UNEARTHING THE ANTIBACTERIAL ACTIVITY OF MEDICINAL CLAYS

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Introduction

A resurgence of inquiry into alternative antibacterial mechanisms has emerged as human pathogens have evolved antibiotic resistance. Documented use of reduced metal-rich clays in healing necrotizing fasciitis (Brunet de Courssou 2002) led to renewed interest in ancient uses of clay for wound healing (Williams et al. 2004). Clays are <2µm minerals of any type (Moore & Reynolds 1997), but largely consist of phyllosilicates, providing an enormous surface area (100's m^2/g) for interaction with environmental water. Antibacterial clays are those that, through physical or chemical interactions, kill certain bacteria, including antibiotic resistant human pathogens (Williams et al. 2008). In the natural depositional environment microbes evolve to thrive in contact with clays; many deriving energy from the minerals (Kostka et al. 1999). However, when clays containing reduced metals are taken out of equilibrium by adding water to make a poultice for topical treatments, the pH and Eh of the water changes due to cation exchange and redox reactions (Morrison et al., 2014). The new aqueous conditions stress human pathogens evolved to thrive in oxidized, circumneutral pH. We observed (Williams et al. 2011) that antibacterial clays display pH <4 or >10, where metals (e.g., Al, Fe) are soluble.

Fe and Al are two of the most abundant elements in the earth's crust with contrasting roles in biological systems. Fe is a limiting nutrient for bacterial growth due to the low solubility of Fe^{3+} (10^{-18}M) at physiological pH, requiring siderophores to access free Fe. However excess Fe is toxic to cells so its uptake is tightly regulated (Touati et al. 1995). Oxidative stress from intracellular Fe^{2+} overload occurs through Fenton reactions with metabolically generated hydrogen peroxide, H_2O_2 (Imlay 1988). In contrast, Al has no known biological function and is thought to exhibit toxicity through membrane interactions (Piña & Cervantes 1996; Williams 1999). The toxicity of individual metals is primarily related to their binding affinity and production of reactive oxygen species (ROS) (Lemire et al., 2013). Here we unearth a new antibacterial process showing that the synergistic activity of metal mixtures generated by antibacterial clays is key to their antibacterial mechanism.

Understanding the antibacterial mechanism begins by knowing geochemical constraints on mineral reactants and the products that abolish pathogenic bacteria. A pristine hydrothermally altered clay deposit formed in porphyry andesite from the Oregon, Cascades was studied in detail to establish the geochemical fundamentals

needed to design a new antibacterial product. Evaluating the natural deposit shed light on key antibacterial components and identified a blue clay assemblage containing Fe^{2+} and Al^{3+} that was 100% effective at killing a broad spectrum of human pathogens (Williams et al. 2011; Morrison et al. 2014). Critically important is the presence of phyllosilicates and pyrite, which buffer solution pH, releasing metals from the mineral assemblage and preserving essential metals in the clay interlayers (Morrison et al. 2014). Using metal toxicity, oxidation and genetic assays, assisted by advanced bioimaging techniques we determined the antibacterial strategy used by this particular clay, that could inform designs for new mineral based antibacterial agents.

Methodology

E. coli (ATCC 25922) was reacted with clay suspensions and clay leachates (aqueous solutions equilibrated with clays for 24 hrs). Inductively coupled plasma mass spectrometry (ICP-MS) was used to measure the elements leaching from the clays. Scanning transmission X-ray microscopy (STXM) imaging was performed on bacteria reacting with leachates to map the redox state of Fe adsorbed to membranes. NanoSIMS (secondary ion mass spectrometry) Al maps were measured to determine the if cytoplasmic uptake occurs. Scanning transmission electron microscopy-electron energy loss spectroscopy (STEM-EELS) was used to investigate the precipitation of intracellular mineral particles. ROS generated by minerals and mineral leachates were measured using a spectrophotometric hydrogen peroxide assay (H_2O_2). Genetic responses to leachates were measured using two lacZ fusion strains, *sulA::lacZ* for genotoxicity and *rpoHP3::lacZ* for membrane damage.

Results

We show that the essential components for the antibacterial activity are soluble Fe and Al, which work synergistically to overcome the highly evolved metabolic functions of human pathogens. Elemental mapping using STXM and nanoSIMS reveal Fe^{2+} and Al^{3+} adsorption to bacterial membranes. The intracellular particles observed upon cell death were determined to be Fe-oxides using STEM-EELS. Hydrogen peroxide assays indicate that antibacterial minerals buffer solutions to continually produce reactive oxygen species over 24 hours. The genetic responses of *E. coli* to envelope stress and genotoxicity show that Fe^{2+}, Fe^{3+} and Al^{3+} work synergistically to stress the outer membrane while intracellular Fe^{2+} levels rise, generating reactive oxygen species and damaging DNA. This is the first documentation of the critical components involved in the antibacterial processes exhibited by this natural clay, and should guide design of new antibacterial agents.

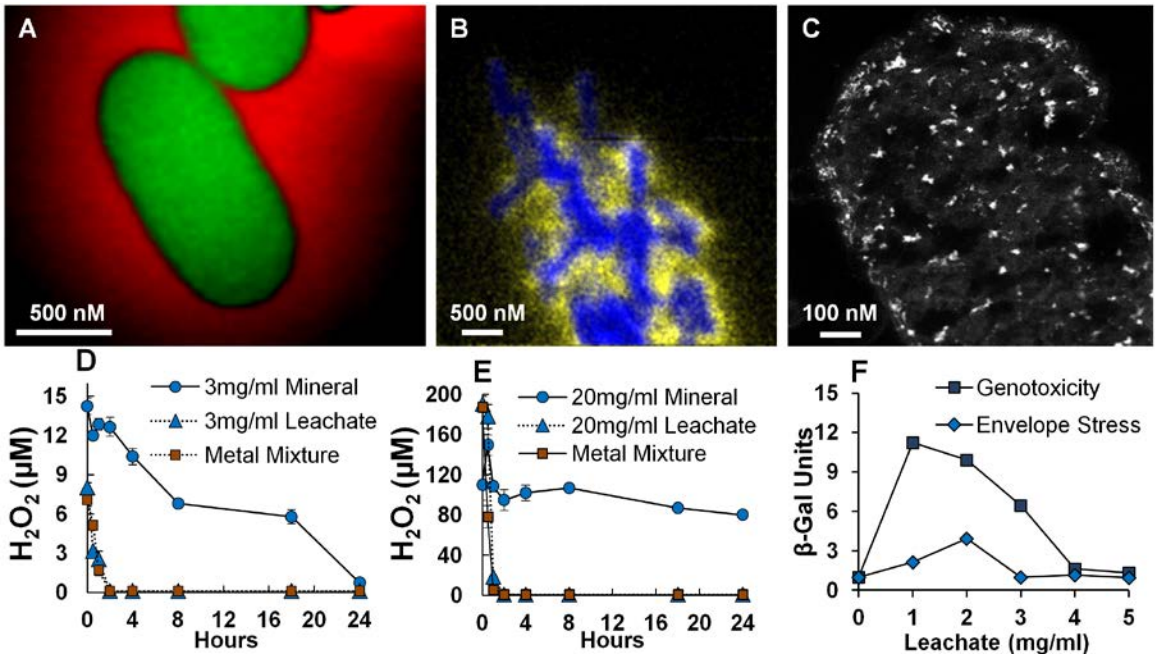

Figure 1. The antibacterial process. *E.coli* cells were reacted with 100 mg/ml mineral suspension and imaged after 12 hours (A to C). STXM elemental maps reveal Fe^{2+}-rich regions (green) associated with bacterial membranes, while the extracellular solution concentrates Fe^{3+} (red) (A). nanoSIMS elemantal maps of $^{12}C^{14}N$ (blue) and $^{54}Al_2$ (yellow), showing Al^{3+} adsorbs to bacterial membranes without entering the cytoplasm (B). STEM-EELS imaging reveales precipitation of Fe-oxide nano particles (C). H_2O_2 levels (µM) measured at minimum inhibitory (D) and bactericidal (E) concentrations for mineral leachates and artificial metal mixtures reacting with *E. coli*. LacZ fusion *E. coli* strains responding to envelope stress (*rpoHP3*::*lacZ*) and genotoxicity (*sulA*::*lacZ*) (F) measured on *E. coli* reacted with leachates. LacZ results are shown as a ratio of the levels measured in the control *E. coli* and expressed as β-Gal Units.

Conclusions

Antibacterial clays release Fe^{2+}, Fe^{3+} and Al^{3+}, which synergistically damage multiple cellular components. Clay mineral interlayer cation exchange serves as a reservoir for Fe^{2+} along with the dissolution and oxidation of pyrite. Plagioclase feldspar and illite smectite dissolution provide Al^{3+} and Ca^{2+}. Mineral suspensions release these elements while reacting toward a new chemical equilibrium, generating ROS as the metals and minerals are oxidized. Pathogenic bacteria exposed to these conditions are initially subject to envelope stress. Outer membranes enriched in Fe^{2+} react with H_2O_2 forming hydroxyl radicals, oxidizing membrane proteins. Aluminum also results in envelope stress, causing protein misfolding in the outer membrane, activating genetic stress responses. Hydrogen peroxide generated extracellularly by minerals can diffuse through the cell envelope and react with intracellular Fe^{2+} causing increased DNA damage. The natural metal based antibacterial process revealed by these clays stresses multiple cellular systems simultaneously unlike traditional antibiotics, which obstruct individual cellular pathways, making resistance through mutations more difficult.

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