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Environmental Significance Statement:

As engineered nanomaterials become increasingly common in consumer and medical products, it is critical to proactively consider the potential long term environmental implications of their production, use, and disposal. Microbes are the foundation of healthy aquatic, terrestrial, and built environments, as well as being critical to human and animal health. However, these organisms have a famed ability to adapt and develop resistance to innumerable molecules and metals. Herein, a critical lens is applied to the current state of knowledge about engineered nanomaterials' impacts on bacterial resistance to antibiotic, the ability of bacteria to develop resistance to nanomaterials, and the challenges that lie ahead.

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Modern materials provoke ancient behavior: Bacterial resistance to metal nanomaterials

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Abstract: The use of engineered nanomaterials, defined as those smaller than 100 nm, in the health, energy, agricultural, and environmental sectors is expanding rapidly. As such, human and environmental exposure to these materials is increasing every day. For example, metal-based nanomaterials, such as nanosilver, have become ubiquitous in antibacterial applications ranging from socks and baby bottles to healthcare materials, such as oral fillings. Engineered nanomaterials are also used as antibacterial agents and adjuvants to improve antibiotic delivery or efficacy. However, even nanomaterials that were not designed to be antimicrobial can possess potent bactericidal activity. Alarmingly, there are clear connections between nanomaterial exposure, metal resistance, and antibiotic resistance and it is crucial that we dramatically improve our understanding of both the toxicity of these materials and their ability to permanently change the organisms that they encounter. Emerging research indicates that microbes are capable of adapting

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to nanomaterial toxicity, often with the same generalizable mechanisms used to overcome antibiotic toxicity. In this perspective, we highlight existing knowledge about microbial response to engineered nanomaterials and the key outstanding questions that must be addressed.

Introduction

Alexander Fleming's discovery of the antimicrobial properties of *Penicillium* mold and the subsequent "Age of Antibiotics" have revolutionized our ability to control and eliminate bacterial infections. While we have learned much about how to discover antibiotics from natural sources, it is only relatively recently that we have come to appreciate that bacteria can evade these treatments through their amazing ability to evolve or acquire new genetic information that encodes adaptation and resistance strategies.[1] Resistance is often due to alteration of the primary target of the antibiotic (e.g., mutation in penicillin-binding proteins to evade penicillin).[2] As such, it has long been postulated that treatment with agents that function through multiple mechanisms of action may elude resistance evolution and increase long-term antibiotic efficacy.[3-5]

One suggested answer to antibiotic resistance is a multi-mechanism arsenal such as engineered nanomaterials (ENMs).[6-10] ENMs have already been incorporated into nearly all sectors of modern technology and are the most common type of nanomaterial produced for commercial use.[11, 12] Metal-based ENMs have been reported to kill bacteria by numerous mechanisms (**Figure 1**), which is no surprise considering that metal(loid)s have long been utilized as antimicrobial agents (e.g., silver and copper in water jugs to prevent fouling, arsenic and mercury to treat syphilis).[13, 14] Metal-based EMNs can affect cell envelope integrity through both physical and chemical disruption, including lipid destruction, membrane permeability changes, potential and fluidity alterations, adhesion to the cell surface, and/or cell wall depolarization.[15-18]

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The surface ligands presented on ENMs can play a major role in these processes by initiating interactions with the cell.[19] ENMs can also act as a delivery mechanism for additional toxins or drugs through the dissolution to metal ions or release of surface ligands, which can be supplied to the bacteria at high local concentrations. Released metal ions are toxic in their own right, affecting homeostasis with essential metals or exhibiting affinity for biomolecules and displacing other cofactors. However, metal ions are not responsible for the entirety of ENM toxicity.[20]

ENM redox properties can cause an oxidative stress response, in addition to exogenous reactive oxygen species (ROS) generation by dissolution of the particles themselves (through Fenton, Fenton-like, Haber Weiss, or light-initiated processes).[21-23] ROS contributions to ENM toxicity are difficult to assess due to their apparent non-preferential targeting of biomolecules. In addition, oxidative damage is likely to disturb the function and expression of other systems, making it extremely difficult to map the initial targets of ENM action in comparison to the subsequent effects. For example, ENMs can cause oxidative damage to DNA, resulting in DNA adducts that can dramatically change gene expression.[24] Indeed, ENMs typically function through multiple mechanisms of action; however, a growing body of research has demonstrated that bacteria can still readily adapt and often rapidly evolve resistance through genome-level changes. Herein, we focus our discussion on adaptation and resistance to metal (oxide) ENMs.

Bacterial Toxicity: Response to Metals

Bacteria have an amazing ability to rapidly respond to their environment, whether it be changes in temperature, ion concentration, or antibiotic exposure. Indeed, bacteria are known for their ability to share genetic material, gene plasticity, and quick replication rates, which enable rapid adaptation. When exposed to antibiotics, resistance is typically evolved or acquired through

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horizontal gene transfer that may result in an alteration of the antibiotic target or membrane permeability, mechanisms to inactivate the active molecule, and/or increased efflux of the toxicant from the cell.[25] While it is now commonly understood that bacteria can readily evolve and change in response to treatment with small molecule antibiotics, adaptation and resistance resulting from other toxicants is much less understood or appreciated. Of particular importance in the study of metal (oxide) ENMs is existing knowledge about the mechanisms of bacterial response to metal ions. Even outside of their application as nanomaterials, rare and precious metals are increasingly used in various technologies, are becoming more prevalent in waste streams,[26] and therefore may also impact metal regulation in microbes.

It is estimated that anywhere from a third to a half of all proteins require a metal ion for functionality, and metals like zinc, manganese, and iron are important for metabolic activity. While metals are essential in many key biological processes (e.g., respiration), when exposed to toxic levels of dissolved metals, most bacteria have mechanisms to readily respond. Metal ion levels are detected by systems of proteins and riboswitches that regulate metal uptake, storage, or efflux.[27] The first line of defense against metal toxicity is cytosolic components such as metal-binding proteins and small molecules like glutathione. If metal stress becomes too high, downregulation of import machinery, expression of additional metal sequestration proteins (e.g., ferritin, metallothionein, heme-containing proteins), and increased metal efflux (e.g., resistance-nodulation-division, p-type ATPase efflux, cation diffusion transporters) mitigate metal toxicity. Specialized microbe classes (e.g., dissimilatory metal-reducing bacteria, sulfate-reducing bacteria) can use a variety of terminal electron acceptors or specialized systems to alter metal oxidation state and solubility, decreasing their bioavailability and toxicity (e.g., merA reduces mercury to volatile Hg^0). Bacteria also use biofilms and extracellular polymeric substances as external protection from

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toxic metal concentrations. If these defense methods are overwhelmed, metal intoxication is particularly damaging to proteins. Metals have high affinity for thiol-containing biomolecules, can disrupt heme-dependent enzymes, and may cause mismetallation of proteins [e.g., Mn(II) replacing Fe(II), Ni displacing Zn(II)), which may inactivate or denature enzymes].[28] High metal concentrations may even affect microbial metabolism and viability before entering the cell by disrupting the electron transport chain. Due to the numerous negative impacts on bacteria that metals can have, it is not surprising that resistance to toxic metals is likely as ancient as antibiotic resistance.[29, 30]

Importantly, there are many examples of co- and cross-resistance between metals and antibiotics in both clinical and environmental settings.[31-34] Co-resistance occurs when antibiotic and metal resistance genes are located on the same mobile genetic element (i.e., plasmid, transposon, integron).[35-42] When the cell experiences either antibiotic or metal selection pressure, this genetic material is passed on via horizontal gene transfer, giving the recipient organism the required machinery to cope with both stressors. Cross-resistance occurs when the same machinery enables the bacteria to cope with two different stressors, such as multidrug efflux pumps.[32, 43]

Resistance to ENMs

Challenges

Until recently, ENM resistance evolution investigations were a rarity perhaps due to the belief that ENM resistance was not possible. The vast majority of studies performed to date have been ***acute*** bacterial exposures to ENMs, often at very high concentrations. While it is challenging to identify relevant EMN doses, especially in environmental settings where concentrations may vary depending on location (*e.g.* proximity to a manufacturing site) and the biotransformations

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that ENMs may undergo, evidence suggests that concentrations >mg/L range are beyond relevance.[44-46] Understanding the extent of ENM toxicity by measurement of cell death and the minimum inhibitory concentration (MIC) of these materials is useful. However, it provides little mechanistic information, does not afford the opportunity to evaluate the potential for resistance evolution, and is not an accurate representation of real-world exposure. Mba and Nweze (2021) have generated a helpful table that lists key findings from a variety of recent microbial-nanoparticle studies.[47]

Mortimer *et al* (2021) conducted a valuable analysis on the application of -omics techniques to elucidate the mechanisms of action of ENM toxicity and included a comparison of the pathways dysregulated by lethal and sub-lethal concentrations of ENMs.[48] This analysis revealed that lethal doses of ENMs primarily trigger oxidative stress pathways in addition to major pathways in energy, carbohydrate, amino acid metabolism, translation, and membrane transport. Although there are *more* dysregulated genes at higher ENM concentrations, there are fewer pathways affected. Treatment with sub-inhibitory ENM concentrations showed *additional* affected pathways including dysregulation of Fe-S clusters, lipid metabolism, replication, cell motility, and community functions (i.e., quorum sensing and biofilm formation). Thus, reducing the concentration of ENMs to enable bacteria to mount a more targeted adaptation response could clarify the numerous, interconnected biomolecular targets of ENMs.

In addition to dosage, ***chronic*** exposures, which inherently require the use of sub-lethal ENM levels, are essential to fully understand the ability of bacteria to adapt and evolve.[49-59] While these experiments are conceptually straightforward, design of investigations that provide information about the pathways that are specifically affected by a given ENM is non-trivial. For example, results are often confounded by ENM dissolution as the generation of metal ions can

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affect bacteria and spur the evolution of resistance (see above).[27, 31] In addition, most ENMs are likely to trigger general stress responses, such as the SOS pathway, masking nanomaterial-specific bacterial response. Thus, temporal evaluation of organismal response is essential to the discovery of other affected pathways, as is the identification of genetic mutations in resistant populations.

These challenges are further exacerbated by the range of possible variations in ENM properties that could be considered: composition (both of core materials and ligands), shape, size (surface-area-to-volume ratio), and surface charge state, as well as consistency of these properties within a given batch of ENMs.[60] Division of ENMs into more distinct classes or investigation of the role of specific properties (size, charge, shape) is likely the best way to gain mechanistic understanding of ENM toxicity.[61] While property-tunable ENMs would be ideal, it can be difficult or even impossible to change only a single property while holding all others constant as can readily be accomplished with a small molecule scaffold (e.g., changes in ENM shape alter surface area and thus, metal ion dissolution).[62] Finally, the environment of the microbial exposure can have drastic effects on the outcome, such as the presence of light, oxygen concentration, media content, and the presence or absence of other biomolecules or organisms. These complications are compounded by inherent differences in the susceptibility of each bacterial species.[63]

Trends in nanomaterial toxicity to microbes generally correlate with surface area as metal dissolution and oxidative stress are primary contributors in aqueous environments.[64] For example, we assessed three morphologies of lithiated nickel manganese cobalt oxide (NMC), a battery cathode nanomaterial, hypothesizing that the different crystal phases may alter dissolution due to the varied levels of transition metal coordination. This investigation demonstrated that NMC toxicity to the bacterium *Shewanella oneidensis* was governed by surface areas across all morphologies and particles sizes, not crystal face.[62] Tuning the stoichiometry of the transition

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metal composition in the NMC nanomaterials was also found to mitigate toxicity. The functionality of these materials for energy storage has not been experimentally evaluated but has been the subject of computational studies.[65, 66] Crystal structures, shapes, and coatings of nanomaterials all affect material dissolution and reactivity in their own right and also affect the degree of aggregation and proclivity to acquire a corona. Furthermore, positively charged ions and materials are generally thought to be more toxic to microbes, perhaps because they are attracted to the negatively charged cell envelope.[67] For further detail the reader is directed to Yougbaré *et al* (2021)[68] and Vimbela *et al* (2017).[69]

Emerging Evidence

There is now substantial evidence of bacterial adaptation to metal (oxide) ENMs, especially those used as antibacterial agents, such as silver nanoparticles (AgNPs).[70-73] These investigations show that bacteria adapt to the toxicity of ENMs by reducing the bioavailability of the particle (e.g., producing molecules that promote settling of the ENM suspension more quickly), increasing efflux, and enhancing the activity of biomolecular repair mechanisms (**Figure 2**).[55, 74] In addition, bacteria can modulate their envelope charge state by altering the amino acid composition on their surface, thereby minimizing interactions with ENMs.[75] ENM exposures also result in the production of ROS and activation of the SOS response.[76] Unchecked ROS can cause DNA damage, as well as activate DNA repair mechanisms that can result in mutation and fitness advantages in the face of selection pressure exerted by ENM toxicity.[39]

Microbes can adapt through tolerance, persistence, or resistance mechanisms. These terms have often been used synonymously in the nanotoxicology field, but should be more carefully delineated as they represent different biological states. An Opinion article by Brauner and colleagues (2016) beautifully discusses the differences between these definitions, as well as

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outlines how one might distinguish between the three survival mechanisms.[77] Briefly, bacterial adaptation yields either tolerant or persistent cells. Tolerance indicates that the organism can temporarily withstand lethal concentrations of an antimicrobial agent (above the MIC), but they do not harbor permanent genetic changes and instead transiently alter their biological processes. Persister cells are a *subpopulation* of bacteria (~1% of the original population) that survive lethal exposure to an antimicrobial agent. If persister cells are subcultured, they generate a heterogenous population that will again be culled to ~1% if re-exposed. Persistence is not heritable. Finally, resistant bacteria are those that actively replicate in the presence of lethal concentrations of the antimicrobial agent due to a heritable, genetic change. These mutations are commonly found in genes that encode for the antimicrobial target (preventing binding) or regulator gene, which causes an observable increase of the MIC in the bacterial population. Although these definitions are important in the discussion of bacterial survival mechanisms, growing evidence suggests that antibiotic resistance can also emerge from the prolonged exposure of tolerant or persister populations to antibiotics, and likely by extension, metals and ENMs.[78, 79] It has been suggested that during extended exposures, tolerant or persister cells can accumulate mutations that give rise to a resistant population. Indeed, all three types of bacterial adaptation–resistance, tolerance, and persistence–play important roles in microbial response and potentially to decreased susceptibility to antibiotics,[77, 80] and likely, to many other environmental toxicants.

While most studies have focused on the characterization of cell adaptation mechanisms and/or ENM doses that result in death, there is increasing evidence that bacteria evolve resistance to metal (oxide) ENMs. For example, extended exposure of *Eschericia coli* and *Pseudomonas aeruginosa* to silver ENMs (for 25 successive cultivations) increased the MIC from 3.4 mg/L to >54 mg/L within 8 to 13 cultivation steps, which was not observed with bulk Ag. During

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exposure to AgNPs, bacteria secreted the protein flagellin, which induced particle aggregation in the culture media, reducing their toxicity. However, this adaptation process did not result in genetic level changes.[55] In a separate study in which *E. coli* K12 was exposed to AgNPs for ~225 generations, mutations were reported in genes at 100 generations that were associated with copper efflux, nucleotide biosynthesis, and the RNA polymerase beta subunit.[57] Upregulation of efflux pumps for expulsion of other metals has also been observed (see above). Prolonged exposure of *E. coli* has also been highlighted in work focused on whether AgNPs accelerate genome-wide mutation rates.[81] Even after >1,000 division cycles in the presence of AgNPs, the authors noted no difference in the frequency of mutation compared to the passaged control. This was unexpected given that ROS typically activates mutational processes.[39] Finally, *S. aureus* has developed stable resistance to AgNPs and ionic silver over 30 to 50 days, which increased the MIC at least four-fold and resulted in two ENM-unique mutations for purine synthesis and cystine import.[82] Because AgNPs have already been extensively employed in consumer products and clinical settings, they are the subject of most studies aimed at understanding microbial resistance to nanomaterials. However, recent studies have revealed that other nanomaterials (*e.g.,* nano-alumina[83] and nano-zinc oxide[39]) also increase mutagenesis and promote horizontal gene transfer.[84, 85]

We have shown that chronic exposure of *Shewanella oneidensis* MR-1 to NMC caused it to filament and enabled growth in concentrations over twenty times the wild-type MIC, even after a prolonged period of non-exposure, indicating a genome-level modification.[86] In a subsequent study, we found that the frequency of mutation within antibiotic resistance-conferring genes of *S. oneidensis* was substantially elevated from NMC exposure.[23] Increases in the mutation of resistance-associated genes has also been observed following chronic exposure to zinc oxide or aluminum oxide ENMs (see above).[87] Conversely, multiple studies have shown no resistance

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evolution following prolonged ENM treatment, such as *E. coli* exposures to boron nitride, copper phosphide, and a variety of gold ENMs.[72] Clearly, we have much to learn about the ability of microbes to undergo genome-level changes upon exposure to various ENMs and the relationship of the resulting resistance mechanisms to known metal and antibiotic resistance pathways.[88-91]

Outstanding Challenges and Broader Implications

What is needed now is an interdisciplinary effort to evaluate the effects of carefully-defined ENMs at the biochemical level. Until we better understand how the myriad of materials will enter the environment and alter microbial function, we are taking a huge gamble not only with the health of our environment, but ultimately, human lives.

Among the most critical questions and challenges to be addressed related to metal and metal oxide ENMs:

- What are the differences in microbial response to metals versus metal (oxide) ENMs?
    - Should regulatory practices be guided by existing knowledge of metal toxicity or are additional considerations needed due to the unique properties of ENMs?
    - How can ENMs be standardized or categorized given the enormous number of possible variables (e.g., size, shape, composition)?
- What are the broader environmental and medical implications of bacterial adaptation and resistance evolution to metal (oxide) ENMs?
    - Can metal (oxide) ENMs be safely used as antibacterial agents or will this continue to accelerate bacterial resistance to both these materials and traditional antibiotics?

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  - Will ENM exposure result in co-selection of metal/ENM/antibiotic resistance genes or co- and cross-resistance to antibiotics as occurs upon metal exposure?
  - How will environmental microbiomes be affected by ENM adaptation and resistance?
- How can we use what we have learned about antibiotic resistance[2] to better inform policies for the regulation of ENM use and disposal?
  - What biological information or scientific strategies can the ENM field adopt from decades of study of antibiotic toxicity and resistance mechanisms?
- Will ENM-resistant bacteria have advantageous properties, such as increased redox, biomineralization, and biomolecular repair capabilities that could prove valuable in microbial fuel cells or support remediation efforts for metal or nanomaterial pollution?

Parallel questions should be considered for other major classes of ENMs, where the relevant biochemical mechanisms will be distinct, based on how microbes interact with the elemental or molecular components of the ENMs or their transformation products.

Studies have conclusively demonstrated that bacteria readily evolve resistance to selected ENMs. It is well-established that high concentrations of metals, such as the pool of ions generated from ENM dissolution, can promote the rapid exchange of antibiotic-resistance genes (co-resistance). Thus, we should have serious concerns that ENM exposure may also increase the mobility of genetic elements. In addition, cross-resistance mechanisms, in which a single gene can regulate or provide resistance to different toxicants,[92-94] are likely to play important roles in the relationship of ENM, metal, and antibiotic resistance. For example, the MdrL efflux pump

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transports both heavy metals and antibiotics like erythromycin.[95] Finally, ROS generated during exposures has been identified to increase transformation and mutation frequency.[23, 96] Thus, the use of ENMs may promote the spread of antibiotic resistance in hospitals and contaminated environmental sites.[97]

ENMs are under consideration as emerging pollutants, so action must be taken to provide regulations and guidance for their appropriate disposal and recycling.[98, 99] This is challenging as regulatory agencies face problems associated with variability in ENM syntheses and characterization and the lack of related suites of ENMs with carefully controlled differences,[100, 101] though there has been recent significant regulatory progress in this area with initiatives such as REACH in the European Union.[102] For these reasons, even meta-analysis and more intricate machine learning algorithms may be misleading.[103] As nanomaterials have diverse roles in various products, guidance for their use and safety would require a concerted effort across several regulatory agencies and could still lead to different regulations for the same material depending on application or analysis method. There is also variability in the perceived risk of the applied ENMs, so decisive action on ENMs has been slow and without clear recommendations.[104] While many of the original calls and concerns for large-scale and ENM-specific regulation have quieted, ENM-induced changes in bacterial resistance should remain an area of investigation and concern. As was the case with antibiotics, it is not trivial to determine an acceptable level of risk in comparison to the potential benefits of nanomaterials. For example, the application of nanoscale battery cathode materials used in electric vehicles would eliminate much of the need for petroleum products and reduce greenhouse gas emissions, but with still-developing regulation for application and recycling, a level of risk is assumed. Finally, human concern over the environmental impact of chemicals has largely been reserved for the endangerment of charismatic megafauna. The risk of ENMs to

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bacterial species is less clear to the public and it will likely to be difficult to promote a better appreciation for the danger of altering environmental microbiomes, which can quickly upset the balance of nutrient availability or bacterial predation, as well as expedite the spread of antibacterial resistance genes.[105] Until the work is put in to truly *understand* the microbial response, we can only guess at the ramification of their continued, and likely increased, exposure to ENMs.

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Author Contributions

E.E.C. and S.L.M. conceived of the idea for this manuscript. S.L.M., N.V.H-S., and D.S. provided the first draft and edits. E.E.C. and C.L.H. provided notable and valuable edits. All authors were involved in the final editing and approved the submitted manuscript.

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Figures

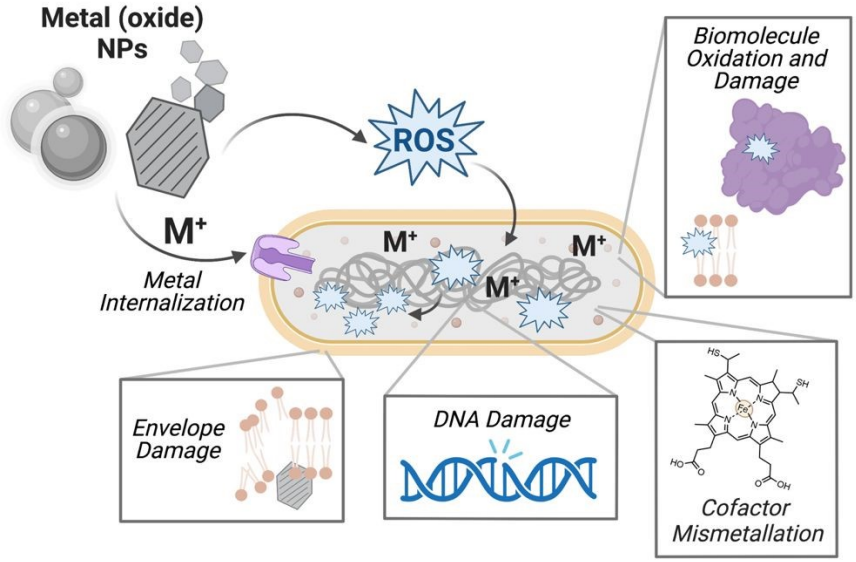

Figure 1. **Mechanisms of metal (oxide) ENM toxicity**. Engineered nanomaterials have unique effects on microbes. Much of this toxicity is the result of the metal ions dissolved from the material.[20] Metals can be bactericidal through mechanisms such as disruption of native metal co-factors in Fe-S clusters, metalloproteins, and the catalytic sites of enzymes.[27] Another main mechanism of nanomaterial toxicity is ROS generation, which causes a cascade of damage and stress.[22] ROS can damage a variety of macromolecules such as DNA and lipids, as well as oxidize sulfur-containing amino acids.[106] Figure generated in BioRender.

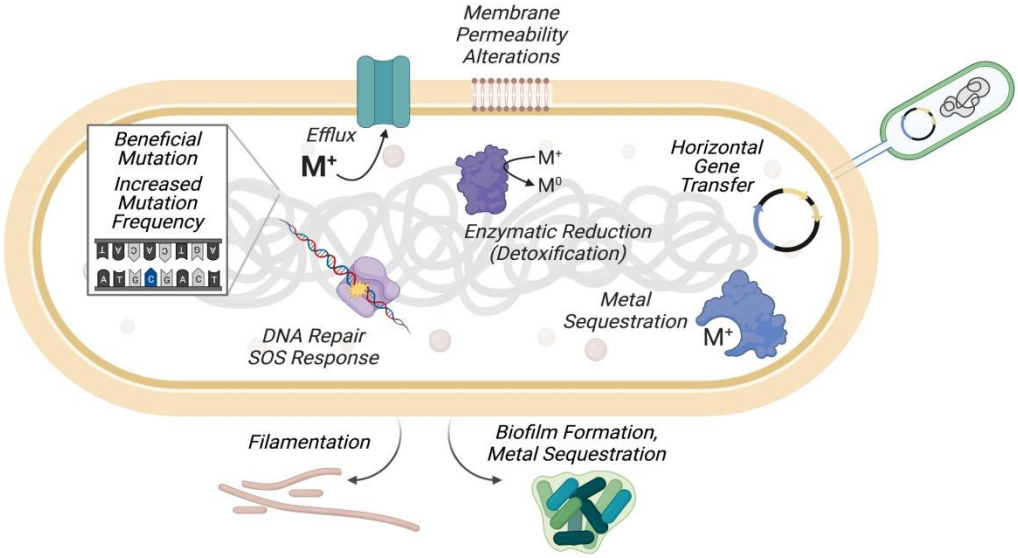

Figure 2. **Mechanisms of bacterial adaptation and resistance to ENMs**. Known nanomaterial resistance mechanisms are largely connected to metal resistance, such as efflux pump expression (e.g., RND protein family, heavy metal efflux family, ATP binding cassettes), which can reduce the toxic load of metals within an organism.[40, 74, 107] The damage and toxicity caused by

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nanomaterials and subsequent ROS generation results in a cascade of expression and regulation changes (e.g., heat shock, envelope stress, general stress). Damage from ROS, whether intrinsically or extrinsically generated, can be repaired by reductases that reduce Cys and Met residues, SOS response, and mutS systems. Biofilm generation protects microbes by sequestering materials, preventing them from interacting with the microbes.[108, 109] Morphology changes such as filamentation result in the sequesteration of damaged DNA to prevent transmission to progeny. Figure generated in BioRender.