The O-antigen mediates differential survival of *Salmonella* against communities of natural predators

Aletheia Atzinger, Kristen Butela† and Jeffrey G. Lawrence

Correspondence
Jeffrey G. Lawrence
jlawrenc@pitt.edu
Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260, USA

Antigenically distinct members of bacterial species can be differentially distributed in the environment. Predators known to consume antigenically distinct prey with different efficiencies are also differentially distributed. Here we show that antigenically distinct, but otherwise isogenic and physiologically indistinct, strains of *Salmonella enterica* show differential survival in natural soil, sediment and intestinal environments, where they would face a community of predators. Decline in overall cell numbers is attenuated by factors that inhibit the action of predators, including heat and antiprotozoal and antihelminthic drugs. Moreover, the fitness of strains facing these predators – calculated by comparing survival with and without treatments attenuating predator activity – varies between environments. These results suggest that relative survival in natural environments is arbitrated by communities of natural predators whose feeding preferences, if not species composition, vary between environments. These data support the hypothesis that survival against natural predators may drive the differential distribution of bacteria among microenvironments.

INTRODUCTION

Bacteria are dominant members of every ecosystem, their $10^{30}$ individuals outnumbering all other cellular life forms (Whitman et al., 1998). Bacteria are major constituents of soil (Trevors, 2010), sediment (Dale, 1974) and marine and fresh waters (Watson et al., 1977). In addition, they colonize both external and internal surfaces of multicellular eukaryotes – where bacterial cells may be 10-fold more abundant than cells of their host (Human Microbiome Consortium, 2012) – and serve as intracellular symbionts to diverse taxa (Baumann, 2005). Turnover of bacteria in natural environments occurs within days (Luna et al., 2002; Roszak & Colwell, 1987), indicating that mortality rates are higher than expected given the natural rate of senescence (Nyström, 2002, 2007). Major arbiters of population control are bacteriophages and amoebae, which limit bacterial population growth in numerous environments (Jjema, 2001; Peduzzi & Schiemer, 2004; Pernthaler, 2005; Ronn et al., 2002); additional grazers (such as nematodes) may be present as well (Trevors, 2010).

Many predators (including components of immune systems) recognize their prey by virtue of the antigenic molecules on the surface of bacterial cells. As the most abundant molecule on the cell surface, the O-antigen LPS is a likely candidate used in prey recognition. In enteric bacteria, LPS is synthesized by enzymes encoded by the *rfb* operon; this region is hypervariable among serovars of *Salmonella* (Grimont & Weill, 2007). LPS components are potent activators of immune responses (Reeves, 1995), including the induction of predatory white blood cells upon systemic infection of a eukaryotic host by pathogenic bacteria. Therefore, it is not surprising that the structure of O-antigen polysaccharides is hypervariable within many species of pathogenic bacteria (Butela & Lawrence, 2010). Often, frequency-dependent selection mediated by the host immune system is invoked as a mechanism maintaining this diversity (Levin et al., 1988; Yuste et al., 2002). Here, the fitness of a variant allele is inversely proportional to its frequency, preventing both the fixation of common alleles and the loss of rare ones.

Yet, diverse O-antigens are also found in species not associated with eukaryotic hosts (Wildschutte et al., 2010). Predators found in natural environments could serve as the selective agents for maintaining antigenic diversity in these cases. In this model, predators in different micro-environments vary in their ability to recognize different antigenic types, thereby conferring high fitness to genotypes they avoid consuming, but only within their resident environment. Consistent with this model, diverse amoebae discriminate among bacterial prey on the basis of their O-antigen polysaccharides (Wildschutte et al., 2004;
Wildschutte & Lawrence, 2007). Moreover, predators found in different environments have different feeding preferences (Wildschutte et al., 2004; Wildschutte & Lawrence, 2007). Regardless of their phylogenetic relatedness, predators isolated from the same environment share feeding preferences (Wildschutte & Lawrence, 2007), so that unrelated predators dwelling in the same environment avoid consuming the same antigenic classes of prey. A model was presented whereby these preferences were driven by molecular mimicry (Wildschutte & Lawrence, 2007). Here, predators may recognize environmental epitopes for adhesion; bacterial cells whose antigens resemble these epitopes would not be recognized as potential prey. While these results are limited to only a few cultivable amoebae, they suggest that bacterial strains could outcompete conspecific, but antigenically different, strains in natural environments by virtue of their ability to avoid being eaten by the communities of resident predators, as opposed to any physiological or metabolic adaptations they might carry. This model stands in contrast to those that cite such physiological traits as the basis for differential distribution of bacterial strains among environments (Gordon & FitzGibbon, 1999; Gordon & Cowling, 2003; Walk et al., 2009). We tested this hypothesis by competing physiologically identical, but antigenically distinct bacteria within natural environments.

**METHODS**

**Bacteria.** Except for strains SARB2 and SARB36, all strains were derived from *Salmonella enterica* serovar Typhimurium strain LT2 (Table 1) and in most cases differed from the WT only in carrying a plasmid expressing a fluorescent protein, and/or having the *rfb* operon replaced so that a different O-antigen was made. A deletion of the *rfb* operon – Δ(*rfb*P-rfbB)3302 – was created by replacing the region between the *gnd* and *galE* genes with the *cat* chloramphenicol resistance gene, all within a *galE*2841::*attA hisD9953::*MudI phs-209::*Tn10Kgn* background. This parental strain was transduced to histidine prototrophy using bacteriophage P1 grown on *galE*8866::*aph* derivatives of antigenically distinct strains of the SARB collection (Salmonella Reference Collection B) (Boyd et al., 1993); these derivatives were created using the method of Butela & Lawrence (2012). Retention of the upstream *phs-209::*Tn10Kgn marker limited the introduction of DNA from upstream of the *his* operon. This strain was back-crossed twice to the parental Δ*rfb*-3302 strain, selecting as above, and the *galE* mutation was repaired via P1 transduction from a Gal*+* Δ*rfb* strain. The identity of the O-antigen in the isogenic strains was verified by antibody agglutination tests. Plasmids pEGFP, pEYFP, pECFP, pDsRed-Express2 and pKAB9 (Lawrence et al., 2013) were introduced by electroporation and express fluorescent proteins GFP, YFP, CFP, DsRed-Express2 and mKalama1, respectively.

**Media and antibiotics.** Bacteria were propagated at 37 °C in LB with 200 μg mL⁻¹ ampicillin to maintain plasmids. MacConkey agar (Difco) was cast with xylose added to 1 % final concentration after incubation prevented growth of eluted *Escherichia coli*. For *Salmonella* competition, strains were grown overnight in LB with 0.01 % glucose, rinsed and mixed in equal proportions prior to spreading 50 μl (~10⁵ c.f.u. total) on NGM plates. An aliquot of several hundred nematodes was introduced via an agar chunk. Plates were incubated for 4 days at 22 °C, allowing the nematodes to consume most of the *Salmonella* lawn. To retrieve remaining *Salmonella*, the agar in contact with the introduced piece was discarded and cells were eluted in 0.9 % NaCl, plated on indicator media and grown at 37 °C to discriminate between the two strains. The high temperature of incubation prevented growth of eluted *C. elegans*.

**Propagation of Caenorhabditis elegans.** *C. elegans* strain N2 Bristol was propagated at 22 °C on nematode growth medium (NGM) plates (Cold Spring Harbor) using *Escherichia coli* as a feeding strain. For *Salmonella* competition, strains were grown overnight in LB with 0.01 % glucose, rinsed and mixed in equal proportions prior to spreading 50 μl (~10⁵ c.f.u. total) on NGM plates. An aliquot of several hundred nematodes was introduced via an agar chunk. Plates were incubated for 4 days at 22 °C, allowing the nematodes to consume most of the *Salmonella* lawn. To retrieve remaining *Salmonella*, the agar in contact with the introduced piece was discarded and cells were eluted in 0.9 % NaCl, plated on indicator media and grown at 37 °C to discriminate between the two strains. The high temperature of incubation prevented growth of eluted *C. elegans*.

**Soil, sediment and fish.** Sediment was obtained from Panther Hollow Lake (40.436838°N, 79.947048°W) and Homewood Cemetery Pond (40.440249°N, 79.911991°W); sites were close to shore but under both leaf litter and ~8 feet (2.4 m) of water. Sediment was filtered through ~0.5 mm nylon mesh. Soil was obtained from the Fruit Room (40.438949°N, 79.948101°W) and Desert Room (40.438533°N, 79.947509°W) of the Phipps Conservatory, Pittsburgh, PA; these areas have had the same plant species growing in them for many years, and have experienced no outside animal traffic. Predators were extracted by passing an equal volume of water through the soil and filtering as above.

Comet goldfish (*Carassius auratus*) were obtained from Carolina Biological and propagated for 1 month in a community tank at 30 °C

### Table 1. Strains of bacteria used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>O-serotype*</th>
</tr>
</thead>
<tbody>
<tr>
<td>LT2</td>
<td><em>Salmonella enterica</em> Typhimurium LT2</td>
<td>4,5,12</td>
</tr>
<tr>
<td>LD901</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; Arfc3303::cat pEGFP</td>
<td>1,4,12</td>
</tr>
<tr>
<td>LD902</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; Arfc3303::cat pDsRed-Express2</td>
<td>1,4,12</td>
</tr>
<tr>
<td>LD903</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; Arfc3303::cat pEYFP</td>
<td>1,4,12</td>
</tr>
<tr>
<td>LD904</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; Arfc3303::cat pKAB9</td>
<td>1,4,12</td>
</tr>
<tr>
<td>LD905</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; Arfc3303::cat pECFP</td>
<td>1,4,12</td>
</tr>
<tr>
<td>LD906</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pEGFP</td>
<td>1,9,12</td>
</tr>
<tr>
<td>LD907</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pDsRed-Express2</td>
<td>6,8</td>
</tr>
<tr>
<td>LD908</td>
<td><em>rfb</em>&lt;sub&gt;SARB2&lt;/sub&gt; pEYFP</td>
<td>3,10</td>
</tr>
<tr>
<td>LD909</td>
<td><em>rfb</em>&lt;sub&gt;SARB2&lt;/sub&gt; pKAB9</td>
<td>8,20</td>
</tr>
<tr>
<td>LD910</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pCFP</td>
<td>6,7</td>
</tr>
<tr>
<td>LD911</td>
<td><em>rfb</em>&lt;sub&gt;SARB2&lt;/sub&gt; pKAB9</td>
<td>1,9,12</td>
</tr>
<tr>
<td>LD912</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pEYFP</td>
<td>6,8</td>
</tr>
<tr>
<td>LD913</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pEGFP</td>
<td>3,10</td>
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<tr>
<td>LD914</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pECFP</td>
<td>8,20</td>
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<tr>
<td>LD915</td>
<td><em>rfb</em>&lt;sub&gt;SARB1&lt;/sub&gt; pDsRed-Express2</td>
<td>6,7</td>
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<tr>
<td>LD916</td>
<td><em>rfb</em>&lt;sub&gt;SARB2&lt;/sub&gt; pDsRed-Express2</td>
<td>6,7</td>
</tr>
<tr>
<td>SARB2</td>
<td><em>Salmonella enterica</em> Agona (Xyl*)</td>
<td>1,4,12</td>
</tr>
<tr>
<td>SARB36</td>
<td><em>Salmonella enterica</em> Newport (Xyl*)</td>
<td>6,8</td>
</tr>
<tr>
<td>HW31</td>
<td><em>salmonella</em> phs-209::Tn10Kgn</td>
<td>1,4,12</td>
</tr>
<tr>
<td>HW19</td>
<td><em>salmonella</em> phs-209::Tn10Kgn</td>
<td>6,7</td>
</tr>
<tr>
<td>LD916</td>
<td><em>rfb</em>&lt;sub&gt;SARB2&lt;/sub&gt; pDsRed-Express2</td>
<td>8,20</td>
</tr>
</tbody>
</table>

*For strains constructed by moving the *rfb* operon from SARB strains into strain LT2, O-serotype is designated according to the serotype of the donor strain.*
until 5–7 cm in length. All animal work was performed in accordance with approved Institutional Animal Care and Use Committee protocols. Fish were sacrificed by overdose of tricaine methyisulfonate. Intestinal contents were released into PBS (135 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄ pH 7.4) and filtered through nylon mesh. Intestine contents from four fish of approximately equal cumulative mass (≈10 g) were pooled. Heat treatment was performed by submerging 30 ml (soil and sediment extracts) or 7 ml (intestinal contents) aliquots in boiling water for 45 min, cooling on ice and equilibrating to room temperature.

**Competition in complex environments.** Overnight cultures of strains were grown at 37 °C in LB with 200 μg ml⁻¹ ampicillin, rinsed and resuspended in 0.9 % NaCl and then added as a mixture to environmental samples to a final concentration of 10⁷ cells ml⁻¹. Samples were taken by thoroughly mixing the suspension, removing 2 ml and allowing it to gravity-filter through a 5 μm pore-size mesh. A 0.012 U aliquot of biotin-conjugated rabbit anti-Salmonella antibody (Gene Tex) was added and the sample was incubated at 4 °C for 30 min. Samples were pelleted at 10 000 g for 5 min, washed in PBS, pelleted again, and resuspended in an equal volume of PBS before adding washed beads from the CELLection Biotin Binder kit (Invitrogen) and following the indirect technique for cell pulldown recommended by the manufacturer. Samples were fixed in 1 % paraformaldehyde and stored at 4 °C.

**Flow cytometry.** The samples were run on a Beckman-Coulter CyAn ADP flow cytometer with a 0.3 neutral density filter controlled by Summit 4.3 software (Dako), or on a BD LSRFortessa cell analyser controlled by BD FACSDiva software (BD Biosciences). SYTO 62 nucleic acid dye (Invitrogen) was added at a final concentration of 50 nM 15 min before cytometry. Flow rate was maintained at approximately 1000 events s⁻¹. Bacteria-only controls were used to differentiate bacteria from debris and larger cells. Data were analysed using FlowJo (Lawrence et al., 2013); Z-scoring was used to reduce miscounting events that arose from non-bacterial sources (Fig. S1, available in the online Supplementary Material).

**Measuring fitness.** Conventional methods for calculating fitness determine relative growth rates between different genotypes in the same environment [e.g. as dP/dt = sP(1 – P)]; here, the proportion of cells (P) is measured over time to calculate the selection coefficient (s). In our case, we wished to measure relative cell fitness of a strain in different environments (with and without predators). To do this, we examined the proportions of each strain at the beginning and end of experimental treatments. The frequency (F) of a strain in its environment was calculated as

\[ F_i = C_i/N \]  

where \( C_i \) is the count of cell type \( i \) and \( N \) is the sum of all countable cell types in the experiment, here fluorescently tagged cells. Relative recovery for strain \( i \) was defined as

\[ R_i = F_i/F_0 \]  

where \( F_0 \) and \( F_0 \) are the frequencies of the cell type at the beginning and end of the experiment, respectively. Fitness in the face of predators can be calculated as

\[ W_i = R_i/R_A \]  

where \( R_p \) and \( R_A \) are the relative recoveries in the presence and absence of predators, respectively.

Variance of both relative recovery and fitness was calculated by resampling. Estimates of genotype frequency using multiple replicates were used to infer a mean and variance, assuming a Gaussian distribution. These distributions were resampled 1000 times and used to estimate the variance of relative recovery and fitness.

**RESULTS AND DISCUSSION**

**Salmonella is removed from natural environments by biological agents**

Many studies have documented the action of predators in the decline of bacterial populations in natural environments (Hahn & Höfte, 2001; Rodriguez-Zaragoza, 1994; Ronn et al., 2002; Serensen et al., 1999). To establish the time frame of predation in our system, we followed the disappearance of *Salmonella* within a sediment sample from Homewood Cemetery Pond. This strain was discriminated from other bacterial cells by GFP-fluorescence and resistance to chloramphenicol, gentamicin and zeomycin. Prior to strain addition, sediment was either left untreated, treated with heat or treated with a cocktail of three antibiotics targeting eukaryotic predators. Samples were recovered over time and the number of tagged *Salmonella* cells remaining was determined by plating on selective media; resulting colonies were verified by fluorescent signal from GFP; no colonies were detected from samples that did not receive *Salmonella* aliquots.

As expected, the numbers of introduced cells recovered from untreated sediment decreased over time (Fig. 1a), with fewer than 2 % remaining after 48 h. However, populations showed no significant decline in sediment exposed to either high temperature or a mixture of antiprotozoal and antihelminthic drugs (Fig. 1a). These data suggest that biological agents are responsible for the decline of *Salmonella* added to the untreated samples. Since drugs targeting eukaryotic predators greatly increased survivorship, we suspect that these organisms are dominant predators in these environments; other environments, such as sea water (Chibani-Chennoufi et al., 2004; Wommack & Colwell, 2000), have dominant bacteriophage predators. Since bacteriophage would be unaffected by antiprotozoal and antihelminthic drugs, they cannot be responsible for the decline in *Salmonella* numbers in untreated samples.

**Diverse antigenic types can be recovered by antibody binding**

For high-throughput enumeration of *Salmonella*, cells were first recovered from natural environments by binding to biotin-conjugated anti-*Salmonella* antibody. To determine if all antigenic types used in this study could be recovered efficiently by this antibody, a mixture of five strains – each expressing a different O-antigen and a different fluorescent protein, but otherwise isogenic – was assembled. Ratios of cell types were determined by flow cytometry for samples collected both before and after binding to the antibody...
and recovery using avidin beads. While subtle differences (about twofold) were seen in the efficiency of binding to the antibody among strains expressing different O-antigens (Fig. 1b), these did not prevent robust recovery and enumeration of the different serovars of *Salmonella*. This antibody recovery protocol was used in all competition experiments.

**Fluorescent tags do not affect enumeration of cells via flow cytometry**

High-throughput enumeration of bacterial cells used flow cytometry, where strains were tagged with different fluorescent proteins. Differential enumeration of fluorescent cells may reflect differences in the longevity of fluorescent proteins rather than changes in relative abundance of the cell types within the original sample. To test the effect of the fluorescent tags on recovery and enumeration of *Salmonella*, strains expressing one of five fluorescent proteins (strains LD901–905) were introduced into sediment from Homewood Cemetery Pond; except for the plasmid expressing different fluorescent proteins, cells were otherwise isogenic. After either 0 or 24 h incubation, cells were recovered using *Salmonella*-specific antibodies and counted via flow cytometry. The same proportions of the five fluorescent strains were found at both time points (Fig. 2a); these results indicate that the half-life of the fluorescent proteins did not significantly differ. Similar results were obtained after 48 h incubation (data not shown), the maximum duration of experiments described here. Fluorescent signal was never found in samples lacking added fluorescent cells.

**Nature of the O-antigen affects relative recovery and enumeration**

To determine if the identity of the O-antigen affects relative survival of bacteria in natural environments, we added a mixture of fluorescently tagged, antigenically distinct, but otherwise isogenic strains of *Salmonella* (LD906–910) to a sediment sample collected from the Homewood Cemetery Pond. Cells were recovered by binding to biotin-conjugated rabbit anti-*Salmonella* antibody after 0 and 24 h incubation and enumerated by flow cytometry. While the nature of the fluorescent tag did not influence relative recovery (Fig. 2a), this was not true for the nature of the O-antigen (Fig. 2b). There were large differences in relative recovery after 24 h (Fig. 2b; \( P = 2.26 \times 10^{-10}, \text{ANOVA} \)). For example, strains bearing the O-antigens originating from SARB52 (O : 1,9,12) and SARB2 (O : 3,10) both increased in abundance over 24 h, while those bearing O-antigens originating from SARB20 (O : 8,20), SARB30 (O : 6,7) and SARB36 (O : 6,8) decreased. Because the strains were isogenic at all loci except the region containing the O-antigen-encoding *rfb* operon, the identity of the O-antigen appears to affect relative recovery of *Salmonella* serovars within natural environments during a time frame when cell numbers decrease (Fig. 1a).

**Relative survival of antigenically distinct strains is affected by heat-labile factors**

Differences in relative recovery of antigenically distinct strains after 24 h incubation in natural environments could be driven by differential binding to silicates or interaction with other, non-biotic aspects of the environment. Alternatively, because predation affects overall survival (Fig. 1a), interaction with predators may lead to differences in cell numbers over time. If heat treatment of the environment influences relative recovery of *Salmonella*, then biotic elements would likely be responsible for differential survival of *Salmonella*, rather than simply differential recovery.
To test this hypothesis, five fluorescently tagged, antigenically distinct but otherwise isogenic strains of *Salmonella* (LD906–910) were competed within sediment samples collected from Homewood Cemetery Pond. The sediment samples either were left untreated or had been heat-treated before equilibration to 25°C. The mixture of *Salmonella* cells was introduced, and cells were recovered by antibody binding after either 0 or 40 h of incubation; cells were enumerated by flow cytometry.

As expected, the fractions of each cell type were the same for untreated and heat-treated sediment at the start of the experiment (Fig. 3a; \(P > 0.18\), ANOVA), indicating that heat treatment did not affect any contribution of differential binding to substances within the sample. However, fractions of each cell type significantly differed between untreated and heat-treated sediment after 40 h (Fig. 3b; \(P < 1.1 \times 10^{-9}\), ANOVA). While strain LD906, bearing the O : 1,9,12 serotype, strongly outcompeted other strains in untreated, predator-bearing samples, this advantage was eliminated when the samples were heat-treated prior to competition, thereby eliminating the predators (Fig. 3b). This suggests that LD906 effectively escaped predation in the untreated samples, while other serotypes were consumed more readily.

These differences in abundance over time led to differences in relative recovery between strains (Fig. 3c; \(P = 4.0 \times 10^{-6}\), ANOVA). However, the nature of those changes was strongly influenced by heat treatment (Fig. 3c). If we posit that differences in relative recovery between untreated and heat-treated samples reflect the presence and absence of predators, then the difference between these samples represents the fitness against these predators. We can estimate fitness \(w\) as the ratio of a strain’s relative recovery in the presence or absence of predators (equation 3) (Fig. 3d), using the variability between the four replicates to calculate a variance (error bars represent 2 SD). Here, LD906 has the highest fitness, meaning it had the greatest increase in relative abundance in the untreated sample relative to the heat-treated sample.

**Heat treatment does not release nutrients allowing growth**

While the decline in bacterial numbers over time in untreated samples is conventionally attributed to the action of predators (e.g. Fig. 1), one could propose that treatment with heat or anti-predator drugs releases nutrients that allow cell growth. If so, then one may observe differences in relative recovery because of differentially enhanced growth. While the strains LD906–910 are isogenic except for the region of the O-antigen-encoding *rfb* locus (Table 1), one may posit that those differences could be sufficient to utilize differentially any nutrients released by boiling.

To test this hypothesis, we gathered four sets of soil samples (from two locations) and four sets of sediment samples (from two locations); samples were heat-treated as above, with an equal volume of water being added to soil samples before boiling. The environmental sample or water control (both buffered to pH 7) was inoculated to 10^7 c.f.u. ml^-1 with LD907. We monitored change in bacterial numbers in each sample or sterile water control in
eight replicate experiments. Two aliquots were removed from each replicate after either 0 or 48 h incubation at 25 °C; each aliquot was independently diluted and plated to estimate c.f.u. The mean change in cell numbers between 0 and 48 h was then computed for each of the eight replicates grown in either environmental extract or water. The significance of any difference between environmental sample and water was determined by a t-test. This was a conservative test in two ways. First, we were testing for any growth advantage for boiled environmental samples compared with water, which measured any nutrients released by boiling as well as any present in the sample prior to boiling. Second, we were measuring growth in an inoculum of 10^7 c.f.u. ml⁻¹, instead of the 10^8 c.f.u. ml⁻¹ added during effective competition experiments (e.g. Fig. 3); with 10-fold fewer cells being added, each cell has 10 times the potential nutrients available for growth.

As shown in Table 2, even under these conditions designed to maximize our ability to detect cell growth, bacterial cells did not show any growth difference between heat-treated environmental samples and water controls, neither of which showed any significant gain or loss of cell numbers during the course of the experiment. Therefore, we conclude that there was no significant release of nutrients due to heat treatment of either soil samples or sediment samples under our experimental conditions.

**Relative survival is affected by drugs targeting eukaryotic predators**

Heat treatment could affect numerous classes of predators, including bacteriophages, bacteria, unicellular eukaryotes such as amoebae, and multicellular eukaryotes such as nematodes. The effect of antiprotazoal and anthelminthic drugs in increasing overall survival (Fig. 1a) suggests that...
**Table 2. Test of growth differences in buffered water or environmental samples**

<table>
<thead>
<tr>
<th>Environment</th>
<th>Environmental sample*</th>
<th>Water control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu$</td>
<td>$\sigma^2$</td>
</tr>
<tr>
<td>Homewood Cemetery</td>
<td>0.164</td>
<td>0.457</td>
</tr>
<tr>
<td>Phipps (Fern Room)</td>
<td>0.380</td>
<td>0.239</td>
</tr>
<tr>
<td>Phipps (Desert Room)</td>
<td>$-0.205$</td>
<td>0.402</td>
</tr>
<tr>
<td>Panther Hollow Lake</td>
<td>$-0.033$</td>
<td>0.284</td>
</tr>
<tr>
<td>All locations</td>
<td>0.075</td>
<td>0.457</td>
</tr>
</tbody>
</table>

*Growth is reported as $\log_2(N_t/N_0)$, where $N_0$ and $N_t$ are cell counts after 0 and 40 h incubation, respectively. Mean values are shown for replicate experiments for each environmental site paired with water controls using the same bacterial inoculum.

eukaryotic predators are primary consumers of bacteria in this system.

To examine the effects of antiprotozoal and antihelminthic drugs on relative survival of *Salmonella*, five fluorescently tagged, antigenically distinct but otherwise isogenic strains of *Salmonella* were competed within sediment samples collected from Panther Hollow Lake (Fig. 4); three replicate experiments were performed. Two treatments were used to attenuate predators: either the sample was heat-treated before equilibration to 25 $^\circ$C, or the sample was exposed to the antiprotozoal drug metronidazole. Drugs used to attenuate eukaryotic predators do not affect growth or recovery of any genotype of *Salmonella* in a laboratory setting (Figs S2 and S3). The mixture of *Salmonella* was introduced and cells were recovered by antibody binding after either 0 or 30 h of incubation; cells were enumerated by flow cytometry.

As expected, the fractions of each cell type were the same between untreated, heat-treated and drug-treated sediment at the start of the experiment, but were significantly different between untreated and heat-treated or drug-treated sediment after 30 h incubation. As expected from Fig. 3(c), relative recovery differed strongly between untreated and heat-treated samples (Fig. 4a; $P = 9.3 \times 10^{-6}$, ANOVA). Notably, relative recovery also differed strongly between untreated and drug-treated samples (Fig. 4a; $P = 1.2 \times 10^{-5}$, ANOVA), indicating that the addition of metronidazole also allowed the survival of strains that were otherwise rapidly removed in the untreated sample. We can calculate fitness in the face of predation by comparing relative recovery in untreated versus heat-treated samples, or by comparing untreated versus drug-treated samples. As seen in Fig. 4b–d, the use of heat treatment or metronidazole treatment resulted in similar patterns of relative survival, indicating that both treatments attenuated predators in similar ways (Pearson $R = 0.794$). Metronidazole inhibits anaerobic amoebae, suggesting that amoebae affect relative survival of antigenically distinct *Salmonella*.

This result was recapitulated in other soil and sediment samples (Fig. S4). In all cases, fitness in the face of predation was similar when using either heat or metronidazole to incapacitate predators in either soil (Fig. S4a) or sediment samples (Fig. S4b). In addition, treatment with tetramisole and ivermectin also led to differential survival of antigenically distinct *Salmonella* (Fig. S4c); both ivermectin and tetramisole are broad-spectrum antihelminthic drugs, suggesting that nematodes both are major predators of bacteria in this environment and can mediate differential survival among antigenically distinct strains. Interestingly, the two classes of anti-eukaryote drugs led to similar fitness profiles (Fig. S4c), suggesting that amoebae and nematodes may share feeding preferences, or that these drugs both target a single class of predator.

**Nematodes discriminate between antigenically distinct strains of *Salmonella***

To test the hypothesis that nematodes differentiate among prey, we grew a mixture of two antigenically distinct strains of *Salmonella* on solid media in the presence or absence of *C. elegans*. As expected, the fractions of each strain did not differ between plates bearing or lacking nematode predators at the start of the experiment ($P > 0.05$ at 0 h, Fig. 5). However, the ratios of bacterial strains differed after 4 days growth with or without *C. elegans* ($P < 10^{-5}$ at 96 h, Fig. 5). When competing WT strains SARB2 (O : 3,10) and SARB36 (O : 6,8), SARB2 increased abundance to a greater degree in the absence of predators, suggesting that *C. elegans* preferentially consumed that serovar. These results were validated in a replicate experiment (Fig. 5).

To determine if *C. elegans* was discriminating based on the O-antigen, we competed near-isogenic strains HW31 (O : 1,4,12) and HW19 (O : 6,7), which differ only at their O-antigens and at the *phs* marker gene. In two replicate experiments, strain HW31 decreased in relative abundance in the absence of predators (Fig. 5), suggesting that *C. elegans* consumed it preferentially. Taken together, these data suggest that the action of antihelminthic drugs in conferring differential survival of *Salmonella* was consistent with nematodes differentially preying upon antigenically distinct strains of *Salmonella*. We discounted any role of nematode chemotaxis (Moens *et al.*, 1999;
Ward, 1973) toward particular serovars because the two serovars were well mixed and abundant on the plates; strains HW31 and HW19 are also nearly isogenic, and would not differentially produce chemoattractants (Chaisson & Hallem, 2012; Prot, 1980) or cAMP (Ward, 1973).

**Fitness of Salmonella against predation varies between natural environments**

We have shown previously that amoebae isolated from different environments show different feeding preferences (Wildschutte et al., 2004), though predators isolated from the same environment show similar feeding preferences (Wildschutte & Lawrence, 2007). Those results predict that Salmonella should show differential fitness when introduced into different natural environments, facing communities of predators with different sets of collective feeding preferences. To test this hypothesis, we repeated these competition experiments across soil and sediment collected from different locations, as well as the intestinal tract of a vertebrate host (Fig. 6). In each case, four replicates were performed for each experimental regime. Predators were attenuated using either heat treatment, exposure to the

**Fig. 4.** Fitness of antigenically distinct strains of *S. enterica* (LD906–910) in natural environments calculated using different treatments to attenuate predators. Fitness was calculated after 30 h incubation in sediment collected from Panther Hollow Lake. (a) Relative recovery was calculated as the ratio of fractions of cells recovered after 0 or 30 h; predators were attenuated by heat treatment (grey bars) or exposure to metronidazole (white bars). Black bars, untreated. (b) Fitness was calculated as the ratio of relative recovery for each serovar without and with heat treatment. Error bars represent 2 SD, which was calculated using the variances of each relative recovery coefficient. (c) Fitness was calculated as the ratio of relative recovery for each serovar without and with treatment with metronidazole. (d) Comparison of fitness values calculated using either heat or metronidazole to attenuate predators. $R=0.794$. 
antiprotozoal drug metronidazole, or exposure to a cocktail of metronidazole, ivermectin and tetramisole. To begin, we expected *Salmonella* competed within replicate samples collected from the same location to show similar fitness values (e.g. Fig. 3); this was upheld for competitions performed in numerous environmental samples, indicating that the method was robust. Because the nature of the fluorescent tag did not affect fitness (Fig. 2a), we expected that the method was robust. We also expected samples collected from the same location, but exposed to predators for different periods of time, to show congruent fitness differences between replicates with and without predators.

We also expected samples collected from the same location, but exposed to predators for different periods of time, to show congruent fitness patterns. This was observed in experiments using one environmental sample wherein strains facing those predators. Taken together, these data demonstrate that communities of predators show an aggregate feeding preference.

**Predator identities are unclear, even though they share feeding preferences**

Soil and sediment environments harbour many predators of bacteria, including amoebae, ciliates, bacteriophage, predatory bacteria and nematodes. Heat treatment, which would eliminate all of these predators, increased bacterial survival (Fig. 1a) and revealed differential survival of antigenically distinct strains in the face of the predator communities (Figs 3 and 6). Treatment with antiprotozoal as well as anthelmintic drugs also increased bacterial survival (Fig. 1a), and all methods of attenuating predators revealed congruent patterns of differential survival (Figs 4 and 6). One conclusion that can be drawn is that the action of heat treatment in increasing survival (Fig. 1a) did not operate by removing bacteriophages, as neither antiprotozoal nor anthelmintic drugs would affect bacteriophages. The similarity of effects between antiprotozoal and anthelmintic drugs suggests that either both protozoa and nematodes share feeding preferences, or that the drugs affect a class of predators susceptible to both classes of drug. Metronidazole targets anaerobic protozoa by interfering with ferredoxin-mediated H2 production (Edwards & Mathison, 1970; Quon *et al.*, 1992). In contrast, anthelmintic drugs act on multicellular eukaryotes. Ivermectin binds to glutamate-gated chloride channels, increasing their permeability to chloride (Cully *et al.*, 1994; Vassilatis *et al.*, 1997); this leads to hyperpolarization. Tetramisole acts as an acetylcholine receptor agonist, disrupting acetylcholine receptors in muscles and neurons (Martin & Robertson, 2007). It is not clear what organism would respond to all compounds. While metronidazole could impact some bacterial taxa, neither tetramisole nor ivermectin is known to affect prokaryotes. Since these drugs have similar effects on attenuating predators (Fig. 5), we conclude that the antibacterial effects of metronidazole were not responsible for the fitness differences between *Salmonella* serovars in our experiments.

We propose that the amoebae and nematode predators within specific environments share feeding preferences. The strains competed herein were isogenic save for the genes encoding the O-antigen biosynthetic proteins; therefore, we conclude that predators recognize prey by virtue of their O-antigen polysaccharides. Yet other structures in these environments,
including intestinal mucins and decaying plant matter, present surface polysaccharides. These structures are not viable food sources for these predators, and attempts at their ingestion would be fruitless. If the predators recognized such structures as environmental attachment sites rather than as food items, then bacteria with surface antigens resembling those structures would be conferred high fitness. This model of molecular mimicry was discussed previously to explain the similarity in feeding preferences shared between amoebae of different phyla (Wildschutte & Lawrence, 2007).

Does differential predation lead to diversifying selection and bacterial speciation?

Different strains of a single bacterial species are often found in different environments. For example, different genotypes of *E. coli* are found associated with different species of mammalian hosts (Gordon & FitzGibbon, 1999; Gordon & Cowling, 2003) or even different age groups in the same host species (Gordon et al., 2005). In such cases, the differential distribution of genotypes may reflect physiological differences between strains that adapt them to each environment. Here we provide evidence that predator escape may also drive the differential distribution of natural isolates between environments.

The relative contributions of predator escape and physiological adaptation in arbitrating the differential distribution of genotypes among environments is unclear. We suggest that antigenic variability may allow bacteria to persist in novel environments by assisting in predator escape. This would allow the accumulation of physiological adaptations to permit more effective competition. Thus antigenic variability may provide the keystone adaptations that allow bacterial invasion of novel environments and, ultimately, speciation.

The relative contributions of predator escape and physiological adaptation in arbitrating the differential distribution of genotypes among environments is unclear. We suggest that antigenic variability may allow bacteria to persist in novel environments by assisting in predator escape. This would allow the accumulation of physiological adaptations to permit more effective competition. Thus antigenic variability may provide the keystone adaptations that allow bacterial invasion of novel environments and, ultimately, speciation. Here, diversifying selection would maintain high antigenic diversity at the O-antigen-encoding *rfb* operon, counter-selecting recombination in the region; this has been observed in enteric bacteria, where sequence divergence is high in the region flanking the *rfb* operon (Butela & Lawrence, 2009; Milkman et al., 2003). Therefore, selection for antigenic diversity at the *rfb* operon would represent the first step in fragmented speciation (Retchless & Lawrence, 2007), where adaptation to different environments imparts genetic isolation to a locus within the bacterial chromosome.

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