous studies have suggested that the mechanism of ZnO killing occurs through multiple mechanisms, and therefore that it would be difficult for bacteria to gain resistance to ZnO NPs (Slavin, 2017). To do this, we passed *S. aureus* in sublethal concentrations of 0.313 mg/ml ZnO and then tested whether the MIC changed over time. We found that within one week, a two-fold increase in the MIC was observed when *S. aureus* was passed under sublethal concentrations of Sigma-Aldrich NPs (fig. 3a). After another three days, a four-fold increase, as compared to day zero, was observed, indicating that *S. aureus* rapidly gains resistance to ZnO NPs. Permanent stocks of our resistant strain were made (hereby referred to as ZnO^R) and we confirmed that this resistance was maintained against the Sigma ZnO particles (fig. 3b) as well as other ZnO sources (fig 3c).

Fig. 3



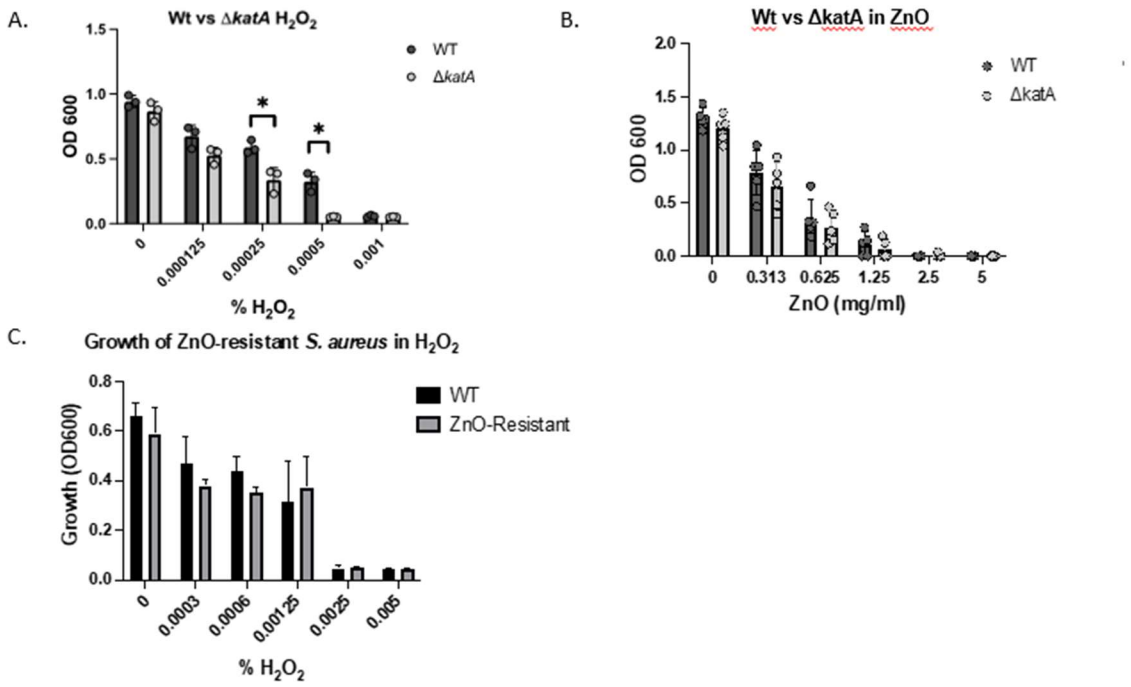
C.

Particle Type	ZnO Susceptible (WT) MIC (mg/ml)	ZnO Resistant Mutant MIC (mg/ml)
Sigma Aldrich	1.25	5
Alfa Aesar	1.25	5
ZoChem	2.5	10

***S. aureus* resistance to ZnO NPs is rapidly acquired and is conserved between different ZnO NPs**. a) Fold change in MIC using Sigma-Aldrich NPs over time. Data represents the mean of 3 independent experiments. b) MIC of Wt and ZnO resistant with Sigma-Aldrich NPs. Data is presented as the mean of at least three independent trials, and SD is represented by the error bars. c) Comparison of MICs of ZnO resistant mutants with indicated NPs.

Since production of ROS was the predominant mechanism suggested by the literature, it was the first proposed mechanism we investigated using a mutant with a deletion in *katA* (Δ*katA*). The *katA* gene encodes the enzyme catalase which is responsible for converting hydrogen peroxide (H_2O_2) into H_2O and oxygen (O_2). We hypothesized that if production of H_2O_2 is responsible for the antibacterial properties of ZnO NPs, then Δ*katA* would be more susceptible to ZnO than the WT strain from which it was derived. To test this, Newman WT stain growth was compared to Δ*katA* growth in the presence of H_2O_2 as well as ZnO NPs. As expected, Δ*katA* is more susceptible to the cytotoxic effects of H_2O_2 (fig 4a) but Δ*katA* did not demonstrate decreased growth as compared to the WT when incubated with Sigma Aldrich NPs (fig. 4b). To further evaluate the role of H_2O_2 in ZnO cytotoxicity, growth of WT *S. aureus* in H_2O_2 was compared to ZnO^R *S. aureus* growth in H_2O_2. If production of ROS is key to the antibacterial mechanism of ZnO NPs, then it is possible that the ZnO^R strain would also be resistant to ROS such as H_2O_2. When incubated with H_2O_2, WT and ZnO^R strains did not demonstrate any difference in susceptibility (fig. 4c).

Fig. 4

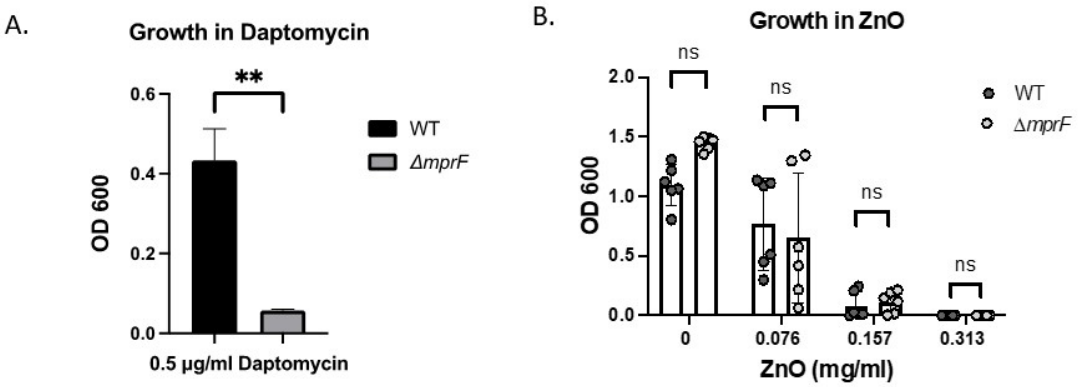


Production of H_2O_2 is not responsible for the antimicrobial activity of ZnO NPs. a) WT Newman and Δ*katA S. aureus* growth in H_20_2. b) WT Newman and Δ*katA S. aureus* growth in Sigma-

Aldrich NPs. c) WT Newman and ZnO Resistant *S. aureus* in H_2O_2. a-c Data is presented as the mean of at least three independent trials, and SD is represented by the error bars.

We next chose to evaluate the role of cell envelope charge in the antibacterial mechanism of ZnO NP. The *mprf* gene encodes a protein that synthesizes and translocates positively charged phospholipids to the cell envelope. A more positively charged cell envelope will more effectively repel positively charged antimicrobial and loss of *mprf* has been linked to decreased antimicrobial resistance to the antimicrobial peptides LL-37 and defensins, which are part of host innate immune defense, as well as to the antibiotic daptomycin [33, 34]. We used a Δ*mprf S. aureus* strain to test whether electrostatic interactions mediate ZnO cytotoxicity. To confirm that the Δ*mprf* strain we received had a difference in surface charge, we first tested the susceptibility of wild-type and Δ*mprf* to daptomycin, which is cationic when incubated in media containing Ca^{2+} ions (CA-MHB). Because the Δ*mprf* mutant has a more negatively charged cell envelope, it is expected to be more susceptible to daptomycin. We find this to be the case with *ΔmprF S. aureus* displaying increased susceptibility to daptomycin verses WT *S. aureus* (fig.5a). We hypothesized that if charged interactions are responsible for the antibacterial properties of ZnO NPs, then Δ*mprF* would be more susceptible to ZnO than the SA113 parental strain. To test this, we compared the susceptibility of the parental and Δ*mprF* to ZnO NPs. Results indicate that the Δ*mprF* did not demonstrate decreased growth as compared to the WT when incubated with Sigma Aldrich NPs (fig. 5b).

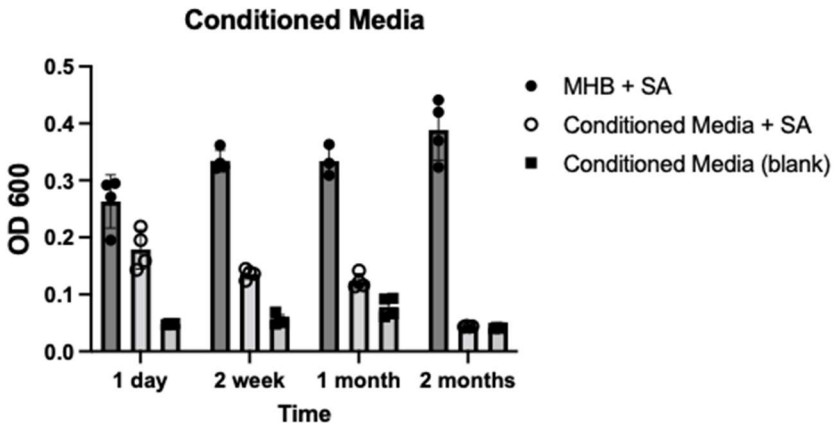
Fig. 5



Charged interactions do not mediate ZnO cytotoxicity a) Absorbance of supernatant of SA113 WT and Δ*mprF* strains after incubated with daptomycin. b) WT SA113 and Δ*mprF S. aureus* growth in Sigma-Aldrich NPs. Data is presented as the mean of at least three independent trials, and SD is represented by the error bars.

We next chose to investigate whether the cytotoxic activity of ZnO NPs is dependent on physical interaction with the bacterial cells. To test this, we created conditioned media that had prolonged exposure to ZnO NPs but from which we removed the ZnO NPs before incubation with *S. aureus*. We hypothesized that if physical contact was necessary, then conditioned media would not inhibit *S. aureus* growth, however if the release of soluble compounds such as ROS or ionic species is responsible, then the conditioned media may retain the ability to inhibit growth. After one day of conditioning, we observed no inhibition of growth compared to growth of *S. aureus* in plain MHB. After one week of conditioning with ZnO particles, inhibition of growth was observed as compared to growth in MHB, but full inhibition was not achieved until one month of conditioning (fig. 6A).

Fig. 6



Media
conditioned with ZnO NPs retains ability to inhibit bacterial growth after removal of NPs.

S. aureus growth in conditioned media.

Discussion

The existing body of knowledge related to the antimicrobial mechanism of ZnO is fraught with controversy and debate. Our primary goal was to address and provide clarity to conflicting reports regarding the mechanism of action for ZnO NPs using mutants with susceptibilities to proposed mechanisms. Although some literature suggested the involvement of multiple pathways working in consort to inhibit bacterial growth [11, 22, 30], our findings that *S. aureus* can quickly gain resistance to ZnO NPs may contradict this. If ZnO NPs inhibited bacterial growth through multiple mechanisms, then we would expect that *S. aureus* would be unable to generate resistance because it would require multiple mutations to be acquired simultaneously. The rate at which *S. aureus* acquired resistance to ZnO NPs suggests that there is a dominant mechanism responsible for ZnO cytotoxicity.

The most widely-proposed mechanism of ZnO antimicrobial activity has been production of ROS, particularly H_2O_2 [11]. ROS can cause oxidative stress and lipid peroxidation which can lead to loss of membrane integrity as well as damage to DNA and proteins. H_2O_2 is the most feasible of the ROS produced due to its ability to easily move across the membrane, while other ROS such as hydroxyl and superoxide are unable to. We saw that H_2O_2 susceptible *S. aureus* (Δ*katA*) were not more susceptible to ZnO NPs than the parental strain indicating that if any H_2O_2 is produced by ZnO NPs, it is unlikely to be responsible for the antibacterial action (fig 4b). Because the Δ*katA* mutant is specifically susceptible to H_2O_2, we are unable to draw conclusions about the role of other ROS using the mutant. The inconsequence of H_2O_2 in the antibacterial mechanism of ZnO NPs, is further supported by the observation that ZnO^R *S. aureus* did not display any resistance to H_2O_2 (fig 4c). Together this suggests that while H_2O_2 may be released into the media by ZnO, the levels that it is released at may not be sufficient to result in inhibition of bacterial growth. Further lending support to this conclusion, Kadiyala, 2018 found that 10 of the 13 oxidative stress genes significantly altered in the microarray were down regulated upon ZnO-NPs exposure, and only 3 were upregulated in methicillin resistant *S. aureus* [20]. If

ROS species are critical to the antimicrobial mechanism of ZnO, we would expect to have seen an increase in the expression of the majority of oxidative stress genes.

Other proposed mechanisms implicated electrostatic interactions with the membrane and production of Zn^{2+} ions in ZnO NP cytotoxicity [16, 22, 24, 35]. As bacteria are normally negatively charged, it has been proposed that positively charged, polar surfaces of the ZnO NPs are attracted to the bacterial membrane. Interaction with ZnO NPs could lead to membrane damage and result in osmotic dysregulation if the membrane is ruptured. If charged interactions with the membrane are responsible, it could explain why ZnO NPs are selectively toxic to bacteria, which are more negatively charged than humans cells. Independent of electrostatic interactions with the membrane, charged species such as Zn^{2+} could also play a role in ZnO toxicity. In high concentrations, Zn^{2+} ions can enter the cell membrane via transport proteins and once inside cause mis-metalation of enzymes [12, 36]. Zn^{2+} can displace Fe^{2+} and other metal ions cofactors on proteins, thus causing protein disfunction. Ultimately, this could lead to lysis of the bacteria cell as enzymes also lose function due to this Zn^{2+} replacement. The role of charged species and interactions was evaluated using the Δ*mprF* mutant that is more negatively charged. We found that changes in the surface charge of *S. aureus* had little effect on the level of bacterial inhibition (fig 5b) indicating that charged interactions with the membrane are not responsible for ZnO cytotoxicity. This may also provide evidence that mis-metalation with Zn^{2+} ions is not responsible for the ZnO antibacterial effect since diffusion of ions across the membrane is likely to be impacted if membrane charge changes.

Previous work by Reeks et al. 2021 found that internalization is not necessary for the antibacterial mechanisms of ZnO [28]. Building off this, we sought to determine whether physical contact was necessary for ZnO NP to exert its cytotoxic effects conditioned media was used. We observed that conditioned media was able to inhibit bacterial growth, even in the absence of ZnO NPs (fig. 6A). This further emphasizes that physical interactions between the membrane and ZnO NPs are not responsible for inhibition of growth. This bacterial inhibition was time dependent (inhibition of bacterial growth

increased as incubation time of ZnO in MHB increased) suggesting that the ZnO NPs are releasing a soluble molecule that is responsible for the antimicrobial mechanism. Data from *ΔkatA* and *Δmprf* mutants indicate that this soluble compound is likely not H_2O_2 and that cellular charge does not affect the mechanism of this unknown compound which potentially rules out other charged ROS as well as Zn^{2+} ions. At present, we are unable to identify what the soluble compound is that results in cytotoxicity to bacterial cells. Although only Sigma Aldrich particles were used in the majority of these experiments, we expect that the mechanism is conserved between all ZnO particles because we saw similar action across particles with different morphologies and because resistance is conserved to particles from different sources (fig 1b). Moving forward, we hope to examine the role of other soluble species in the antibacterial mechanism of ZnO as well as continue to investigate how the type of media affects the conditioned media. Underscoring the importance of soluble species to the mechanism, we observed highly variable killing based on the type of media used. Specifically, we observed that ZnO is bacteriostatic in MHB, bactericidal in saline, and does not seem to have any cytotoxic effect in PBS (fig. 2b). The lack of activity could likely be due to reactions between ZnO NPs and phosphates present in the media that produce innocuous compounds, thereby ameliorating that normal activity of ZnO as suggested by Herrmann et. al 2014 [32]. Johnson et al. 2022 further implicates a link between the media type and the mechanism of ZnO NPs by showing that ZnO particles have different properties depending on the media type they have been exposed to.

Although the anti-bacterial properties of ZnO NPs have exciting applications in medicine, food packaging, and first aid, the fundamental mechanisms driving this activity must first be uncovered before they can be applied large scale. Understanding these mechanisms is key to designing particles that are more efficacious. This is particularly important as pathogens continue to acquire antibiotic resistance and discovery of novel antibiotics plateaus. We are quickly approaching a breaking point in which we have insufficient antibiotics to treat infections that have been readily treatable over the last century. Examining

non-traditional antibiotics such as zinc oxide could provide new antibacterial agents that are widely effective against many pathogens.

References

1. Hutchings, M.I., A.W. Truman, and B. Wilkinson, *Antibiotics: past, present and future.* Current Opinion in Microbiology, 2019. **51**: p. 72-80.
2. Pulingam, T., et al., *Antimicrobial resistance: Prevalence, economic burden, mechanisms of resistance and strategies to overcome.* European Journal of Pharmaceutical Sciences, 2022. **170**.
3. Lee, A.S., et al., *Methicillin-resistant Staphylococcus aureus.* Nature Reviews Disease Primers, 2018. **4**(1): p. 18033.
4. Sharma, D.K., et al., *A review on ZnO: Fundamental properties and applications.* Materials Today: Proceedings, 2022. **49**: p. 3028-3035.
5. Shetti, N.P., et al., *ZnO-based nanostructured electrodes for electrochemical sensors and biosensors in biomedical applications.* Biosensors and Bioelectronics, 2019. **141**: p. 111417.
6. Klingshirn, C., *ZnO: Material, Physics and Applications.* ChemPhysChem, 2007. **8**(6): p. 782-803.
7. Dhanasegaran, K., et al., *Antibacterial properties of zinc oxide nanoparticles on Pseudomonas aeruginosa (ATCC 27853).* Scientia Iranica, 2022. **28**: p. 3806-3815.
8. Gudkov, S.V., et al., *A Mini Review of Antibacterial Properties of ZnO Nanoparticles.* Frontiers in Physics, 2021. **9**.
9. Liu, Y., et al., *Antibacterial activities of zinc oxide nanoparticles against Escherichia coli O157:H7.* Journal of Applied Microbiology, 2009. **107**(4): p. 1193-1201.
10. Modi, S., et al., *Nanostructured Antibiotics and Their Emerging Medicinal Applications: An Overview of Nanoantibiotics.* Antibiotics, 2022. **11**(6): p. 708.
11. Siddiqi, K.S., et al., *Properties of Zinc Oxide Nanoparticles and Their Activity Against Microbes.* Nanoscale Research Letters, 2018. **13**(1).
12. Sirelkhatim, A., et al., *Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism.* Nano-Micro Letters, 2015. **7**(3): p. 219-242.
13. Li, Q., et al., *Antimicrobial nanomaterials for water disinfection and microbial control: Potential applications and implications.* Water Research, 2008. **42**(18): p. 4591-4602.
14. Akbar, A., et al., *Synthesis and antimicrobial activity of zinc oxide nanoparticles against foodborne pathogens Salmonella typhimurium and Staphylococcus aureus.* Biocatalysis and Agricultural Biotechnology, 2019. **17**: p. 36-42.
15. Ali, S.S., et al., *Efficacy of metal oxide nanoparticles as novel antimicrobial agents against multi-drug and multi-virulent Staphylococcus aureus isolates from retail raw chicken meat and giblets.* Int J Food Microbiol, 2021. **344**: p. 109116.
16. Dadi, R., et al., *Antibacterial activity of ZnO and CuO nanoparticles against gram positive and gram negative strains.* Materials Science and Engineering: C, 2019. **104**: p. 109968.
17. Tiwari, V., et al., *Mechanism of Anti-bacterial Activity of Zinc Oxide Nanoparticle Against Carbapenem-Resistant Acinetobacter baumannii.* Front Microbiol, 2018. **9**: p. 1218.
18. Soltanian, S., et al., *Biosynthesis of Zinc Oxide Nanoparticles Using Hertia intermedia and Evaluation of its Cytotoxic and Antimicrobial Activities.* BioNanoScience, 2021. **11**(2): p. 245-255.
19. Joe, A., et al., *Antibacterial mechanism of ZnO nanoparticles under dark conditions.* Journal of Industrial and Engineering Chemistry, 2017. **45**: p. 430-439.
20. Kadiyala, U., et al., *Unexpected insights into antibacterial activity of zinc oxide nanoparticles against methicillin resistant Staphylococcus aureus (MRSA).* Nanoscale, 2018. **10**(10): p. 4927-4939.
21. Zudyte, B. and Z. Luksiene, *Visible light-activated ZnO nanoparticles for microbial control of wheat crop.* Journal of Photochemistry and Photobiology B: Biology, 2021. **219**: p. 112206.
22. Slavin, Y.N., et al., *Metal nanoparticles: understanding the mechanisms behind antibacterial activity.* Journal of Nanobiotechnology, 2017. **15**(1).

23. Raghupathi, K.R., R.T. Koodali, and A.C. Manna, *Size-Dependent Bacterial Growth Inhibition and Mechanism of Antibacterial Activity of Zinc Oxide Nanoparticles.* Langmuir, 2011. **27**(7): p. 4020-4028.
24. McDevitt, C.A., et al., *A Molecular Mechanism for Bacterial Susceptibility to Zinc.* PLoS Pathogens, 2011. **7**(11): p. e1002357.
25. Johnson, D., et al., *Influence of Surface Properties and Microbial Growth Media on Antibacterial Action of ZnO.* Coatings, 2022. **12**(11).
26. Li, M., L. Zhu, and D. Lin, *Toxicity of ZnO Nanoparticles to Escherichia coli: Mechanism and the Influence of Medium Components.* Environmental Science & Technology, 2011. **45**(5): p. 1977-1983.
27. Manzoor, U., et al., *Antibacterial, Structural and Optical Characterization of Mechano-Chemically Prepared ZnO Nanoparticles.* PLOS ONE, 2016. **11**(5): p. e0154704.
28. Reeks, J.M., et al., *Microscale ZnO with controllable crystal morphology as a platform to study antibacterial action on Staphylococcus aureus.* Biointerphases, 2021. **16**(3): p. 031003.
29. Park, B., V. Nizet, and G.Y. Liu, *Role of Staphylococcus aureus Catalase in Niche Competition against Streptococcus pneumoniae.* Journal of Bacteriology, 2008. **190**(7): p. 2275-2278.
30. Peschel, A., et al., *Staphylococcus aureus resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with l-lysine.* J Exp Med, 2001. **193**(9): p. 1067-76.
31. Everett, W.N., et al., *Phosphate-enhanced cytotoxicity of zinc oxide nanoparticles and agglomerates.* Toxicol Lett, 2014. **225**(1): p. 177-84.
32. Herrmann, R., F.J. García-García, and A. Reller, *Rapid degradation of zinc oxide nanoparticles by phosphate ions.* Beilstein Journal of Nanotechnology, 2014. **5**: p. 2007-2015.
33. Matsuo, M., et al., *Growth-phase dependence of susceptibility to antimicrobial peptides in Staphylococcus aureus.* Microbiology, 2011. **157**(6): p. 1786-1797.
34. Andrä, J., et al., *Multiple Peptide Resistance Factor (MprF)-mediated Resistance of Staphylococcus aureus against Antimicrobial Peptides Coincides with a Modulated Peptide Interaction with Artificial Membranes Comprising Lysyl-Phosphatidylglycerol.* Journal of Biological Chemistry, 2011. **286**(21): p. 18692-18700.
35. Lemire, J.A., J.J. Harrison, and R.J. Turner, *Antimicrobial activity of metals: mechanisms, molecular targets and applications.* Nature Reviews Microbiology, 2013. **11**(6): p. 371-384.
36. Lallo Da Silva, B., et al., *Relationship Between Structure And Antimicrobial Activity Of Zinc Oxide Nanoparticles: An Overview.* International Journal of Nanomedicine, 2019. **Volume 14**: p. 9395-9410.