EVALUATING THE ANTIBACTERIAL ACTION OF A CLAY FROM THE COLOMBIAN AMAZON

Londono, Sandra-Carolina, Williams, Lynda

School of Earth and Space Exploration, Arizona State University. Tempe, AZ, 85287-1404 USA

Sandra.Londono@asu.edu, Lynda.Williams@asu.edu

Introduction

The overuse and misuse of antibiotics threatens both environmental and human health. Antibacterial clays are an alternative that can reduce even antibiotic resistant bacteria and can be safely disposed of in the environment after use. The known mechanisms of action of antibacterial clays involve metal species, which can react to produce deleterious effects on bacteria, or directly interact with cell molecules (Williams et al. 2004, 2008, 2009, 2011; Cunningham et al. 2010; Morrison et al. 2014). However, clays can host a variety of metals and determining which ones are pivotal for the bactericide is key to identifying natural antibacterial clays or to develop products based on them. In addition, elucidation of the pathways or chemical reactions of the metals from clay to bacteria remains an active field of research. Here, we report on a natural antibacterial clay of lacustrine origin from the Colombian Amazon (AMZ). Preliminary results show that the source of aluminum is clay dissolution under acidic pH conditions (pH 4.5). Al seems to work by inactivating enzymes in the membrane that are key for bacterial cellular metabolism.). In addition, there is evidence that bacterial nutrients are depleted in model E. coli treated with the antibacterial clay. Adsorption of nutrients by the clay minerals smectite and halloysite, dominant in the clay, may compound the antibacterial action. This study shows that not only exchangeable transition metals but also a structural metal can contribute to fight bacteria.

Methodology

Characterization of clays

The chemical composition of the clay fraction (<2µm), leachates, and cation exchange solution were determined using spectroscopic and spectrometric methods. Major elements in AMZ were determined using X-ray fluorescence (XRF). Analyses were conducted at the U.S. Geological Survey (Denver, CO) and at the National University of Colombia in Bogotá. Minor and trace elements in cells, clay separates, cation exchange solution, aqueous and LB leachates, and growth media (LB), were quantified by inductively coupled plasma mass spectrometry (ICP-MS) in a 2% HNO3 matrix. A Thermo electron X-series quadrupole (Q-ICP-MS) in the Keck lab at Arizona State University (ASU) was used. The anions in the leachate were determined using ion chromatography (IC) in a DX600 Ion Chromatography system

(Dionex). To determine the elemental concentration sourced by the clay in the LB leachate, the elemental composition from LB was subtracted from the LB leachate.

Antibacterial assays

Bacterial strains were grown overnight in Luria Bertani broth (LB; 5 or 25 g LB/L) at 37°C on a rotating drum. In vitro antimicrobial susceptibility tests were conducted following procedures described in Williams et al. (2008). Briefly, 400 µl of liquid culture (~10^8 CFU/mL) in exponential growth phase was incubated overnight at 37°C with 100 mg of autoclaved clay (250 mg/mL). The incubated mixture of bacteria and clay in LB suspension was serially diluted and plated on LB agar (25g/L LB) to count viable colonies (NCCLS 2000). Controls for bacterial growth in the absence of clay and in the presence of reference clays (Kaolinite API #5) were included (Figure 1)

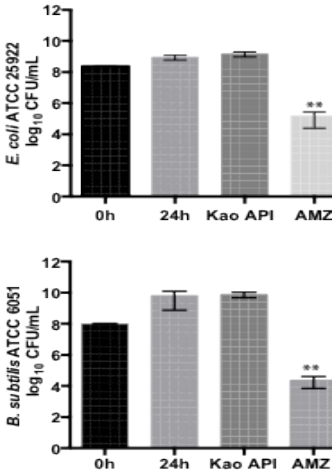

Figure 1. Effects of clay on cell growth *CFU: Colony forming units. 0h, 24h: initial and bacterial concentration without clay, Kao API: standard, non-antibacterial clay, AMZ: unknown.*

Bacteria-mineral separation

To study the chemical composition of E. coli and clay after the antibacterial assay, a cell-mineral separation procedure was used (Neveu et al. 2014). Bacteria and clay were incubated following the antibacterial testing protocol described above. More dilute media (5g LB/L) was used in order to minimize metal precipitation due to the presence of high concentrations of particular medium components. The clay and bacteria were separated following methods of Neveu et al. (2014). In brief, 5mL of extraction buffer (5mL phosphate buffer saline (PBS) at pH 7.4; 1mg Na-pyrophosphate; 0.25mL Tween detergent) were added to the *E. coli* - clay suspension. The samples were stirred at 720 rpm for 30 min, and sonicated in a low

energy water bath for 1 min. The samples were transferred on top of a 5mL layer of Nycodenz density gradient media (Axis-Shield Cat. No. 1002424, density adjusted to 0.8mg/mL). Final separation of phases was achieved by centrifugation in a swing-arm centrifuge (Eppendorf Benchtop Centrifuge 5804R, 5000g, 50min, 4°C). The sediments (clays > 800 mg) settled at the bottom and the bacteria (<80 mg) floated on top of the Nycodenz layer. The presence of intact bacterial cells was confirmed under the light microscope. The recovered bacterial cells were washed 3 times with EDTA-oxalate to remove metals adsorbed on the cell membranes (Tovar-Sanchez et al. 2003). A control of *E. coli* grown in LB was included. All experiments were performed in triplicate.

Element exchange between clay and bacteria

Results showed that a single dose of AMZ clay (250mg/mL) induces a 4-6 order of magnitude reduction of cell viability over 24h. (Figure 1) The minimum inhibitory concentration of AMZ (minimum dose that produces 90% cell death) is 70mg/mL. The minimum bactericidal concentration (minimum dose that produces 100% cell death) is 300mg/mL.

To test for exchange of elements between clays and bacteria, the chemical composition of each was analyzed using ICP-MS. After bacteria - mineral separation, the separated phases (reacted clay and bacteria) were dried in a clean hood and weighed. A control of E. coli grown overnight in LB (5g/L) was included. Samples were re-suspended in 5mL concentrated HNO_3 and transferred into Teflon beakers for acid digestion. Bacterial cells were digested overnight in concentrated HNO_3 and H_2O_2 on hot plate at sub-boiling temperature (130°C) followed by additional digestion rounds in concentrated HNO_3. Clays were digested in 4mL concentrated HNO_3 and 1mL concentrated HF. A second digestion was performed using concentrated HCl at 130°C. Finally, acids were evaporated and the samples were diluted in a 2% HNO_3 matrix for multi-elemental analyses by ICP-MS.

Results

To study the chemical interactions between clay and bacteria, we compared the elemental composition of *E. coli* and the clays after reaction (incubation at 37˚C for 24 hr) relative to control bacteria grown without clay. Hereafter, we refer to the *E. coli* that was incubated with AMZ clay as '*E. coli* reacted with AMZ ' or for experiments with Kao API#5 '*E. coli* reacted with Kao', and to the clay that was mixed with bacteria as the "reacted clay". *E. coli* incubated without clay is the 'control *E. coli'*.

E. coli reacted with AMZ showed a decrease in the Mg, and P concentrations relative to the control *E. coli* (Table 1). The concentration of Al, Se, and V is notably high (2 orders of magnitude greater than the control) in *E. coli* reacted with AMZ, and the concentration of metals including Fe, Co, Ni, Cu, Cd, Pb, and Cs also increased.

No change was observed for Ca, Rb, Mo, and Mn, relative to the control (within error) (Table 1). Na is excluded from the analysis because it is a major component of reagents used, and not a true indicator of exchange between clay and bacteria. The *E. coli* reacted with Kao consistently showed a ~1 fold increase in the concentrations of elements except for V and Al that increased 2 and 1 orders of magnitude, respectively. Realizing that the separation of clay from bacteria is not perfect, we estimated the contribution of clay to the bacterial population considering elements found only in the clay (e.g., Ba, Pb, W) to estimate a percentage of clay-related elements to subtract (Table 1).

Discussion

The differences observed between the chemical composition of *E. coli* reacted with AMZ and the control *E. coli* suggest that the viability loss of *E. coli* reacted with AMZ may be linked to the clay robbing nutrients from the bacteria. A correction of 3.2% clay contribution based on Ba content resulted in removal of many insoluble elements (e.g. Ti, W) most certainly associated with the clay. This correction allows evaluation of metals potentially important in the antibacterial process. Following the same rationale, the *E. coli* reacted with Kao API#5 was corrected for a 1% contribution from the clay. The separation of kaolinite and *E. coli* is more effective than AMZ separation perhaps due to a weaker attraction between kaolinite and bacteria than smectite and bacteria (Walker et al. 1989). In this analysis, *E. coli* reacted with AMZ accumulated two orders of magnitude more Al, Se, and V than the control.

While Se and V are essential elements, Al does not play a role in biology (Borrok 2003). Al^{3+} forms complexes with phosphate ligands found both in bacteria and minerals (MacDonald & Martin 1988). This chemical affinity provides an avenue for membrane damage as Al^{+3} alters lipid-protein interactions when it is bound to phospholipids (Garcidueñas & Cervantes 1995). It also interferes with the membrane electrical potential, and inhibits membrane transport proteins (Xu et al. 2012). In the membrane, Al^{+3} can substitute for Ca^{+2} and Mg^{+2}, the cations responsible for stabilizing the membrane by binding LPS (Hancock 1984). At acidic conditions buffered by the AMZ clay (pH 4.5), *E. coli* susceptibility to metal attack increases due to the displacement of the structural cations by H+. Thus, Al^{3+} can compromise the integrity of the membrane causing leakage of cytoplasm (Beveridge and Koval 1981; Garcidueñas & Cervantes 1995; Williams 1999).

Conclusions

The chemical data suggests two main factors that reduce bacterial viability in *E. coli*: 1) the uptake or adsorption of metals by bacteria, primarily Al^{3+}, may derive from dissolution of components in the AMZ and 2) The AMZ clay may reduce the bioavailability of nutrients and abundance of nutrients (Mg, P) to the bacteria, which further diminishes cell function. It is possible that the clay absorbs cation nutrients

and likely that phosphates precipitate in the growth media, thus starving the bacteria. Furthermore, the cell membrane could have been compromised by the low pH buffered by the clay, and the impaired ability of *E. coli* to maintain the structural stability of the outer membrane due to Mg^{2+} loss. The availability of transition metal ions may further challenge the bacteria by oxidative damage to the membrane or interfering with metabolism by altering enzyme specificity.

Table 1. Elemental composition of *E. coli* cells before and after clay treatment

	***E. coli* EDTA washed**		***E. coli* reacted with AMZ**		**Corrected *E. coli* for 3.2% AMZ**		**Corrected *E. coli* reacted with Kao API#5 (1%)**	
Element	**Average (ppm)**	**S.D.**	**Average (ppm)**	**S.D.**	**Average (ppm)**	**S.D.**	**Average (ppm)**	**S.D.**
Mg	562	43	264	63	163	59	580	98
Al	34	35	7166	986	2112	1028	-676	517
P	8826	507	4663	1495	4620	1500	11701	2055
K	1965	31	741	116	377	114	5132	895
Ca	15	4	73	4	35	5	52	2
Ti	13	1	24	5	-96	7	-119	9
V	0.05	0.0	7.54	1.2	2.31	1.3	0.02	0.1
Cr	0.49	0.1	5.57	0.6	2.30	0.7	-0.32	0.0
Mn	2.54	0.2	8.38	2.6	4.18	2.1	3.23	0.6
Fe	82	13	1231	115	464	136	91	20
Co	0.30	0.1	1.29	0.3	1.13	0.3	0.30	0.0
Ni	0.86	0.1	2.39	0.1	1.94	0.1	0.81	0.1
Cu	3.88	0.1	24.87	8.7	17.50	5.5	3.49	0.8
Zn	14.52	0.6	30.84	4.2	25.73	1.3	15.52	2.4
As	0.08	0.0	0.54	0.1	0.35	0.1	0.35	0.0
Se	0.09	0.0	5.27	1.4	4.37	1.4	0.38	0.1
Rb	0.69	0.1	3.54	0.9	0.86	0.8	1.63	0.4
Sr	0.16	0.0	3.08	0.5	0.44	0.5	0.78	0.2
Zr	0.18	0.1	3.51	0.7	0.44	0.6	-0.07	0.1
Mo	0.97	0.1	0.85	0.1	0.71	0.1	2.11	0.6
Cd	0.06	0.0	0.25	0.1	0.18	0.0	0.46	0.2
Ba	0.45	0.1	6.93	1.7	-0.24	0.2	-0.06	0.1
Pb	0.38	0.1	2.46	0.6	1.48	0.8	0.10	0.1

References

BORROK, D., TURNER, B.F., FEIN, J. 2005. A Universal Surface Complexation Framework for Modelling proton binding onto bacterial surfaces in geologic settings. *American Journal of Science,* **305**, 826-853.

BEVERIDGE, T. & KOVAL, S. F. 1981. Binding of Metals to Cell Envelopes of Escherichia coli K-12. *Applied and Environmental Microbiology,* **42**, 325-335.

CUNNINGHAM, T. B., KOEHL, J. L., SUMMERS, J. S., HAYDEL, S.E. 2010. pH-dependent metal ion toxicity influences of the antibacterial activity of two natural mineral mixtures. *PLoS-ONE, 5, e9456*.

GARCIDUEÑAS-PINA, R., & CERVANTES, C. 1995. Microbial interactions with Aluminum. *BioMetals*, **9**, 311-316.

HANCOCK, R. E. 1984. Alterations in outer membrane permeability. *Annual Review Microbiology.* **38**, 237–264

MACDONALD, T.L., & MARTIN, R.B. 1988. Aluminum ion in biological systems. *Trends in Biochem Science,* **13**, 15-19.

MORRISON, K.D., UNDERWOOD, J., METGE, D., EBERL, D., & WILLIAMS, L. 2014. Mineralogical variables that control the antibacterial effectiveness of a natural clay deposit. *Environmental Geochemistry and Health*, doi: 10.1007/s10653-013-9585-0

NCCLS. 2000. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*: *Approved Standard*. NCCLS Document M7-A5 (ISNB 1-56238- 394-9) fifth. NCCLS, Pennsylvania, USA.

NEVEU, M, PORET-PETERSON, A, LEE, Z, ANBAR, A, & ELSER, J. 2014. Prokaryotic cells separated from sediments are suitable for elemental composition analysis. *Limnology and Oceanography methods,* **12**, 519-529.

TOVAR-SANCHEZ, A., SANUDO-WILHELMY, S. A., GARCIA-VARGAS, M., WEAVER, R. S., POPELS, L. C., & HUTCHINS, D. A. 2003. A trace metal clean reagent to remove surface-bound iron from marine phytoplankton. *Marine Chemistry*, **82**, 91-99

WALKER, S., FLEMMING, C., FERRIS, F., BEVERIDGE, T., & BAILEY, G. 1989. Physicochemical interaction of *Escherichia coli* cell envelopes and *Bacillus subtilis* cell walls with two clays and ability of the composite to immobilize heavy metals from solution. *Applied and Environmental Microbiology, 55,* **11**, 2976-2984.

WILLIAMS, L.B., HAYDEL, S.E., GIESE, R.F., & EBERL, D.D. 2008. Chemical and mineralogical characteristics of french green clays used for healing. *Clays and Clay Minerals*, 56 **4**, 437-452.

WILLIAMS, L.B., HAYDEL, S.E., & FERRELL, R.E. 2009. Bentonite, bandaids, and borborygmi. *Elements: An international magazine of mineralogy, geochemistry and petrology*, 5, **2**, 99-104.

WILLIAMS, L. B., HOLLAND, M., EBERL, D. D., & DE COURRSOU, L. B. 2004. Killer Clays! Natural antibacterial clay minerals. *Mineralogical Society of London Bulletin*, **139**, 3-8.

WILLIAMS, L.B, HAYDEL, S.E. 2010. Evaluation of the Medicinal use of clay minerals as antibacterial agents. *International Geology Reviews.* **52**, 745-70

WILLIAMS, L., METGE, D. W., EBERL, D. D., HARVEY, R. W., TURNER, A. G., PRAPAIPONG, P., & PORET-PETERSON, A. T. 2011. What makes a natural clay Antibacterial? *Environmental Science and Technology*, **45**, 3768–3773.

WILLIAMS, R. J. 1999. What is wrong with Aluminum*? Journal of inorganic biochemistry*, **76**, 81-88.

XU, J., CAMPBELL, J. M., ZHANG, N., HICKEY, W., & SAHAI, N. 2012. Did mineral surface chemistry and toxicity contribute to evolution of microbial extracellular polymeric substances? *Astrobiology*, 12, **8**, 785-798.