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Experimental evidence that clay inhibits bacterial decomposers, with implications for the preservation of organic fossils

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ABSTRACT

Exceptionally preserved organic fossils are commonly associated with clay-rich horizons or directly with clay minerals. It has been posited that interactions between clay minerals and organic tissues inhibit enzymatic reactions or protect carcasses in such a way that decay is inhibited. However, interactions between clay minerals and the biological agents of decay, especially bacteria, may be at least as important to preservation potential. Here we show that clays of particle size < 2 μm in suspensions exceeding 10 mg/ml in concentration inhibit the growth of *Pseudoalteromonas luteoviolacea*, a marine heterotrophic bacterium involved in the decay of marine animals. Such clay–microbe interactions can contribute to exceptional preservation and specific examples may play a role in shaping the distribution of Konservat-Lagerstätten through time.

INTRODUCTION

Exceptionally preserved fossil assemblages (Konservat-Lagerstätten *sensu* Seilacher, 1970) that yield the remains of soft tissues constitute an invaluable source of information about the history of life on Earth. In diverse lagerstätten of all ages, organic remains are associated with aluminosilicate clay minerals (e.g., Butterfield, 1994; Anderson et al. 2011; Laflamme et al., 2011; Cai et al., 2012; Pan et al., 2014; Wacey et al., 2014). A large and paleobiologically important subset of these lagerstätten exhibit Burgess-Shale type (BST) preservation, in which organic material is retained and flattened parallel to bedding in fine-grained siliciclastic lithologies often with high clay-to-organic ratios (Butterfield, 1990; Butterfield et al., 1994, Butterfield, 1995; Gaines, 2014). Indeed this is the principal mode of preservation for eukaryotic remains in Proterozoic successions (e.g. Cohen and Macdonald, 2015; Yuan et al., 2011; Butterfield et al., 1994), as well as soft-bodied Cambrian marine metazoan faunas (e.g., Butterfield, 1990; Gaines et al., 2008; Gaines 2014). Many workers have argued that temporal and environmental distributions of clay minerals impart patterns and biases to the fossil record (e.g. Butterfield, 1995). Butterfield (1990) attributed the preservation of organic material to the effect of clays in adsorbing and deactivating autolytic enzymes, Orr et al. (1998) proposed that early diagenetic clays templated original organic tissues, and Petrovich (2001) suggested that Fe^{2+} ions stabilized the tissues and acted as nucleation sites for clay minerals. Wilson and Butterfield (2014) proposed that Al^{3+} ions derived from a clay-rich substrate protected the tissues from decay in a process analogous to tanning and speculated that Fe-rich clays could have a similar effect. Independent of the association

of clays and fossils, there is abundant evidence that clay minerals interfere with bacterial growth (e.g. Wong et al., 2004; Williams et al., 2011; Morrison et al., 2016).

We tested the hypothesis that clay mineral particles inhibit the growth of bacterial decomposers by comparing the growth of *Pseudoalteromonas luteoviolacea* (a heterotrophic marine γ-proteobacterium) in the presence of various clay minerals, calcite, and in a mineral-free control. Bacteria of this widespread genus are commonly found in biofilms associated with live marine animals, have the capacity to degrade a range of tissue types, and constitute a large proportion of the bacterial assemblage in submerged carcasses (Skovhus et al., 2007; Raff et al., 2008; Dickson et al., 2011). The strain used here was shown to decompose sea urchin embryos *in vitro* (Raff et al., 2013).

MATERIALS AND METHODS

Five minerals were used in the experiments: berthierine (Scarborough, UK; Yale Peabody Museum (YPM) MIN 100586), calcite (Budapest, Hungary; YPM MIN 056322), glauconite (Odessa, Delaware; YPM MIN 043086), illite (Silver Hill, Montana; Clay Minerals Society #IMt-2), and kaolinite (Santa Cruz Biotechnology Company, USA). Specimens were rinsed in distilled water, ground with an agate mill and sonicated to obtain fine powders, which were then rinsed again by centrifuging in distilled water at 2773 × g (4000 rpm) for 15 minutes, three times. Following 10 minutes of sonication to disaggregate particles, grains of up to two microns in diameter were obtained by centrifuging in distilled water at 97 × g (750 rpm) for 5 minutes, discarding the pellet and retaining the supernatant, which was removed and dried at 45 °C. This ensured that all minerals were similar in particle size to natural mud and to each other.

Bacterial growth medium (Difco Marine Broth [MB]) was prepared in sterile conditions, filtered to remove particles >20 μm, and added in aliquots of 950 μl to glass culture vials (8 ml; Wheaton/Fisher Scientific) to which the clays were added immediately prior to autoclaving. Additional sonication was employed to disaggregate mineral particles prior to inoculation. Vials were prepared with suspended mineral concentrations of 5, 10 and 25 mg/ml (Fig. 1) for each of the five mineral species and for a control with no suspended mineral particles.

The inoculum was taken from a stock culture on agar plates of *Pseudoalteromonas luteoviolacea* (ATCC33492) and grown in MB to an optical density (OD_{600}) of ~0.5 (above sterile MB). Vortex-mixed aliquots of 50 μl were transferred under sterile conditions to each of the clay-bearing vials, which were incubated on a rotary shaker at 25 °C. The use of continuously agitated suspensions rather than settled sediment ensured thorough and homogeneous mixing of mineral particles and bacteria. Preliminary results suggested that the presence of mineral particles interfered with normal techniques for measuring bacterial growth. We therefore subsampled the experimental cultures (post vortexing) after 24 hours' growth and diluted them in fresh medium so that both clay particles and bacteria were 100 times less concentrated. The bacteria multiplied in the fresh medium so that geometric differences in population size were inherited from the original cultures, while clay concentration remained minimal. Turbidity at 600 nm (a standard proxy for bacterial cell density) was measured using a spectrophotometer (Hach DR/2010) in mid-exponential phase (after six hours of growth), at which time the disparity in population sizes was clearly evident. Initial values were subtracted so that any turbidity due to clay was excluded. All experiments were performed in quadruplicate.

92 Statistical analyses were performed using the R programming language (R Core
93 Team, 2016). For each mineral concentration turbidity was normalized by the mean of
94 the clay-free control treatment. Using the control-standardized data, two-way analyses of
95 variance (ANOVA, Type III Sum of Squares) were performed on turbidity levels in the
96 'car' R package (Fox and Weisberg, 2011), with mineral concentration and mineral
97 species as factors. Planned post-hoc comparisons were performed in the phia R package
98 (De Rosario-Martinez, 2013) using the Holm (1979) correction for multiple comparisons.
99 Such pair-wise comparisons were made to test for statistical differences between factor
100 levels (e.g., kaolinite versus control) and were made independently both within and
101 across the three mineral concentrations. Effect sizes were calculated using the etaSquared
102 function in the lsr R package (Navarro, 2015), which reveals the proportion of the
103 variance in the dependent variable that is attributable to the factor in question. We also
104 examined the effect of mineral species and mineral concentration on bacterial growth by
105 considering the four clay minerals (berthierine, glauconite, illite, and kaolinite) as a
106 single category. The above analyses were repeated using this binned dataset. All data
107 were normally distributed, homoscedastic, and showed linear structure based on Q-Q,
108 scale-location, and residuals vs. fitted values plots, respectively.
109
110 **RESULTS**
111
112 A two-factor analysis of variance showed significant main effects of mineral
113 concentration, $F(2, 53) = 58.3$, $p < 0.05$ ($\eta^2 = 0.40$) and mineral species, $F(5, 53) = 2.9$, p
114 < 0.05 ($\eta^2 = 0.29$) on turbidity, but these effects were qualified by an interaction between

the two variables, $F(10, 53) = 11.56$, $p < 0.05$ ($\eta^2 = 0.20$) (Supplementary Table 1), discussed below.

Post-hoc comparison tests revealed no significant difference in bacterial growth among mineral species compared to the control at 5 mg/ml, with the exception of glauconite (Fig. 1A; Supplementary Table 2). The significance of the response to glauconite at 5 mg/ml suggests an unusually low minimum inhibitory concentration with just a slightly increased effect at higher concentrations. Mineral suspensions of 10 and 25 mg/ml resulted in significantly depressed bacterial growth for all mineral species compared to the clay-free control, with the exception of calcite (Figs. 1A and 1B). The response at 25 mg/ml is much more pronounced to berthierine and kaolinite than to the other clay minerals—a fourfold decrease in turbidity compared to the control and threefold compared to calcite. We observed an inverse relationship between mineral concentration and turbidity for all mineral species except calcite, but the strength and significance of this relationship varies with mineral species (Supplementary Table 3). Although calcite appears to impede bacterial growth more strongly at 10 mg/ml than at 25 mg/ml, this is not significant.

In order to examine the impact of clays in general, we compared the effect of the clay minerals as a group with that of calcite and the control (Fig. 2). The two-factor analysis of variance showed significant main effects of mineral concentration, $F(2, 62) = 5.98$, $p < 0.05$ ($\eta^2 = 0.40$) and mineral species, $F(2, 62) = 1.23$, $p < 0.05$ ($\eta^2 = 0.30$) on turbidity, qualified by their interaction, $F(4, 62) = 12.246$, $p < 0.05$ ($\eta^2 = 0.13$) (Supplementary Table 1). Post hoc comparisons revealed no significant effect of clay or calcite on bacterial growth at 5 mg/ml. Both clay and calcite minerals, however, affected growth significantly at 10 and 25 mg/ml, but in both instances clay minerals were

associated with significantly less growth than calcite (Supplementary Table 2). Increasing clay concentration resulted in significantly depressed bacterial growth, but the same was not true for increasing calcite concentration (Supplementary Table 3). The marginal means and standard deviations of all within-concentration analyses are presented in Supplementary Table 4.

In summary, the results of our experiments show that the growth of *Pseudoalteromonas luteoviolacea*, a marine heterotrophic bacterium known to degrade animal tissues, is significantly inhibited by the presence of clay-sized mineral particles. With increasing mineral concentration, this inhibition becomes more pronounced and shows greater variation between mineral species. Clay minerals produce a stronger effect than calcite particles of similar size.

DISCUSSION AND CONCLUSIONS

Our experimental results support the hypothesis that clay minerals impede the growth of bacterial decomposers, providing a way to facilitate the preservation of soft tissues. Hypotheses to explain particular instances of exceptional fossil preservation have implicated both detrital clays, and authigenic clays that formed in sedimentary pore spaces and in association with decaying organic matter (e.g., Butterfield, 1990; Orr et al. 1998; Gabbott et al. 2001). Our results are relevant in both cases, and furthermore they suggest that certain clays are more likely to inhibit bacterial activity than others.

Our results are consistent with the usually high clay content of Cambrian Burgess Shale-type fossil-hosting sediments (Curtin and Gaines, 2011; Forchielli et al., 2014; Gaines et al., 2011; Powell, 2003). However, there is currently limited documentation of

associations with specific clay compositions. An exception is the Mount Cap Formation, where glauconite is associated with fossiliferous mudrock horizons (Aitken et al., 1973; Butterfield, 1994). Indeed, macrostratigraphic data from Laurentia reveal a spike in glauconite production during the Cambrian (Peters and Gaines, 2012). More generally, it has been argued that the rise and fall of BST preservation tracked the weathering flux of Al-rich clays as well as particular biogeochemical conditions that affected the composition of clay in marine sediment (Butterfield, 1995; Wilson & Butterfield 2014). Preliminary data through a Cryogenian succession in Mongolia provide a further example of clays of specific composition (in this case berthierine) in fossiliferous horizons (Anderson et al., 2014).

In both BST and non-BST lagerstätten, clay minerals can also be found intimately associated with organic fossils, often coating their external surfaces (e.g. Gabbott, 1998; Orr et al., 1998; Gabbott et al., 2001; Anderson et al., 2011; Laflamme et al., 2011; Cai et al., 2012; Pan et al., 2014; Wacey et al., 2014). A more refined mineralogical characterisation of clays within fossiliferous laminae and surrounding individual fossils awaits the application of emerging microscopic techniques (Tosca et al., 2015).

Our results are also consistent with the higher quality of organic tissue preservation associated with clay minerals as opposed to carbonate throughout the geological record. Fine-grained carbonates tend to preserve organic tissues more rarely and with less fidelity than clays (e.g., Butterfield et al., 1994). Organic fossils in Proterozoic carbonates, for example, tend to be dominated by robust testate forms (e.g., Bosak et al., 2011a; Bosak et al., 2011b; Bosak et al., 2012; Dalton et al., 2013) in contrast to more delicate forms preserved in clay-rich shales (e.g. Butterfield et al., 1994; Butterfield & Rainbird, 1998).

However, other factors may contribute to these differences, such as the higher sedimentation rate represented by clastic beds (Canfield, 1994).

The antibacterial properties of clays are attributed mostly to the toxicity of metal cations, particularly Al^{3+} and Fe^{2+} (e.g., Wong et al., 2004; Morrison et al., 2016). Diverse bacteria are susceptible to Al^{3+}, while excessive Fe^{2+} in aerobic conditions causes oxidative damage to bacterial cells (Guida et al., 1991; Kapoor and Arora, 1998; Amonette et al., 2003; Imlay et al., 2008). It is therefore striking that kaolinite and berthierine, the most aluminum-rich and ferrous-iron-rich clays in our experiments respectively, were associated with the strongest suppression of bacterial growth at 25 mg/ml. Kaolinite, which has been shown to preserve experimentally buried invertebrate carcasses better than quartz, calcite, and montmorillonite, also inhibits the growth of sulfate-reducing bacteria, autotrophic methanogens, and a heterotrophic soil bacterium (Wong et al., 2004; Wu et al., 2013; Wilson and Butterfield, 2014; Liu et al., 2016). Natural 'blue' clays, which release both Al^{3+} and Fe^{2+} from illite-smectite into solution, are effective antibiotic agents (Morrison et al., 2016); these two ions work synergistically to disrupt and oxidatively damage bacterial cells.

Our results show that clays impede the growth of decay bacteria, providing clear evidence that they are likely to have played a role in promoting organic preservation (Butterfield, 1990; Petrovich, 2001; Wilson and Butterfield, 2014). However, clay–bacterial interactions are highly specific with respect to both clay mineral composition and bacterial strain. Na-montmorillonite, for example, inhibits sulfate reducers but increases the longevity, growth rate and metabolic activity of several other groups of decomposers, probably due to its tendency to adsorb trace metals from the environment (Kunc & Stotzky, 1974; Hwang and Tate, 1997; Wong et al., 2004; Wu et al., 2013). The

chemical changes associated with decomposition are likely to affect the leaching and adsorption behavior of the clay as well as the speciation and toxicity of leached ions (Guida et al., 1991; Andrews et al., 2003; Morrison et al., 2014). Further experiments involving a wider range of relevant strains, mineral species, and environmental conditions are required to unravel the role of clay–microbe interactions in exceptional preservation.

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FIGURE CAPTIONS

Figure 1. Normalized optical density at 600 nm (ΔOD_{600}) of six-hour-old *Pseudoalteromonas luteoviolacea* subcultures taken after 24 hours growth with A: 5, B: 10 and C: 25 mg/ml <2-3 um mineral particles (B = berthierine, C = calcite, G = glauconite, I = illite, K = kaolinite) and with no mineral particles (Control). Floating points are outliers. Experiments were conducted in quadruplicate. Values shown represent increases above t=0 and are normalized to the control.

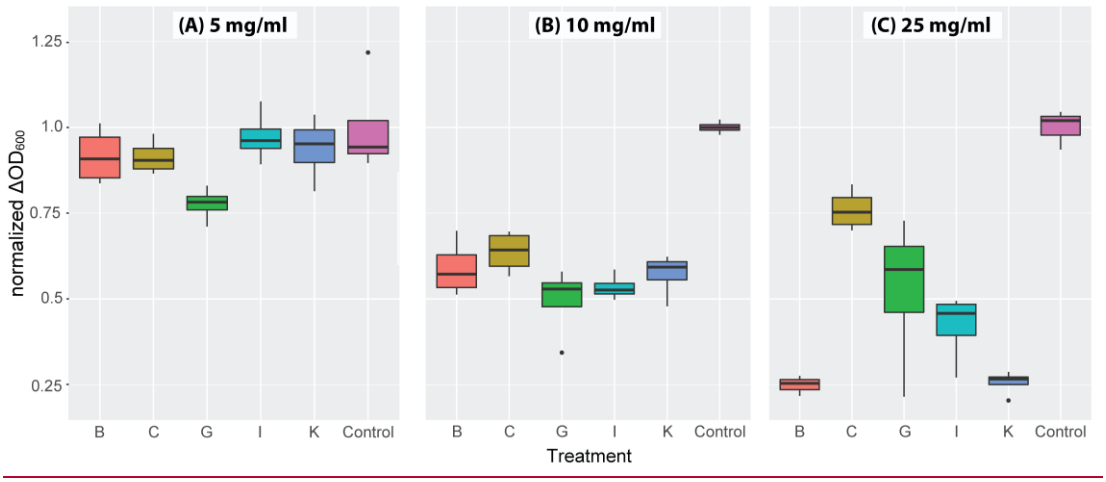

Figure 2 Normalized optical density at 600 nm (ΔOD_{600}) of six-hour-old *Pseudoalteromonas luteoviolacea* subcultures taken after 24 hours growth with 5, 10 and 25 mg/ml <2-3 um mineral particles and with no mineral particles (Control). Berthierine, glauconite, illite and kaolinite are grouped together as Clay. Experiments were conducted

in quadruplicate. Values shown represent increases above t=0 and are normalised to the control.