Evaluation of the medicinal use of clay minerals as antibacterial agents

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Abstract

Natural clays have been used to heal skin infections since the earliest recorded history. Recently our attention was drawn to a clinical use of French green clay (rich in Fe-smectite) for healing Buruli ulcer, a necrotizing fasciitis ('flesh-eating' infection) caused by *Mycobacterium ulcerans*. These clays and others like them are interesting as they may reveal an antibacterial mechanism that could provide an inexpensive treatment for this and other skin infections, especially in global areas with limited hospitals and medical resources.

Microbiological testing of two French green clays, and other clays used traditionally for healing, identified three samples that were effective at killing a broad-spectrum of human pathogens. A clear distinction must be made between 'healing clays' and those we have identified as antibacterial clays. The highly adsorptive properties of many clays may contribute to healing a variety of ailments, although they are not antibacterial. The antibacterial process displayed by the three identified clays is unknown. Therefore, we have investigated the mineralogical and chemical compositions of the antibacterial clays for comparison with non-antibacterial clays in an attempt to elucidate differences that may lead to identification of the antibacterial mechanism(s).

The two French green clays used to treat Buruli ulcer, while similar in mineralogy, crystal size, and major element chemistry, have opposite effects on the bacterial populations tested. One clay deposit promoted bacterial growth whereas another killed the bacteria. The reasons for the difference in antibacterial properties thus far show that the bactericidal mechanism is not physical (e.g., an attraction between clay and bacteria), but by a chemical transfer or reaction. The chemical variables are still under investigation.

Cation exchange experiments showed that the antibacterial component of the clay can be removed, implicating exchangeable cations in the antibacterial process. Furthermore, aqueous leachates of the antibacterial clays effectively kill the bacteria. Progressively heating the clay leads first to dehydration (200°C), then dehydroxylation (550°C or more), and finally to destruction of the clay mineral structure by (~900°C). By identifying the elements lost after each heating step, and testing the bactericidal effect of the heated product, we eliminated many toxins from consideration (e.g., microbes, organic compounds, volatile elements) and identified several redox-sensitive refractory metals that are common among antibacterial clays. We conclude that the pH and oxidation state buffered by the clay mineral surfaces is key to controlling the solution chemistry and redox related reactions occurring at the bacterial cell wall.

Keywords

antibacterial clay; bentonite; smectite; medicinal minerals; reduced iron; skin disease; French green clay

Introduction

The role of clays in human health has experienced a revival in interest due to advances in modern instrumentation [e.g., transmission electron microscopes (TEM), field emission scanning electron microscopes (FESEM), atomic force microscopy (AFM), and secondary ion mass spectrometers (SIMS)], that allow us to study surfaces of nano-scale minerals *in situ* within their natural environmental matrix. By identifying the special characteristics that make a particular clay antibacterial, we may elucidate some of the reasons these common nano-minerals not only have potential applications in medicine, but may also contribute to the general understanding of antibacterial mechanisms lending insights to potential cures.

Recent reviews regarding uses of clay in achieving and maintaining human health have focused on the ancient practice of *geophagy*, which is the practice of eating earth materials containing clay minerals (Wilson, 2003; Ferrell, 2009). The purpose of geophagy is to elicit a healing response in humans through ingesting the easily available materials that may physically soothe an infected and inflamed gastrointestinal lining (Droy-Lefaix et al., 2006). Alternatively, clays have been used topically in mud spas (pelotherapy) to adsorb toxins from skin and provide heat to stimulate circulation for rheumatism treatment (Carretero et al., 2006; Gomes et al., 2007). Historical accounts of humans using 'healing clay' began with Aristotle (384-322 BC) (Mahaney et al., 2000) and Pliny the Elder (23–79 AD) later recounted the cure of intestinal ailments by ingestion of volcanic muds (Carretero et al., 2006). Of the many historical accounts of clays, muds, and soils used by people for 'healing', the scientific evidence of the action of clays for treating and healing ulcers, tumors, cysts, cancers, osteoporosis, etc, is lacking.

Nonetheless, the observations of humans who have been 'cured' of illness by clay applications and the correlating photographic documentation (Brunet de Courssou, 2002; Williams et al., 2004) were the stimuli for our research into the healing mechanism of clays. We provide a general compilation of the descriptions of healing and the common mineralogical attributes of clays and clay minerals used in the past.

In 2002, Line Brunet de Courssou reported to the World Health Organization (WHO), a summary of ~10 years of work in the Ivory Coast of Africa (east Africa), where she documented the use of specific clay minerals as therapeutic treatment of advanced Buruli ulcer disease (Brunet de Courrsou, 2002). However, she was unable to conduct additional experiments of traditional and accepted scientific studies due to lack of financial support. There have been several reports describing the antibacterial properties of natural and synthetic clay minerals (Herrera et al., 2000; Hu et al., 2005; Tong et al., 2005; Williams et al., 2004; Wilson, 2003; Haydel, 2008). Despite these studies and the clinical evidence suggesting that clay minerals promote healing in individuals infected with *Mycobacterium ulcerans*, the chemical interaction occurring at the clay mineral – bacterial interface, and the mechanism by which the clay minerals inhibit bacterial growth remains unknown.

Our research into the healing process lead us to propose *in vitro* testing of the effect of 'healing clays' on a broad spectrum of human bacterial pathogens. We make an important distinction between 'healing clays' and 'antibacterial clays'. While clays may heal various ailments by their unique physical properties (e.g., high absorbance, surface area, heat

capacity, exchange capacity, etc.), we have identified only a few natural clays that kill pathogenic bacteria. Building on the recent literature on healing and antibacterial minerals and compounds, this paper reviews the methods and analytical protocols we have established for investigating the antibacterial properties of clays.

Traditional Uses of Clays for Human Health

Healing practices of ancient cultures, as well as modern society, have depended on clay minerals with powerful adsorptive and absorptive properties to treat a variety of topical maladies. *Adsorption* is the process of attraction, binding, and accumulation of molecules or particles to a solid surface in a condensed layer. *Absorption* results when a substance diffuses or penetrates into a liquid or solid forming a transition zone or layer, often with a new composition, adjacent the substrate. Clay minerals are ubiquitous in nature and their adsorptive and absorptive capabilities have been exploited in a variety of cosmetics and pharmaceutical formulations. Traditionally, clay minerals are mixed with water for various periods of time (days to years) to form clay gels or pastes that can be applied externally for cosmetic or skin protective purposes (Carretero, 2002; Carretaro et al., 2007; Gomes et al., 2007). The high adsorption and absorption capacities, cation exchange capacity, as well as the extremely fine particle size of certain clays, e.g. smectites (expandable clay minerals) and kaolin group minerals are important reasons why these minerals are used to remove oils, secretions, toxins, and contaminants from the skin. By adsorbing and absorbing moisture and impurities from the skin, the clays also serve to cleanse and refresh the skin surface and to aid in the healing of topical blemishes, the major selling point for many cosmetics. Although consumers generally consider clays to be safe when applied topically, it is important to recognize that cosmetic firms must substantiate the safety of their products and that the U.S. Food and Drug Agency does not subject these products to pre-market approval.

The intentional consumption of earth materials, such as clays, by humans and animals is known as geophagy (Wilson, 2003). This complex and poorly understood practice is largely attributed to religious beliefs, cultural practices, psychological disorders, dietary/nutritional needs, and medicinal benefits (Abrahams et al., 1996; Aufreiter et al., 1997; Hunter, 1973). Although often viewed as an abnormal behavior by medical practitioners (i.e., pica), geophagy is believed to be an adaptive phenomenon in mammals and primates and a learned behavior in various societal cultures (Klaus and Schmidt, 1998; Krishnamani and Mahaney, 2000). Historically, geophagy is believed to be practiced to remedy a physiological response to mineral nutrient deficiencies, such iron or zinc, to satisfy a dietary craving, and to ease psychosocial problems, including anxiety, stress, and obsessive-compulsive disorder (Lacey, 1990; Sayetta, 1986). However, several studies and medical reports indicate that ingesting large amounts of clay (>200 grams per day), particularly clay with a high cation-exchange capacity, can impede absorption of iron, zinc, and potassium, leading to iron, zinc, or potassium deficiencies (Arcasoy et al., 1978; Cavdar and Arcasoy, 1972; Cavdar et al., 1977a,b; Gonzalez et al., 1982; Mengel et al., 1964; Minnich et al., 1968; Severance et al., 1988; Ukaonu et al., 2003).

The ingestion of dried clay minerals or a clay suspension is commonly used as a source of dietary elements, as a detoxifying agent, and as an allopathic treatment of gastrointestinal illnesses and acute and chronic diarrhea (Carretero, 2002). For example in Ghana, the iron, copper, calcium, zinc, and manganese consumed in clays were in the range of 2 to 15 percent of recommended dietary allowances (Hunter, 1973) and it was concluded that moderate ingestion of clays lacking high cation-exchange capacities could serve as a nutritional supplement for these essential elements. In the acidic environment of the stomach, the clay minerals could bind to positively charged toxins and serve as detoxifying agents to reduce bioavailability interfering with gastrointestinal absorption of the toxin

(Hladik and Gueguen, 1974; Johns and Duquette, 1991; Mahaney et al., 1996; Phillips, 1999; Phillips et al., 1995). Over-the-counter pharmaceuticals that originally contained kaolinite, attapulgite, or clay-like substances (i.e. Kaopectate®) represent classic examples of the use of clay minerals by human populations to treat diarrhea and intestinal illnesses (Vermeer and Ferrell, 1985) and soothe gastrointestinal ailments. (Note: Kaopectate was reformulated in 2002 and now contains bismuth subsalicylate instead of kaolinite or attapugite.) Kaolinite has many medically beneficial attributes primarily related to its ability to adsorb lipids, proteins, bacteria, and viruses (Adamis and Timar, 1980; Lipson and Stotzky, 1983; Schiffenbauer and Stotzky, 1982; Steel and Anderson, 1972; Wallace et al., 1975).

Geology and Geochemistry

The study of medicinal applications of minerals requires collaborative efforts by many specialists with diverse expertise and educational backgrounds including, for example, geology, geochemistry, microbiology, environmental science, soils and agriculture, medicine, statistics, and pharmacology to name a few. Understanding the interactions of natural minerals and microbial systems is an immense undertaking, so there is no limit to the efforts of diverse scientific disciplines in these arenas. Because of this it is important to establish a basis of communication among the diverse scientific groups. Here we present some back ground and define some common terms used by geologists/mineralogists and clinical microbiologists in attempt to alleviate misconceptions across disciplinary boundaries.

Clays and Clay Minerals

First and foremost, it is essential to define what clay is and how it differs from mud, soil, and minerals. In the field of geology clay is a size-based term for very fine-grained minerals with an estimated spherical diameter <2.0 µm and approximate density of 2.65 g/cm^3 as defined by Stokes Law (Jackson, 1979; Moore et al., 1997). As gravel, sand, and silt are terms for sedimentary grain sizes, clay is the term for the finest fraction of sediments that all consist of accumulations of different minerals (e.g., quartz, feldspar, carbonates, etc.) and organic matter. Clay, when moistened with water, creates a mud-like consistency, comparable to that used in spas for pelotherapy. Soils, often comprise many minerals and especially clay-sized minerals and organic matter (humus), as this medium must be able to support life by exchange of ions through water and gas that fills the spaces between the solid particles (Voroney, 2006). Mud is a slurry of water and sediment dominated by clay and silt sized particles. A mineral is a natural solid with a generally uniform composition and repeating internal crystalline order. When the crystalline domains are nanometers in scale, they are sometimes referred to as disordered or poorly crystalline. Clay minerals exhibit a wide range of order/disorder and crystallinity.

Most of the clay-sized fraction of sediments consists of clay minerals or *phyllosilicates* (defined below). Clay minerals are formed by weathering of other silicate minerals on and in the earth's crust, or they may precipitate directly from a solution. Minerals that form deep in the earth are likely to be unstable under the lower temperature and pressure conditions on earth's surface. When water and carbon dioxide from the atmosphere and from soil respiration interact with these minerals, they may dissolve often if the waters are acidic (carbonic acid) and the leached components precipitate as a variety of clay minerals (Giese and van Oss, 2002).

Bentonites

Most of the healing clays described in the literature (e.g., Carretero and Lagaly, 2007 and papers therein) are bentonites. However, bentonite is not a mineral but is a generic term for rocks derived from 'altered volcanic ash beds'. The ash is a layered sedimentary deposit that accumulates during volcanic eruptions. This ash produces mostly disordered solids but glassy particles formed by rapid quenching (cooling) of magma when the liquid material is thrown into the atmosphere. The volcanic ash layers, when compacted and infiltrated with water, alter primarily to kaolinite in acidic environments, smectite in mildly alkaline (seawater) environments, or zeolites in highly alkaline environments (Bohor and Triplehorn, 1993). The mineralogical variations occur depending on the chemical characteristics of the volcanic glass and the local water chemistry (Christidis, 1998).

Smectite is a group of expandable clay minerals with a variety of structural and chemical differences that affect their surface charge and chemistry. Montmorillonite, commonly identified in 'healing clays', is one type of smectite. It has a structure of 2 tetrahedral sheets that sandwich a single octahedral sheet (Fig 1). The 2:1 structure forms layers that stack like cards with the space between cards called the 'interlayer'. The collective of interlayer, octahedral, and tetrahedral sites of montmorillonite forms a mineral with an ideal chemical formula; $R_{0.33}^+$ ($Al_{1.67}$ $Mg_{0.33}$) Si_4 O_{10} $(OH)_2$ (where R represents interlayer cations; Moore and Reynolds, 1997). Many elements can substitute into these various structural sites. In addition water and organic compounds may be found in the interlayer sites and/or adsorbed on the edges and exterior surfaces of the crystal structure. Zeolites have properties similar to clays, but form tubes or cage-like structures that can also incorporate a variety of molecules and ions. Over time, if the bentonite is deeply buried and subjected to an increase in temperature, or if hot volcanic water percolates through the ash layer, the smectites will alter to form illites or other minerals that are stable under the higher temperature conditions. The point here is that a bentonite usually does not consist of a single pure smectite, and each natural deposit is mineralogically variable.

Bentonite deposits are found worldwide wherever volcanic eruptions have taken place and preservation of the ash has exceeded erosion. Ten billion tons of bentonite are mined worldwide each year with about 35% produced in the western United States (primarily Wyoming). The second largest producer of bentonite is Greece (Grim and Güven, 1978), however, significant reserves are also found in many countries; Italy (Sardinia), India, China, and Australia to name a few. Many of the clays used in pelotherapy come from these deposits and localities (Cara et al., 2000; Veniale et al., 2007).

Clay Mineralogy

While natural clay samples contain a variety of minerals and organic compounds, *clay minerals* can often be isolated from the sample by selecting the finest size fraction (<0.2 µm). It is very difficult to remove silica from the finest clay fractions, but other detrital minerals (feldspars, micas, carbonates, etc) are usually concentrated in the coarser fractions (1–2 µm) (Jackson, 1979; Moore and Reynolds, 1997).

Clay minerals are *phyllosilicates* (*phyllo* is Greek for 'leaf' or layers) consisting of layers of silicates arranged in tetrahedral and octahedral sheets (Fig. 1). The clay silicate layers consist of sheets containing hexagonal rings of SiO_4 (silicate tetrahedra) stacked on octahedral sheets containing primarily Al, Mg, and/or Fe bound to two planes of closest packed oxygen atoms and/or hydroxyl groups. The edge sharing octahedra may be filled with two trivalent (di-octahedral) or three divalent (tri-octahedral) cations for a total charge of +6 (Giese and van Oss, 2002). This charge is partially balanced by the −2 charge on

oxygens, but the total charge balance depends on the structural arrangement and precise elemental substitutions within the silicate framework.

Kaolinite (the predominant mineral employed for making porcelain) and its polymorphs are 1:1 clay minerals i.e. they have layers consisting of one tetrahedral sheet and one octahedral sheet. Smectite is one example of 2:1 clay minerals with layers containing an octahedral sheet sandwiched between two tetrahedral sheets (Fig. 1). The stack of two tetrahedral sheets to one octahedral sheet, has an interlayer with water, cations, and molecules of highly variable chemical composition. Layers of the 2:1 clay minerals may be stacked in a variety of orientations and held together by electrostatic and van der Waals forces (Brindley and Brown, 1980;Moore and Reynolds, 1997). When trivalent ions (e.g., Al^{3+}) substitute for tetravalent Si^{4+} in the tetrahedral sheets, or divalent ions (Fe^{2+}) replace Al^{3+} in the octahedral sheets, the basal surface (bottom plane of the tetrahedral sheets) develops a net negative charge. The magnitude and distribution of this charge can vary depending on where the substitutions take place (Güven and Pallastro, 1992;Johnston, 1996). Smectites are the most common alteration product of bentonites, and many varieties of smectite are present in the antibacterial clays. Cations are attracted to the negative surface of the silicate sheets, whereas anions are attracted to the positively charged sites where bonding take place at the edges of the crystalline structure (Moore and Reynolds, 1997). If this region collapses (water is removed) due to a high surface charge attracting cations (primarily K^+) to interlayer sites, it forms an illite.

Chlorite is another group of layered silicates but with hydroxide sheets (e.g., $MgOH_3$) between the 2:1 silicate layers, and are known as 2:1:1 minerals. Giese and van Oss, (2002) present excellent diagrams of the crystallography of the major classes of clay minerals. Most natural clay samples contain mixtures of these four major groups of phyllosilicates; smectite, illite, kaolinite and chlorite. Table 1 summarizes the basic mineralogical classification of phyllosilicates. Within the 2:1 clays, the layer charge (interlayer cation occupancy) is used to distinguish the major clay sub-categories. Nonetheless, there are vast chemical substitutions and structural re-arrangements in clay minerals that result in the wide variety found in nature.

Crystal Growth and Chemical Variability

The growth of clay crystals and changes that may occur in their chemistry and structure is complex (see reviews in Altaner and Ylagan, 1997; Srodon, 1999). However, it has been shown for sedimentary environments that clay minerals precipitate as small particles (<10 nm) and grow in diameter over time as water provides a continuous supply of components or 'building blocks' to the structure (Eberl et al., 1998; Nadeau et al., 1984). The crystallite size distribution of clay minerals in most natural samples is log-normal, meaning there are more small crystals than large crystals (Eberl et al., 2002). This distribution has been shown to result from the stunted growth of a large number of nucleated crystals from which a limited number of the clay crystallites continue to grow. Clay crystals can grow in a variety of morphologies depending on the temperatures and pressures of their geologic environment. At low temperatures (~50°C,) the crystallites tend to be irregular flakes. As temperature increases, rectangular laths may be found, and finally hexagonal plates may form at >100°C (Inoue et al., 1988; Lanson and Champion, 1991). The crystallite growth usually incorporates other dissolved elements into the clay structure, therefore a change in the water chemistry over time may be recorded in different sized crystals of clay (Clauer et al., 1997; Srodon, 1999; Williams et al., 2006).

Medicinal Uses of Clays

Most uses and research emphasis on healing clays has focused on the physical characteristics of clay minerals that benefit digestion or protect and cleanse the skin (Carretero, 2002). The adsorptive and absorptive properties of clay minerals have historically been the driving force behind the traditional use of healing and therapeutic clays. Initially, negatively-charged interlayer sites of clays will absorb positively-charged substances to their extensive surface area. Over time, many clay minerals may absorb substances in between the stacked silicate layers of the mineral, allowing for expansion and swelling or contraction. While the physical adsorption of water and organic matter is the most common attribute of healing clays, the geochemical mechanisms controlling antibacterial properties of clays have received significantly less attention.

It is well known that metallic ions, such as silver, copper, and zinc, have strong inhibitory and bactericidal effects on a broad spectrum of bacteria (Berger et al., 1976; Domek et al., 1984; Gordon et al., 1994). Various forms of silver ions have been used to treat burn wound infections, osteomyelitis, urinary tract infections, and central venous catheter infections (Becker and Spadaro, 1978; Davenport and Keeley, 2005; Fox, 1968; Fox and Modak, 1974; Jansen et al., 1994; Liedberg and Lundeberg, 1990). In low concentrations (4 µg/ml), silver ions produced inhibitory and bactericidal effects with no obvious toxic effect on human blood cells (Berger et al., 1976). Although required by most living organisms at low concentrations, elevated levels of copper can inhibit the growth of some microorganisms and exhibit bactericidal activity (Domek et al., 1984; Gordon et al., 1994). The use of copper-coated products or copper alloys has been proposed for surfaces exposed to human contact to reduce the transmission of infectious microbial agents. Other metallic oxides, including zinc oxide, magnesium oxide, and calcium oxide, have antibacterial activity with demonstrated effectiveness against *E. coli* and *S. aureus* (Sawai, 2003). The nanometer particle size of these oxides, as well as titanium and silicon dioxide (Yamamoto et al., 2000; Yamamoto, 2001; Adams et al., 2006), have proven to be important for antibacterial activity. Zinc oxide has been used in a variety of dental composites to treat or prevent dental caries and as an endodontic sealer (Turkheim, 1955; Moorer and Genet, 1982; Siqueira and Goncalves, 1996). Nonetheless, the antibacterial mechanism has not been identified.

The high cation exchange capacity of different clay minerals has been targeted in the creation of inorganic antibacterial materials. Synthetic antibacterial clay minerals are prepared by exchanging their native ions with known antibacterial ions such Ag (Ohashi, 1992; Ohasi et al., 1998; Marini et al., 2007). The rational being that the novel exchanged ions will be gradually released from the synthetic clay for long-term antibacterial effectiveness. Thus far, silver-loaded clays have been pursued more aggressively than other antibacterial chemical ion options, although copper-loaded mineral substrates have been recently investigated (Gant et al., 2007).

The mineral group zeolites, also have vast adsorptive and absorptive capabilities through a rigid, porous, three-dimensional channel or tube in their basic cryalline structure. They are compositionally similar to clay minerals and also have a high cation exchange capacity (Baerlocher et al., 2007). With strong affinity for oxidized silver ions (Ag^+), up to 40% (w/w), silver-exchanged zeolites have demonstrated antibacterial effectiveness against aerobic and anaerobic Gram-negative and Gram-positive bacterial pathogens, including *Pseudomonas aeruginosa*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Staphylococcus aureus*, *Streptococcus mutans*, and *Streptococcus sanguis* and have been used in various dental applications (Hotta et al., 1998; Kawahara et al., 2000; Matsuura et al., 1997). Another study investigated the mechanism of action of silver-exchanged zeolite and determined that physical contact with the bacterial cell, silver transfer into the cell, and/

or the generation of reactive oxygen species attributed to the bactericidal activity of silver zeolite (Matsumura et al., 2003). However, while these inorganic products have antibacterial activity and low toxicity, issues related to the reduction of silver ions to elemental silver and subsequent loss of antibacterial effectiveness could be problematic (Li et al., 2002). Therefore, an oxidized form of Ag appears to be important for bactericidal activity of silver-exchanged zeolites.

Vermiculite is another clay mineral that has been targeted for medicinal uses. Vermiculite is a smectite-like mineral, but with a high layer charge, thus a high attraction for cations (Mathieson and Walker, 1954; Mathieson, 1958). As an alternative to silver, it is speculated that copper-loaded vermiculite could offer reduced costs, improved stability, and better antifungal activity (Li et al., 2002). As demonstrated by antimicrobial testing, a copper-exchanged vermiculite (containing 5.5% Cu^{2+}), as well as 200°C– and 400°C-heated copper-exchanged vermiculite, inhibited growth of *E. coli* (Li et al., 2002).

Natural Antibacterial Clays

Unlike the synthetic clays and chemical manipulations of clay minerals manufactured to kill certain types of environmental and clinical bacteria, we focus now on natural, untreated clay minerals that are effective at killing a broad spectrum of human pathogens. The detailed results have been published elsewhere (Haydel et al., 2008; Williams et al., 2008), but herein we outline the approach we use to evaluate the antibacterial activity of clays. Figure 2 schematically summarizes our approach to investigating the antibacterial properties of natural clays. We evaluated the effect of various clays and clay minerals on a broad spectrum of bacteria and determined whether the bactericidal effect was via a physical or chemical attack on the various pathogens. Again, our overarching goal is to understand how clay minerals kill bacteria in order to create new topical treatments for antibiotic-resistant skin infections and for large, necrotic skin ulcers. If we can identify the mechanism by which natural clay minerals kill a wide range of bacterial species, then it is possible that either natural clay deposits could be located and made available as an inexpensive treatment modality for topical infections or that materials with similar properties could be designed to provide a safe alternative to current antibiotics and antimicrobials.

Clinical Observations of Natural Clays Healing Patients with Buruli ulcer

Within the past decade, the incidence of Buruli ulcer has dramatically increased in several countries in sub-Saharan Africa, Australia, Asia, Mexico, and Peru, leading the WHO to declare this disease a global health threat in 2004 (WHO, 1998). Despite being the third most common mycobacterial disease in immunocompetent humans after tuberculosis and leprosy (Weir, 2002), Buruli ulcer, considered a "neglected tropical disease" by the WHO, does not garner the attention given to other infectious diseases. Although the exact prevalence and burden of the disease are difficult to determine, Buruli ulcer is endemic in central and western Africa with more than 40,000 Buruli ulcer cases recorded in the African countries of Ivory Coast, Ghana, and Benin from 1978 to 2006 and some villages reporting rates as high as 16–22% (Amofah et al., 1993; Marston et al., 1995; WHO, 2007). The WHO estimates that the incidence of Buruli ulcer will surpass that of leprosy and could become more problematic than tuberculosis in some African regions (van der Werf et al., 1999; WHO, 2007).

M. ulcerans is a slow-growing environmental mycobacterium with an unknown source or natural reservoir (Dobos et al., 1999). Human transmission is believed to occur via the skin by direct inoculation or an insect vector (Portaels and Meyers, 1999; Weir, 2002). Most individuals infected with *M. ulcerans* initially develop a small, painless, pre-ulcerative skin nodule or plaques with larger areas of indurated skin and edema (van der Werf et al., 1999).

As the disease progresses over 1–2 months, the infected skin will begin to ulcerate with characteristic necrosis of the subcutaneous fatty tissues, deeply undermined edges, and vascular blockage. These necrotic ulcers can lead to very extensive skin loss, damage to nerves, blood vessels, and appendages, and deformity and disability, particularly in children (van der Werf et al., 1999; Weir, 2002). One study reported that 26% of patients with healed Buruli ulcers suffered from chronic functional disability (Marston et al., 1995).

Currently, treatment of Buruli ulcer, depending on the size of the lesion, includes antibiotic therapy and/or surgical excision of the ulcerative lesion. Thirty years ago, variable successes in uncontrolled trials were demonstrated with heat treatment and hyperbaric oxygen treatment (Krieg et al., 1975; Meyers et al., 1974). However, the impracticality and costs of these treatments abrogate its usefulness in endemic and underprivileged populations. Phillips et al. (2004) used topical aqueous creams releasing nitrogen oxides to decrease the size of ulcers with minimal adverse side effects. Recently, Chauty et al. (2007) determined that a combination of rifampin (taken orally) and streptomycin (injected intramuscularly) successfully treated 47% of the cases and was more effective against small Buruli ulcer lesions (<5 cm in diameter). In addition, rifampin and streptomycin treatment converted early *M. ulcerans* lesions (nodules and plaques) from culture positive to culture negative (Etuaful et al., 2005). According to WHO guidelines (2004), combined use of streptomycin and rifampin is the recommended treatment of early Buruli ulcer lesions (nodules, papules, plaques, and ulcers less than 5 cm in diameter). For treatment of ulcerative lesions greater than 5 cm in diameter, combined antibiotic therapy for four weeks, followed by surgical excision of the lesion and another four weeks of antibiotic treatment is recommended (WHO, 2004). While surgery is a standard treatment for large ulcerative lesions, antibiotic therapy reduces the extent of surgical excision and infection recurrence (WHO, 2004). Since surgical treatment is often not available or practical in rural, endemic regions and possibly subjects patients to other infections, development of an effective and affordable drug treatment and new treatment modalities is a research priority for the control of Buruli ulcer (WHO, 2007).

The treatment of Buruli ulcer by Line Brunet de Courssou employed two clay samples provided by different suppliers of 'French green clay' (Brunet de Courrsou, 2002). These clays are thought to be altered volcanic ash deposits from Central France. The dry clay is mixed with water and applied as a paste directly to the ulcerated lesions and extended healthy skin of infected patients. The course of treatment is to remove and renew the clay packs at least once a day. Within days of initiating treatment with clay poultices, the therapeutic properties of the clay minerals were demonstrated, with the initiation of rapid, non-surgical debridement of the destroyed tissue. Extended treatment with the clay poultices resulted in continued debridement of the ulcer, regeneration of healthy tissue, and wound healing. After several months of daily clay applications, the Buruli ulcer wounds healed with soft, supple scarring, allowing return of normal motor function (Brunet de Courrsou, 2002; Williams et al., 2004). These observations are highly relevant since antibiotic treatment is only effective for small early lesions (nodules and plaques <5 cm in diameter) and has generally been unsuccessful as the sole treatment for larger, ulcerative forms of Buruli ulcer disease (Chauty et al., 2007; van der Werf et al., 1999).

Microbiology Introduction

The mechanism by which natural clay minerals kill bacteria should be understood in order to search for the least expensive cure modalities and specifically to maximize effective antibacterial agents. Therefore, we have been investigating the antibacterial properties of several different natural clays, including the two French clays used by Brunet de Courssou, referred to as CsAr02 and CsAg02. We have tested these clays on a series of Gram-negative,

Gram-positive, and mycobacterial pathogens using the technique of broth culture susceptibility testing. The cell envelopes of these bacteria greatly differ and these differences could influence their vulnerability and response to inorganic materials. Compositionally, the three groups of bacteria, are similar with a cytoplasmic membrane and a rigid polysaccharide-peptide cell wall called peptidoglycan (Fig. 3). However, the Gram-positive cell wall is considerably thicker than the Gram-negative cell wall. The thinner Gram-negative cell wall is covered by an outer membrane, composed of phospholipids and lipopolysaccharides, and has a periplasmic space between the cell membrane and the outer membrane (Fig. 3). Genetically, mycobacteria are Gram-positive bacteria. However, the mycobacterial cell envelope is more structurally similar to Gram-negative bacteria. In addition to having a thinner cell wall than traditional Gram-positive bacteria, the mycobacterial peptidoglycan layer is linked to a waxy lipid coating by an arabinogalactan layer. This outer waxy layer is predominantly composed of special lipids called mycolic acids, lipoarabinomannan, and glycolipids (Fig. 3). The different cell types have distinct cell envelopes that play a significant role in the effectiveness of various antimicrobial agents

Antimicrobial Agents

The use of antibiotics and chemotherapeutic agents during the past century represents one of the greatest advances in human health and has led to a remarkable reduction of morbidity and mortality related to bacterial infections. In modern medicine, antibacterial, antimicrobial, and chemotherapeutic agents are terms used to describe chemical agents effective at treating infectious diseases. Most of these agents are antibiotics, which by definition are low molecular weight byproducts of microorganisms that kill or inhibit the growth of other and susceptible microorganisms. The term 'antibiotic' is often incorrectly used to describe antibacterial or chemotherapeutic agents that are synthetically manufactured or modified by chemical processes, independent of microbial activity, to optimize their activity. Although the antibacterial clay minerals discussed herein are natural substances, they are not produced by microorganisms and are not considered antibiotics. In an ideal situation, antimicrobial agents disrupt microbial processes or structures that largely differ from those of the host. The majority of known antimicrobials function by affecting cell wall synthesis, inhibiting protein and nucleic acid synthesis, disrupting membrane structure and function, and inhibiting key enzymes essential for various microbial metabolic pathways.

Chemotherapeutic or antibacterial agents can be either bacteriostatic or bactericidal. A bacteriostatic agent reversibly inhibits microbial growth and microorganisms will resume growth upon removal of the bacteriostatic agent. Since bacteriostatic antimicrobials do not kill the bacteria, elimination of the infection is dependent on the host's resistance and immune response. When administered at sufficient levels, a bactericidal agent kills the targeted bacterial pathogen. However, it is important to realize that the effectiveness of the antimicrobial is largely dependent on the targeted bacterium. An antimicrobial agent that is bactericidal for one particular bacterial species may be bacteriostatic for another. Moreover, various antibacterial agents vary considerably in their range of effectiveness. A narrow-spectrum antibacterial is effective against a limited number of pathogens, usually Gram-positive or Gram-negative bacteria, but not both. A broad-spectrum antimicrobial is generally effective at destroying or inhibiting the growth of a wide range of Gram-positive and Gram-negative bacteria.

Effects of two natural clay minerals on bacterial growth

The extensive use of antibiotics has led to an increase in antibiotic resistance in many pathogenic and clinically-relevant bacteria, including *Mycobacterium tuberculosis*, *S.*

aureus, *Enterococcus faecalis*, and *Streptococcus pneumoniae* (Menichetti, 2005; Shah, 2005; Sharma et al., 2005; Zetola et al., 2005). Therefore, modern and innovative research approaches are needed to identify and generate new antimicrobials for treating infections that are resistant to existing antibiotics or for which there is no known effective therapeutic agent. Using antibiotic-sensitive and antibiotic-resistant bacterial strains obtained from the American Type Culture Collection (ATCC) and a local diagnostic laboratory, we have been investigating the antibacterial properties of the two clays, CsAg02 and CsAr02, used to treat Buruli ulcer patients. The CsAr02 mineral promoted or had no effect on bacterial growth (Haydel et al., 2008). In contrast, CsAg02 exhibits an extraordinary ability to kill pathogenic *E. coli*, *Salmonella enterica* serovar Typhimurium, *P. aeruginosa*, ESBL *E. coli* (which is resistant to 11 antibiotics), and *Mycobacterium marinum* (a species genetically closely related to *M. ulcerans* that also causes a cutaneous infection) as well as inhibit the growth of pathogenic *S. aureus*, penicillin-resistant *S. aureus* (PRSA), methicillin-resistant *S. aureus* (MRSA; and also resistant to 10 antibiotics) (Haydel et al., 2008). During the course of her observations, Brunet de Courssou suggested that the CsAg02 clay was not as effective in killing *M. ulcerans* as the CsAr02 clay (although this was not demonstrated microbiologically), but was more suited for promoting skin granulation after the mycobacteria were killed (Brunet de Courrsou, 2002). The scientific basis of the therapeutic differences or the healing characteristics of these two clay minerals is still under investigation.

Broad-spectrum *in vitro* Susceptibility Testing

In vitro broad-spectrum antimicrobial activities of clay minerals were tested against bacterial strains that are recommended by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) as quality control strains for laboratory testing of antimicrobials (NCCLS, 2004). Bacteria are grown overnight in common laboratory liquid media and diluted with fresh medium to achieve an approximate initial density of ~10^7 bacteria per ml. Adjustment of each bacterial inoculum is performed using a spectrophotometer since the experimental microorganisms exhibit varying replication times in liquid media. To confirm the initial bacterial counts, serially-diluted bacterial cultures are plated on appropriate agar plates and colony-forming units are counted after incubation at 37°C. Before use in any susceptibility testing, all clay mineral samples are sterilized by autoclaving at 121°C (at 15 psi) for 1 h to assure removal of airborne, inherent, or contaminating microbes. To achieve a consistency similar to the hydrated clay poultices used to treat Buruli ulcer patients, 200 mg of clay minerals is added to 400 µl of the initial inoculum of bacteria in the appropriate growth medium. After the addition of the clay minerals, the bacteria - clay mineral mixtures are incubated in capped test tubes at 37°C in ambient air for 24 hours (NCCLS, 2004) with constant rotary agitation to ensure contact with the clay minerals and to prevent sedimentation. Positive controls for growth of bacteria in the absence of clay minerals are included in each series of independent experiments. To ensure that the clay samples were sterilized after autoclaving and maintained sterilization during storage, negative control growth experiments with clay minerals in liquid media are performed several times throughout the course of the study. These conditions revealed complete killing of *E. coli*, *S. typhimurium*, *P. aeruginosa*, and *M. marinum* by CsAg02 (Haydel et al., 2008).

Given that the clays were hydrated with water for treating Buruli ulcer lesions and are generally hydrated with water for therapeutic use, we have initiated "use-derived" antibacterial testing of the clay minerals whereby the clay and the bacteria are incubated together in a sterile, deionized water solution. After growth in a liquid medium, bacteria are washed twice and suspended (~10^7 CFU) in 1 ml of sterile deionized water before the addition of clay minerals and subsequent incubation at 37°C. Depending on the type of clay, poultice consistencies used for therapeutic purposes are generally ratios of 1:2 or 1:3

(clay:water). To determine the effect of quantity, various amounts of the clays can be added to one ml aliquots of the aqueous bacterial suspension. Bacteria-clay mineral suspensions are incubated rotating at 37°C for 24 hours at which time serial dilutions of all samples are plated to determine bacterial viability. Minimal bactericidal concentration (MBC) is defined as the lowest concentration of a particular antibacterial agent that kills ≥99.9% of the bacterial population in a liquid medium.

Mineralogical and Geochemical Assessment of Antibacterial Clays

Initial testing of the two French green clays (in 2003) showed that only one of the clays (CsAg02) killed a nonpathogenic, laboratory strain of *E. coli* even though the two clays, CsAg02 and CsAr02, were very similar in general mineralogy with the dominant portion of the sample consisting of clay minerals as set out in Table 2 along with quartz, calcite, and feldspar (Williams et al., 2004). Subsquent testing of CsAg02 and CsAr02 against a pathogenic strain of *E. coli* revealed similar results (Haydel et al., 2008) and their mineralogical composition was very similar. The clay fraction was dominated by smectite, a group of expandable clay minerals, a common component of all of the antibacterial clays we have investigated. However, a substantial amount of illite is also present in the French clays. Notably, the illite-smectite crystals in the French clays are of extremely small size relative to natural illite and smectite reference materials from the Clay Minerals Repository (www.clays.org) at Purdue University (Fig. 4).

Size Fractionation of Clay Minerals

In order to eliminate the possibility that other mineral phases were responsible for killing the bacteria, we separated sequentially smaller size fractions of the mineral mixture using centrifugation (Jackson, 1979). Evaluating the antibacterial effect of the coarse (1.0 –2.0 µm), medium (0.2–1.0 µm) and fine (<0.2 µm) size fractions independently against *E. coli* allowed us to eliminate detrital minerals (quartz, carbonate, feldspar) from the clays as potential participants in the antibacterial effect. We found that only the finest fraction (<0.2 µm) was effective against *E. coli*, while the coarser fractions had no effect on bacterial growth (Haydel et al., 2008; Williams et al., 2007). Furthermore, X-ray diffraction analyses of the finest clay fraction confirmed that the coarser detrital mineral phases had been largely eliminated. Smectite dominated the <0.2 µm fraction. Figure 5 shows two different morphologies of crystals in the <0.2 µm fractions of the French clays. The antibacterial clay has crystals as small as 20 nm, and this enormous relative surface area, controls the water chemistry when wet.

Others have shown that particle size effects antibacterial activity for various oxides (e.g., ZnO, MgO), such that bactericidal activity increases with decreasing particle size (to 0.1 µm) (Yamamoto, 2001; Sawai, 2003). Adams et al. (2006) showed that nanoparticles of TiO, SiO, and ZnO are photosensitive, with the light promoting formation of reactive oxygen species. Further evaluation is needed to determine the potential role of oxides associated with the clay mineral surfaces in chemical reactions that could influence the survival of pathogens. One way to do this is to selectively chelate metal oxides out of the sample and evaluate the resulting antibacterial effect, or to remove hydroxyl and superoxide radicals before testing. Mossbaüer spectroscopy is another useful tool for evaluating oxide versus clay mineral–metal bonds in clay samples (Stucki et al., 1996).

Next, we need to determine if the fine clay fraction was killing *E. coli* by a physical or chemical effect. Were the clays physically impeding a metabolic process of the bacteria by surface attractive forces causing the clay to wrap around, penetrate, or otherwise destroy the cell walls? Or, was the clay producing a toxic chemical that precipitates on the cell walls or that enters the bacterial cell inducing cellular death? By inserting a dialysis tube filled with

an antibacterial clay suspension into a bacterial population in liquid growth media, Metge et al. (2007) showed that the bacteria were killed without physically contacting the clay mineral surfaces. Given this observation, we leached the antibacterial clay with distilled, deionized water. Two grams of the antibacterial clay was ultrasonified (using a Braun microtip ultrasonic disaggregator) in 40 ml water, then shaken for 24 hrs to equilibrate. The clays were removed from suspension by centrifuging for 3 hrs at 20,000 rpm (SS-34 rotor). The leachate was then tested against bacteria. Compared to control samples of *E. coli* in distilled water, the clay leachate also killed *E. coli*. This evidence suggested that the antibacterial clay killed by providing a toxin. Nonetheless, after water leaching, the clay sample continued to kill *E. coli*. In fact, separation of the different size fractions of the clay also required multiple washings and centrifugation cycles in deionized water, which did not eliminate the bactericidal effects of the minerals. These results indicate that the antibacterial agent is not highly soluble.

Cation Exchange of Clay Minerals

The next test was to evaluate if the antibacterial agent(s) could be removed by cation exchange. Ions and molecules can be bound to the interlayer surface of the expandable clays or adsorbed on the edges. Cation exchange is performed by soaking the clay fraction of interest in a concentrated (1M) solution containing 'preferred cations' or ions with the best fit in ionic radius and charge for the interlayer exchange sites (Jackson, 1979). One can remove any chemical species that are not tightly bonded (fixed) in the clay structure. This is the commonly used procedure to determine the 'cation exchange capacity' of clay (Moore and Reynolds, 1997), but the process also removes ionic or molecular species that might be involved in the antibacterial mechanism. After cation exchange, we found that the smectite-rich clay samples (<0.2 µm fraction) no longer killed *E. coli* (Haydel et al., 2008), indicating that the antibacterial agent is linked to ions that are presumably in the exchangeable sites of the clay. However, the cation exchange will also affect the surface energy of the clay mineral and may affect the pH, of the clay surface (zero point of charge), so we attempted to evaluate these complicating factors.

Heating experiments

One common way to remove interlayer water and hydroxyl groups from clays is by progressive heating. In general, the clay becomes dehydrated, as the interlayer water is removed, by heating to 200°C. At 550°C or more (depending on chemical bonding), most iron-rich smectites dehydroxylate, meaning that all hydroxyl bonds in the octahedral layer are broken (Heller-Kallai and Rozenson, 1980). However, it is important to note that the temperature for dehydroxylation varies depending on the clay mineral structure and composition (Bish and Duffy, 1990). Upon heating to 900°C, the clay mineral structure is effectively destroyed, leaving only the oxide components.

Progressive heat treatments were applied to the French antibacterial clay in order to eliminate some of the possible bactericidal elements from consideration. Heating to 200°C (24 hrs in air) removes volatile elements in addition to water, (including H, O, N, F, Cl, Br, I). Although it is important to consider how these elements are bound in the mineral structure, certainly their presence in the hydrated interlayer would be affected by temperatures leading to volatilization. Tests of the clay after heating to 200°C show that it still killed *E. coli* (Williams et al., 2008; Haydel et al., 2008). This heating step provides evidence that the antibacterial agent in the clay is not an inherent microorganism, as they would certainly be killed at this temperature, even if the clay had previously physically protected them.

Heating to 550°C (24 hrs in air) showed significant oxidation of the French antibacterial clay as it turned from green to orange-red due to oxidation of Fe in the octahedral sites of the clay (Williams et al., 2008). However, the clay still killed *E. coli* (Haydel et al., 2008). This high temperature would be expected to volatilize elements including S, P, and Hg if they were not tightly bound in the phyllosilicate structure. Furthermore, this step of the process verifies that the antibacterial agent is not an organic compound as they are certainly eliminated at this high temperature.

Heating to 900°C highly oxidized the French antibacterial clay (CsAg02) turning it a deep red and caused the clay to lose its antibacterial effectiveness (Williams et al., 2008; Haydel et al., 2008). The lost antibacterial effect could point to elements lost above 550°C as the active antibacterial agents, however the highly oxidized state may alter the toxicity of remaining elements, or recrystallization may change the availability of elements that were bactericidal before heating. The major oxides of both clay samples including Si, Al, Ca, Fe, Mg, Mn, and Ti remain, but the clays lose their coherent crystalline structure. This temperature leaves many refractory elements in oxidized form, but those lost between 550°C and 900°C include Na, K, As, Se, Rb, Cd, and Cs. Table 3 compares the abundances of the refractory elements in the two French clays before heating.

The conclusion from this heating analysis is that the more volatile elements or compounds are not necessary for the antibacterial action displayed by this particular clay. The experiments do not conclusively identify a toxic substance, but are a method for eliminating some of the variables from consideration.

Chemical Speciation

All of the tests and chemical manipulations of the French antibacterial clay (CsAg02) leave elemental components in abundances that are well below the minimal inhibitory concentrations (MIC) reported to be toxic to *E. coli* (Dopson et al., 2003; Nies, 1999; Wackett et al., 2004) and other bacteria tested (Haydel et al., 2008). However, MICs are usually tested at a neutral or near neutral pH, which does not attest to the fact that the pH and oxidation state of the metals must also be considered. Metal speciation is critical to their bioavailability and the subsequent interaction with bacteria (Reeder and Schoonen, 2006). Therefore, in the future we aim to establish what chemical species are soluble under the pH and oxidation state buffered by the clay mineral surfaces in order to evaluate the element mobility of antibacterial clays (Tateo and Summa, 2006). The whole process of transferring elements from a clay surface through water to a cell membrane involves numerous chemical reactions and variables that can be affected not only by the source clay and water chemistry, but also by the surface complexation of chemical species on the bacteria (Barrok et al., 2005).

Additional Considerations

Cellular processes important for metabolism, nutrient transport, movement, and cell division are localized at the cell membrane, which is the reactive surface controlling chemical accommodation, and may vary with environment (Konhauser, 2007; Lalonde et al., 2008 a,b). The ability of a given bacterial species to modify its surface chemistry in order to adapt to various environmental stresses depends on the growth phase, regulatory networks, metabolic pathways, and chemical variables in the environment (Warren and Ferris, 1998; Lalonde et al., 2008 a,b). There are three general mechanisms by which bacteria can accommodate high concentrations of ions that may be toxic to the species (*i*) the ions may be expelled from the cell by efflux (Nies and Silver, 1995); (*ii*) the metal ions may complex into non-toxic molecules such as thiols (S-compounds) in the surrounding solution, or (*iii*) the metal ions may be reduced to a less toxic oxidation state in the cell (Nies, 1999).

Unwanted reduced metal ions usually are eliminated from cells by an efflux system. However by application of antibacterial clay or its soluble compounds to the bacteria, there is the potential for precipitation of compounds that would inhibit efflux of toxins from bacteria. A geochemically-induced gradient in pH or oxidation state, imposed by the presence of the antibacterial clay mineral could clearly be damaging to the functions of even the most adaptive bacteria. Reactive oxygen species are another frontier for investigation of the possible inhibitors active against bacteria. Fenton-mediated reactions, for example, drive the oxidation of mineral-bound Fe^{2+} to generate hydroxyl radicals (Fenton, 1894; Schoonen et al., 2006; Cohn et al., 2006) that can damage cells. Furthermore, during active infections, it is important to consider the complexity of host-pathogen interactions, largely influenced by *in vivo* chemistry, in addition to the chemical interactions identified in vitro (Sahai and Schoonen, 2006). If we can integrate analytical protocols in the study of bacterial metabolism, adaptation, and pathogenesis with methods of mineral and chemical manipulation and characterization (see reviews in Banfield and Nealson, 1997; Reeder and Schoonen, 2006), the forward motion of identifying new antibacterial agents or processes will be much better served.

Concluding Remarks

In this era when bacteria are developing antibiotic resistance to existing pharmacological agents, the potential for discovery of new broad-spectrum antibacterial agents, such as natural clay minerals, to combat pathogenic bacteria would be particularly advantageous. Analysis of the chemical interaction occurring at the clay mineral – bacterial interface is being explored and will be pertinent in understanding the mechanism by which the clay minerals can inhibit bacterial growth. Initial investigations indicate that particular natural clay minerals can have striking and very specific effects on microbial populations. These effects can range from enhanced microbial growth to complete growth inhibition, and these opposite effects can occur with clay minerals of similar structure and bulk crystal chemistries. The key antibacterial agent is likely a trace element or transition metal groups stabilized by the ability of particular clay minerals to buffer the aqueous speciation of those elements involved in the antibacterial process.

During the past 25 years, approximately 70% of newly discovered drugs introduced in the U.S. have been derived from natural products (Newman and Cragg, 2007). Topical treatments by clay minerals have considerable advantages over surgery or generalized antibiotic therapy due to the practical simplicity of the application in the area specifically affected, rather than ingestion of drugs with potential side effects. The broad-reaching impacts of antimicrobial mineral research with applications in topical antimicrobial dressings, wound care management, personal care, and animal care markets are obvious. The discovery that natural minerals harbor antibacterial properties should provide impetus for exploring terrestrial sources for novel therapeutic compounds. Often natural products, such as clays, which are heterogeneous in chemical composition and physical character, are rejected as therapeutic agents by regulatory agencies. Nevertheless, in comparison to organic antimicrobial agents, inorganic minerals are likely to be considerably more stable and heat resistant, making the development and use of inorganic antimicrobial agents particularly advantageous. Combining the availability of natural, potentially bioactive, resources with powerful combinatorial chemistry optimization methodologies could result in the development of new antibacterial agents to fight existing antibiotic-resistant infections and diseases for which there are no known therapeutic agents, such as advanced *M. ulcerans* infections.

Clearly understanding the antibacterial mechanism of natural clay is complex, and it is possible that no single mechanism or set of reaction pathways is uniquely responsible for the

observed bactericidal activity. Our future work will focus on identifying general themes displayed by the interaction presented herein between problematical human pathogens (e.g., MRSA) and the natural clay minerals that have now been shown to kill such bacteria (Williams et al., 2008). To progress towards understanding how antibacterial clays can be effective for treating bacterial infections will require integrated mineralogical, chemical, and microbial studies

The work of Konhauser (2007) and his co-workers describe novel methods for studying environmental bacteria and their chemical interactions with minerals in sedimentary environments. Similar methods must be employed in clinical microbiological research on antibacterial minerals. For example, the forces of attraction between bacterial species and mineral surfaces (Bank and Giese, 2007), the formation of membrane vesicles and other mechanisms for accommodating environmental stresses (Mashburn-Warren et al., 2008), the response of various bacteria to oxidative and reductive variables, in addition to pH, should be evaluated. This new focus in medical geomicrobiology should grow exponentially, just as environmental microbiology has developed exponentially over the last three decades (Konhauser, 2007). Although the use of clays in human health has been promoted empirically and traditionally, perhaps since the beginning of mankind, our knowledge of natural mineral impacts on human pathogens is in its infancy. New technology for determining physical and chemical interactions in situ on the nano-scale will provide the keys to opening these doors (Skinner, 1997).

Acknowledgments

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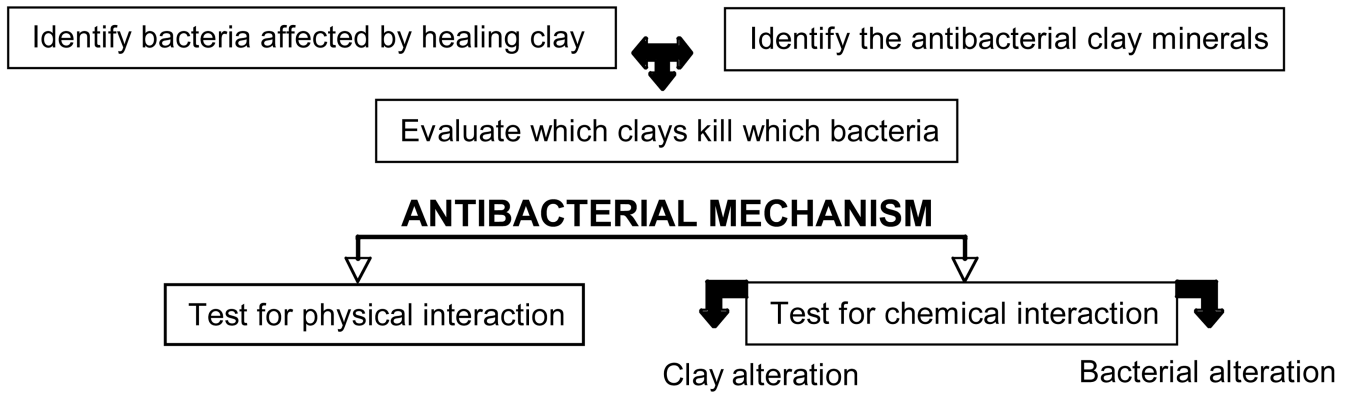Sayetta RB. Pica: an overview. American Family Physician 1986;v. 33:181–185. [PubMed: 3486579]

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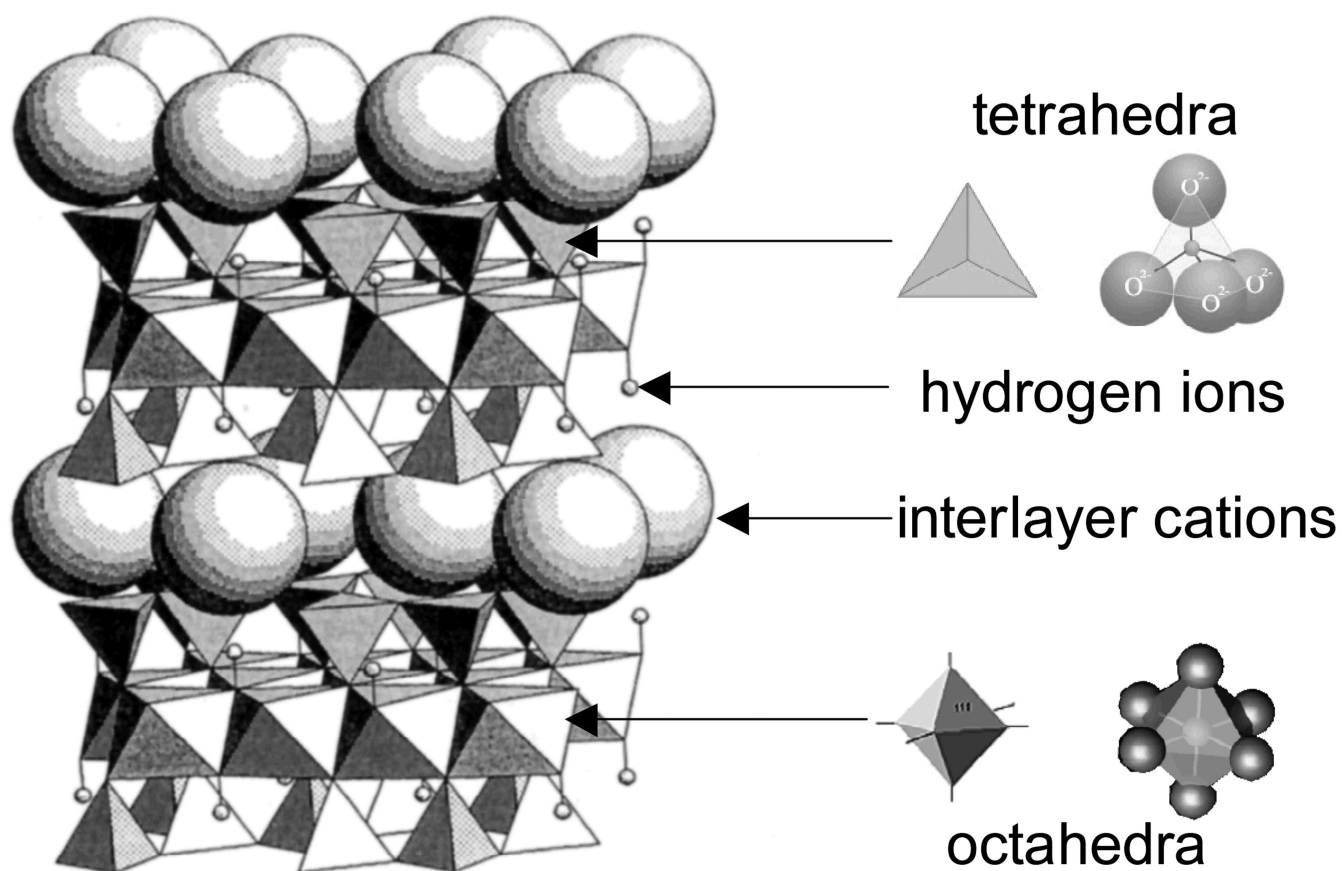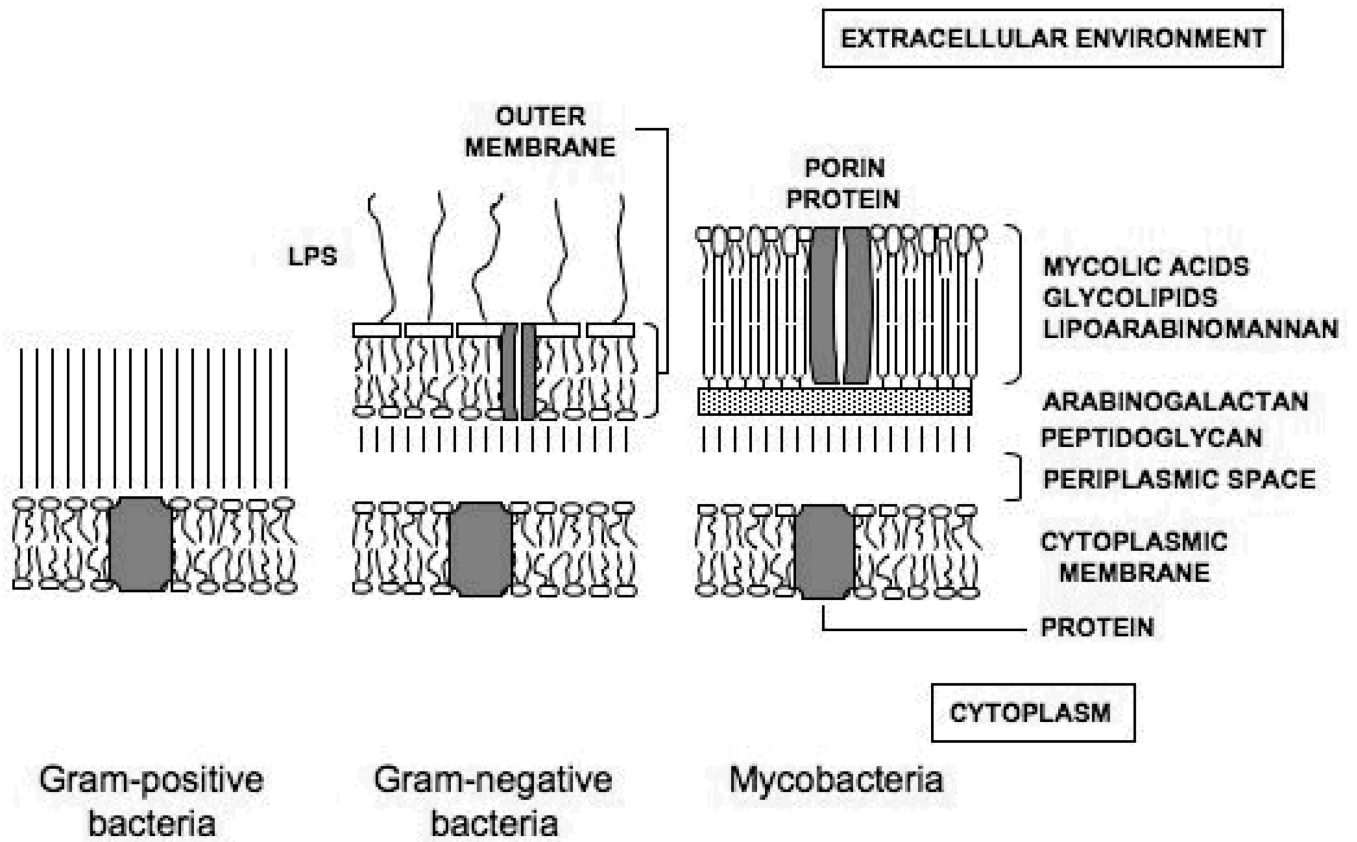
Figure 1.
Schematic representation of the basic structure of a clay mineral, showing tetrahedral and
octahedral arrangements of atoms in sheets, separated by cations (large spheres) and water
in the interlayer between the silicate sheets. Small spheres are H^+. (modified from Giese and
van Oss, 2004)

ANTIBACTERIAL EFFECTIVENESS

Identify bacteria affected by healing clay   Identify the antibacterial clay minerals

Evaluate which clays kill which bacteria

ANTIBACTERIAL MECHANISM

Test for physical interaction   Test for chemical interaction

Clay alteration   Bacterial alteration

Figure 2.
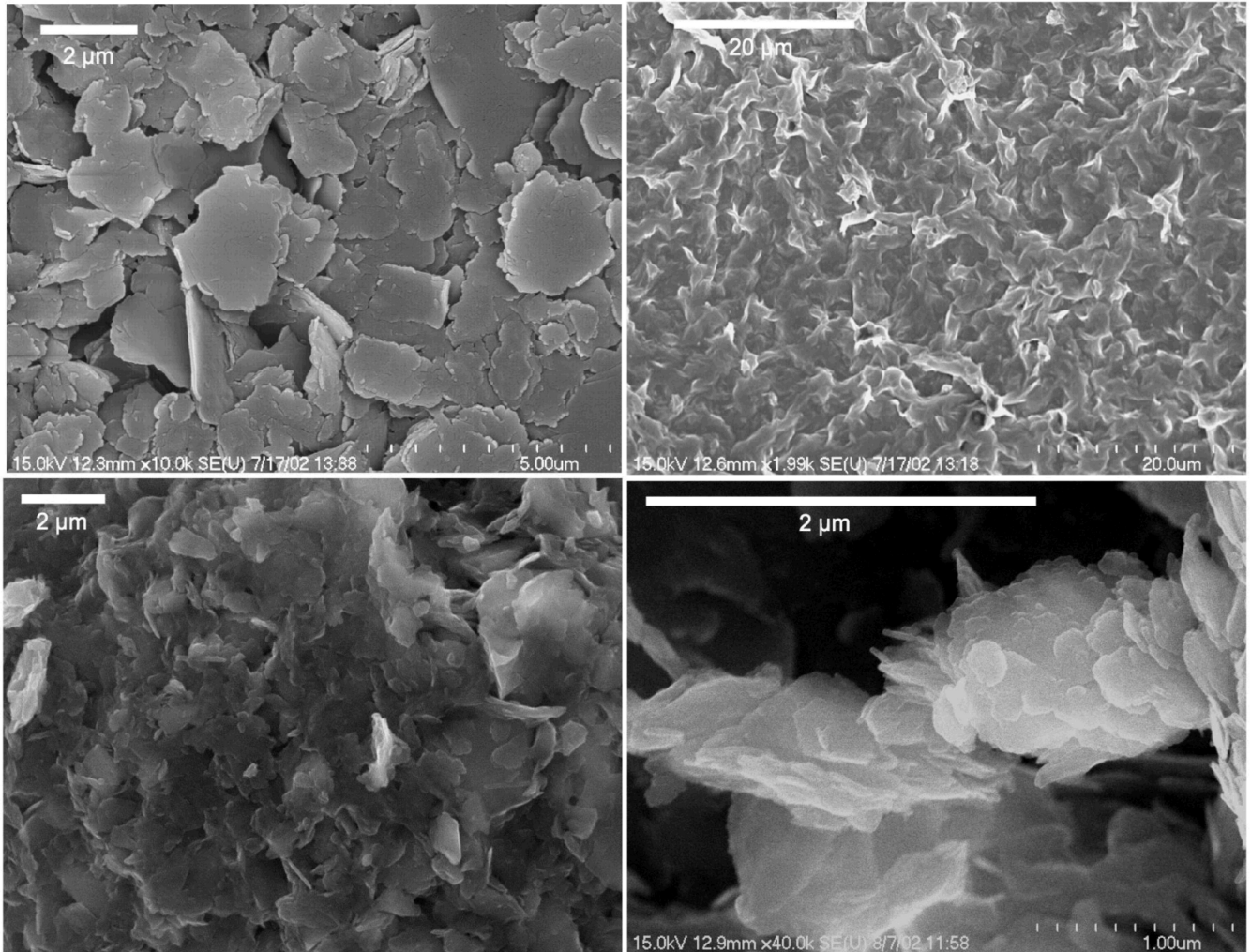General scheme for evaluating and testing antibacterial clays.

Figure 3.
Schematic representation of the cell envelopes of Gram-positive bacteria, Gram-negative bacteria, and mycobacteria. LPS, lipopolysaccharide. SOURCE?
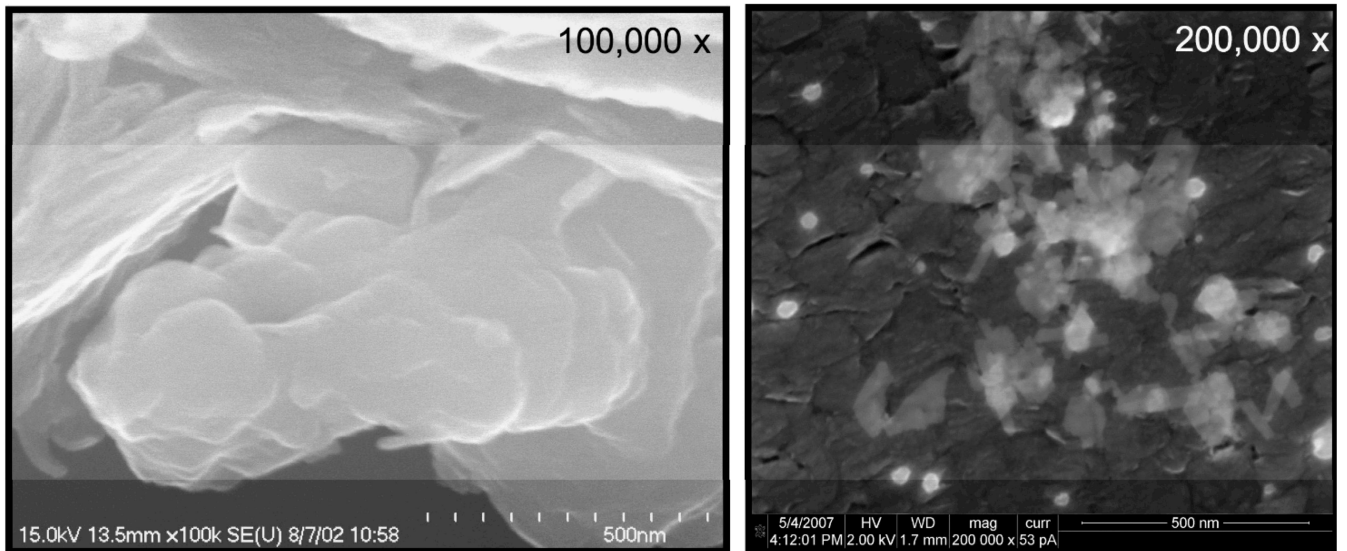
illite (IMt-1)

smectite (SWy-1)

CsAr02

CsAg02

Figure 4.
Comparison of illite and smectite textures and crystal size. The standard reference materials (top) are much coarser than the French clays (containing both illite and smectite). The very small crystal size may play a role in the antibacterial mechanisms of clays (SEM images by Lynda Williams).

Figure 5.
The antibacterial French clay (CsAg02) has two clay crystal morphologies; 200nm diameter hexagonal plates (left sample analyzed by SEM, gold coated by L. Williams) and 20nm × 40nm rectangular laths (right sample uncoated on carbon planchet; by L. Garvie). The uncoated sample allows imaging of the very finest crystallites in the clay.

Table 1

Basic classification of phyllosilicate minerals based on the layer type and charge (electro static units). Compiled from Brindley and Brown, 1980; Moore and Reynolds, 1997; Giese and van Oss, 2002).

Layer Type	Charge (esu)	Di-octahedral	Tri-octahedral
1:1	~0	kaolinite halloysite	serpentine minerals (antigorite, crysotile, lizardite)
2:1	~0	pyrophyllite	talc
	0.2–0.6	smectites	smectites
	tetrahedral charge	beidellite	saponite
	octahdral charge	montmorillonite	hectorite
	0.6–0.9	illite	vermiculites
	~1.0	true	micas
	~2.0	brittle	micas
2:1:1			
	variable (hydroxide)		chlorites

Table 2

Comparison of the mineralogy of the two French clays.

Sample name:	CsAr02		CsAg02
Mineral	**Weight %**	**Mineral**	**Weight %**
NON-CLAYS		NON-CLAYS	
Quartz	18.3	Quartz	2.7
Calcite	15.9	Calcite	3
intermediate Microcline feldspar	3.0	intermediate Microcline feldspar	3.9
Orthoclase feldspar	1.4	Orthoclase feldspar	3.2
Albite feldspar (Cleavelandite)	0	Albite feldspar (Cleavelandite)	1.8
Total non-clays	38.6	Total non-clays	14.6
CLAYS		CLAYS	
1Md illite (+ dioct mica & smectite)	23.1	1Md illite (+ dioct mica & smectite)	24.5
Ferruginous smectite	14.5	Ferruginous smectite	32.6
1M Illite (R>2; 88%I)	10.6	1M Illite (R>2; 88%I)	15.6
Chlorite	4.2		
Mg-Chlorite (clinochlore)	1.9	Phlogopite (2M1)	3.7
Muscovite (2M1)	6.1		
Total clays	60.3	Total clays	76.4
TOTAL	98.9	TOTAL	91

Table 3

Comparison of refractory elements in the antibacterial and non-antibacterial French clay samples. Highlighted elements are those removed by heating above 550°C.

Element	CsAg02 ppm	CsAr02 ppm
23Na	10800	1290
24Mg	13750	10100
39K	24000	3110
55Mn	405	203
56Fe	44750	56300
63Cu	31.3	23.7
66Zn	160	104
69Ga	30.5	23.5
72Ge	1.8	2.6
75As	43.3	5.5
77Se	1.0	0.1
85Rb	53	43
88Sr	198	145
89Y	13.5	5.7
90Zr	53.8	85.8
93Nb	11.5	8.7
95Mo	0.8	0.4
109Ag	1.3	1.1
111Cd	0.2	0.3
118Sn	8.3	3.2
123Sb	2.3	0.7
133Cs	13.3	5.2
138Ba	100	226
139La	16.3	8.4
140Ce	43.0	19.8
141Pr	5.0	2.2
146Nd	18.5	8.3
147Sm	4.0	1.9
153Eu	0.8	0.5
157Gd	4.0	1.9
159Tb	0.8	0.3
163Dy	3.3	1.6
165Ho	0.5	0.3
166Er	1.8	1.1
169Tm	0.3	0.2
172Yb	1.5	1.1
178Hf	2.0	3.2
181Ta	2.5	1.1

Element	CsAg02 ppm	CsAr02 ppm
182W	3.3	1.4
197Au	1.3	0.1
205Tl	1.3	0.8
208Pb	32.8	18.0
209Bi	1.3	0.1
232Th	13.5	4.2
238U	9.0	3.2