Elucidation of Facilitated Uptake of Small Molecule Antibiotics by Rare Earth Modified Bacteria

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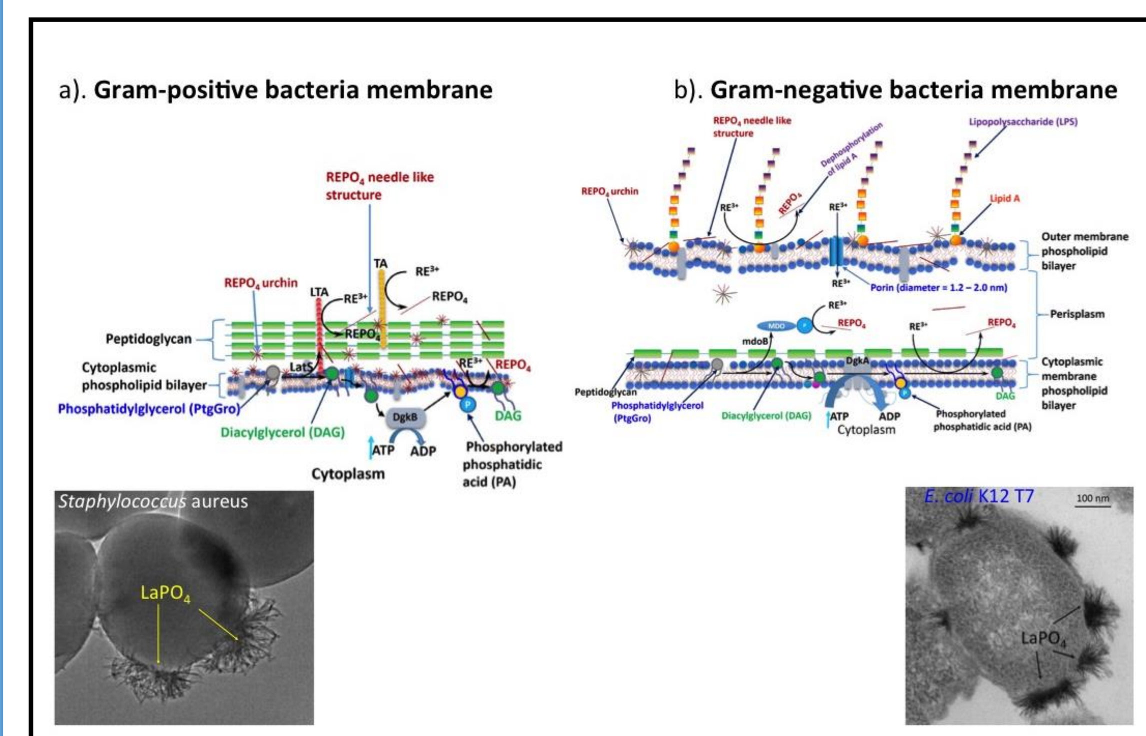
Abstract

Rare Earth Elements (REEs) possess unique chemical and physical characteristics that account for their use in high capacity batteries[1,2], magnets[1,3], and fuel cells[1,4]. Recent evidence found that REEs can universally dephosphorylate Gram-positive and Gram-negative bacteria based on their high affinity for phosphate ions.[4] Under phosphate limited conditions, it was recently established that, bacterial growth is significantly inhibited by REEs, coincident with increased levels of intracellular adenosine triphosphate (ATP), intracellular polyesters, alkaline phosphate enzyme and reactive oxygen species (ROS).[4] Emerging from these evidences in dephosphorylation from LaPO4 crystals on the bacterial membrane have biomedical implications on membrane integrity and permeability. The growing amount of antibiotic resistant strains of bacteria is increasing annually. Each year over 20,000 people in the United States alone die from drug resistant bacteria with billions of dollars spent annually to deal with direct and indirect costs associated with these microorganisms.[5] It is therefore essential to develop new techniques and mechanisms that can help address the problem of drug resistant pathogenic bacteria. To approach this issue chemical and biological assays were created to test facilitated uptake as well as observe if the bacterial DNA has retained its integrity under LaPO4 modification. Gram-negative bacteria (E.coli K12 T7) have shown facilitated uptake when treated with aminoglycosides. Gram-positive bacteria (Staphylococcus aureus) have yet to show significant signs of facilitated uptake due to LaPO4 modification and is possibly from the more permeable phospholipid bilayer that is results in antibiotics to travel inside the bacterial without facilitation. DNA content analyses on LaPO4 modified E. coli K12 T7 suggested possible phosphate leakage from the E. coli K12 T7 cell interior, however, no relative activation of Raw264.7 cells by genomic DNA from LaPO4 modified E. coli K12 T7 was observed, confirming that the integrity of the residual E. coli K12 T7 genomic DNA was not interfered with.



RE^{3+} - dephosphorylation occurs for both Gram-positive (a) and Gram-negative (b) bacteria. The resulting $REPO_4$ crystal membrane modification suggests possible facilitated uptake for small molecule antibiotics. [4]

Motivation

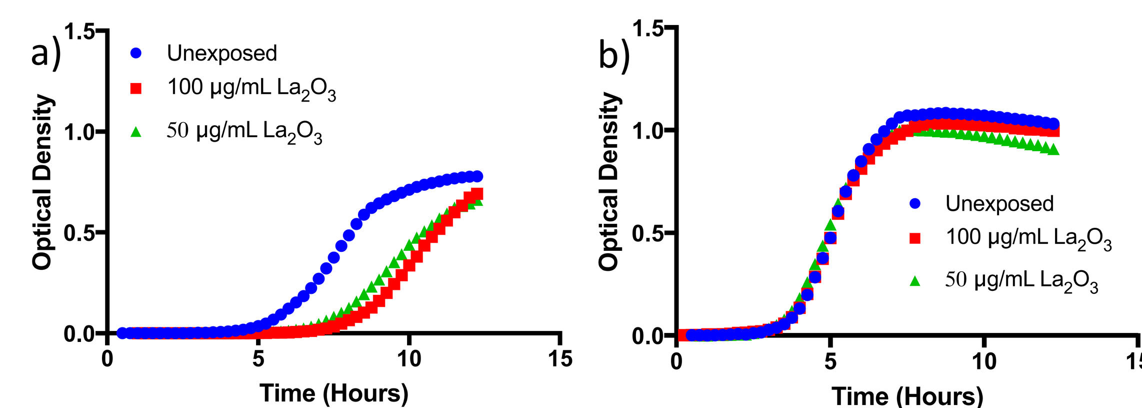
- Problem of antibiotic resistant bacteria is a global threat and continues to endanger the efficacy of many conventional antibiotics.
- Over 2 million people get infections resistant to antibiotics every year in the USA with at least 23,000 people dying as a result
- Antibiotic resistance adds excess of US$20 billion in direct health care costs per year in the USA.

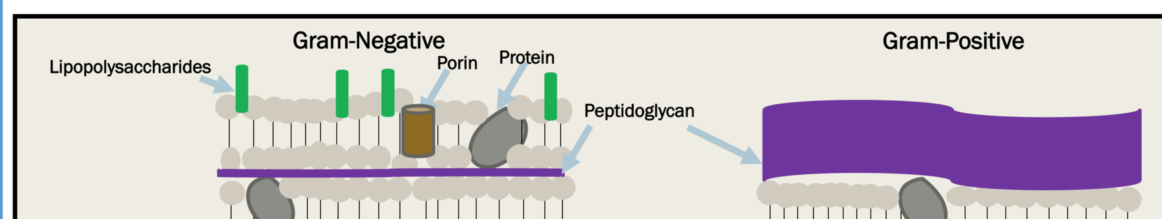
Materials & Methods

The effects of REEs on facilitated uptake of small molecules into bacterial organisms can be assessed using assays that quantify changes in growth kinetics. This can be coupled by mechanistic biochemical assays that assess the impact of REEs on the overall content and integrity of the genomic DNA in REE exposed bacteria. Gram-negative (E. coli K12 T7) and Gram-positive (Staphylococcus aureus) were incubated with 50μg/mL and 100μg/mL of La_2O_3 Nano Particles in LPM for two hours to initiate La-mediated dephosphorylation that yield needle or urchin-like $LaPO_4$ crystals4 on the bacterial membrane. The $LaPO_4$ modified bacteria were then tested for facilitated antibiotic uptake in Luria-Bertani (LB) for the E. coli K12 T7 and Tryptic Soy Broth (TSB) for the Staphylococcus aureus. A total of 1x105 bacterial cells were incubated with increasing concentrations of a library of conventional antibiotic drugs in a 96-well format using a plate reader set at 600 nm and 37 ^{0}C. Optical densities (ODs) were then read over 12hr period and then processed using GradPad Prism software to generate the kinetic of killing growth curves. Appropriate controls including bacteria not exposed to antibiotics and blank growth medium were included and subtracted where necessary. To further understand the phenomenon of the $LaPO_4$ modification and how it impacts bacteria, DNA content in $LaPO_4$ modified E. coli K12 T7 was quantified using propidium iodide (read at ex 535 nm and em 617 nm). In brief, $LaPO_4$ modified E. coli K12 T7 was fixed in cold 70 % ethanol overnight in -20 ^{0}C freezer, washed three times with 0.9 % saline, and then incubated for 30 minutes with RNase to degrade RNA before staining with the propidium iodide to qualitatively assess genomic DNA content using plate reader. In order to assess if the integrity of genomic DNA in LaPO4 modified E. coli K12 T7 was affected or impacted by the La-dephosphorylation, genomic DNA was isolated using commercially sourced DNA extraction kit and then used to assay the activation status of the murine mouse macrophage Raw264.7 cells. Levels of nitrous oxide released in the Raw264.7 cell culture medium (DMEM) were quantified using Griess reagent as per the manufacturer's prescriptions. Upon diluting culture DMEM medium with Griess reagent at 1: 1 ratio and incubating for 15 minutes at room temperature, absorbance readings were obtained at 540 nm using a plate reader set at 25 ^{0}C.

Results

Growth Recovery Kinetics of $REPO_4$ modified Bacteria is Different according to Bacterial Membrane Physiology

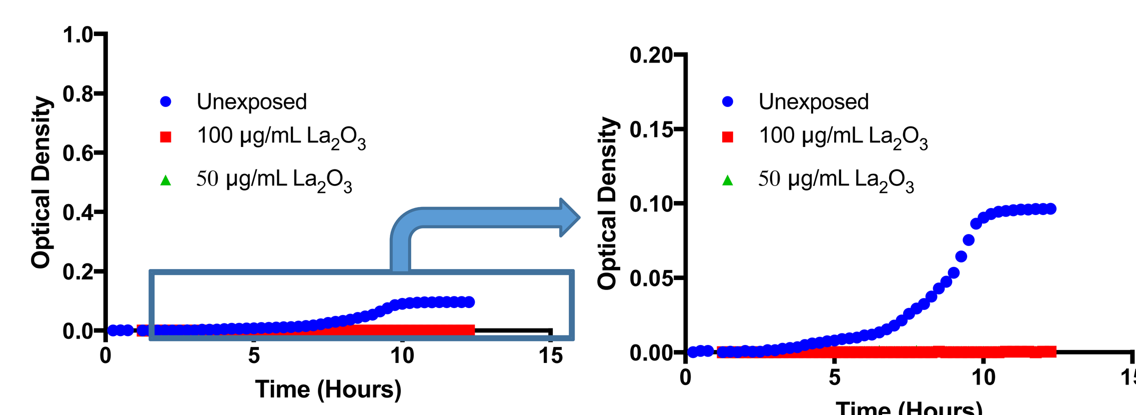




** Gram-positive S. aureus (b) has shorter lag time as compared to the Gram-negative, suggesting the S. aureus has a faster membrane recovery time. This phenomenon is possible due the more permeable membrane of Gram-positive bacteria, thus resulting in a efficient nutrient uptake. The double phospholipid bilayer of Gram-negative bacteria prevents fast uptake of nutrients.

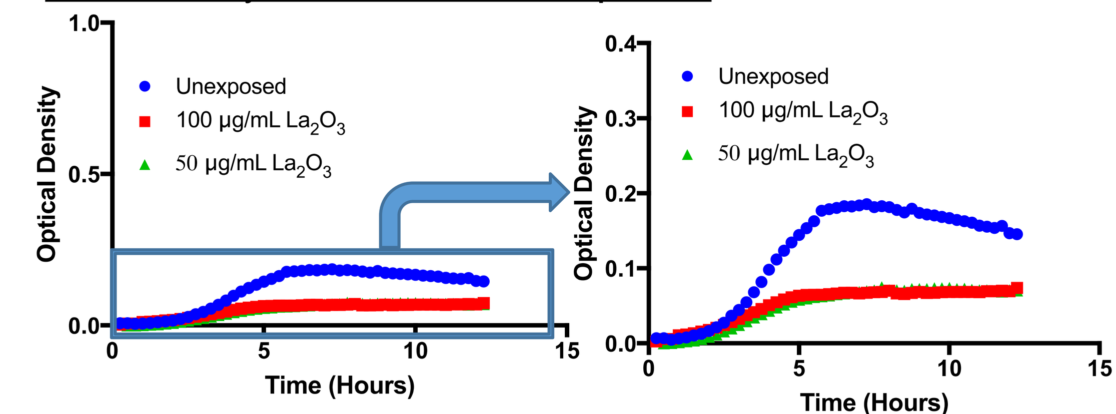
Results cont.

Rare earth modification facilitates uptake of gentamicin (1 μg/mL) into *E. coli* K12 T7



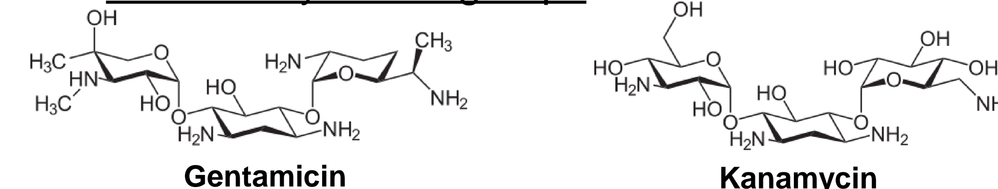
Gentamicin binds to Mg^{2+}-binding sites on the lipopolysaccharide (LPS**) during uptake. Kills bacteria by inhibiting protein synthesis.[6]

Rare earth modification facilitates uptake of kanamycin (4 μg/mL) into *E. coli* K12 T7. Aminoglycosides consistently show facilitated uptake.



**Kanamycin, although similar in structure to gentamicin, does not have as intense inhibitory properties; which suggests a different uptake mechanism.

Aminoglycosides are characterized as glycosides modified by amine groups



Most antibiotics tested resulted in no facilitated uptake into $LaPO_4$ modified E. coli K12 T7

Antibiotics Tested for Gram-negative bacteria

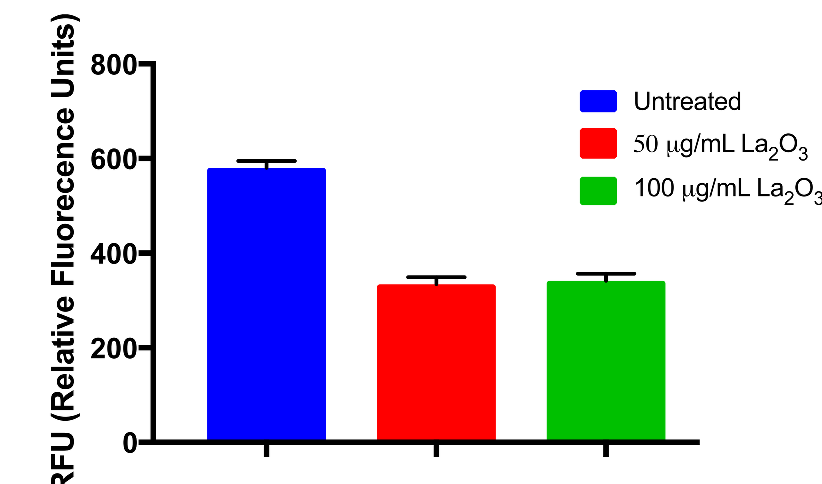
Antibiotic	Concentration (μg/ml)	Result
Kanamycin	2-16	Facilitated Uptake
Gentamicin	0.25-4	Facilitated Uptake
Erythromycin	8-128	Inconsistent
Azithromycin	2-16	Inconsistent
Rifamycin	0.5-4	No facilitated Uptake
Clindamycin	8-64	No facilitated Uptake
Penicillin	1-32	No facilitated Uptake

Gram-positive bacteria did not show signs of facilitated uptake into $LaPO_4$ modified bacteria with the tested antibiotics.

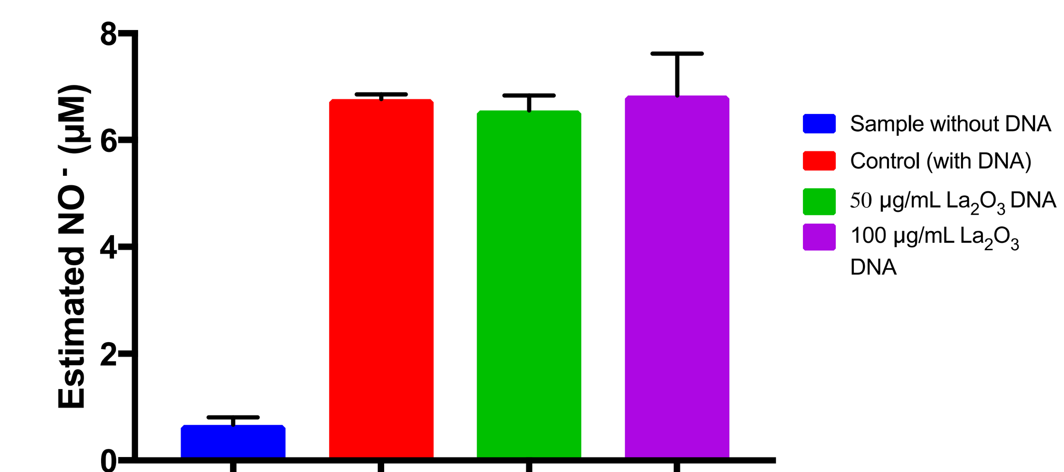
Antibiotics Tested for Gram-positive bacteria

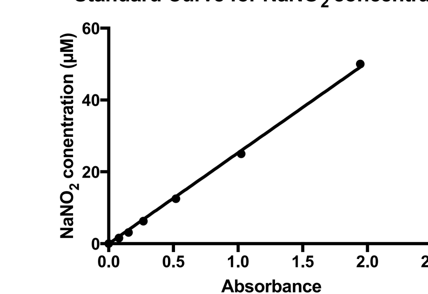
Antibiotic	Concentration (μg/mL)	Result
Novobiocin	0.03125-2	No facilitated uptake
Penicillin	0.125-2	No facilitated uptake
Erythromycin	0.25-4	No facilitated uptake
Gentamicin	0.25-4	No facilitated uptake

DNA content is lower in $LaPO_4$ modified E. coli K12 T7 relative to the control



Genomic DNA derived from $LaPO_4$ modified E. coli K12 T7 does not preferentially activate macrophage Raw264.7 cells





** A standard curve for estimating molar concentration of NO^- was derived from a serial dilution of $NaNO_2$ solution

Conclusions

- La-dephosphorylation of *E. coli* K12 T7 leads to slower growth recovery due to possible interference with nutrient uptake mechanisms.
- La-dephosphorylation of *E. coli* K12 T7 leads to facilitated uptake of aminoglycoside antibiotics of gentamicin and kanamycin.
- La-dephosphorylation of *Staphylococcus aureus* has not shown any sign of facilitated uptake of the tested antibiotics possibly due to the more permeable membrane of the Gram-positive bacteria

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