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ENVIRONMENTAL MICROBIOLOGY

Shigella sonnei Does Not Use Amoebae as Protective Hosts

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ABSTRACT *Shigella flexneri* and *Shigella sonnei* bacteria cause the majority of all shigellosis cases worldwide. However, their distributions differ, with *S. sonnei* predominating in middle- and high-income countries and *S. flexneri* predominating in low-income countries. One proposed explanation for the continued range expansion of *S. sonnei* is that it can survive in amoebae, which could provide a protective environment for the bacteria. In this study, we demonstrate that while both *S. sonnei* and *S. flexneri* can survive coculture with the free-living amoebae *Acanthamoebae castellanii*, bacterial growth is predominantly extracellular. All isolates of *Shigella* were degraded following phagocytosis by *A. castellanii*, unlike those of *Legionella pneumophila*, which can replicate intracellularly. Our data suggest that *S. sonnei* is not able to use amoebae as a protective host to enhance environmental survival. Therefore, alternative explanations for *S. sonnei* emergence need to be considered.

IMPORTANCE The distribution of *Shigella* species closely mirrors a country's socioeconomic conditions. With the transition of many populous nations from low- to middle-income countries, *S. sonnei* infections have emerged as a major public health issue. Understanding why *S. sonnei* infections are resistant to improvements in living conditions is key to developing methods to reduce exposure to this pathogen. We show that free-living amoebae are not likely to be environmental hosts of *S. sonnei*, as all *Shigella* strains tested were phagocytosed and degraded by amoebae. Therefore, alternative scenarios are required to explain the emergence and persistence of *S. sonnei* infections.

KEYWORDS amoeba, *Shigella sonnei*, intracellular survival

Shigella is a genus of Gram-negative enteric pathogens comprised of four species. All species can cause severe diarrhea, and *Shigella* is estimated to cause 165 million infections and 120,000 deaths annually, accounting for 10% of deaths due to diarrheal disease worldwide (1, 2). *Shigella flexneri* and *Shigella sonnei* cause the majority of infections, but the ratio of species dominance is highly dependent on the socioeconomic conditions of the area. In countries with a low per capita income, including those of sub-Saharan Africa and some countries in Asia, *S. flexneri* is the dominant cause of shigellosis, responsible for over 60% of infections. However, in areas with a high human development index, such as Europe and North America, *S. sonnei* causes around 80% of shigellosis cases (3). Transitioning countries currently undergoing socioeconomic improvements are experiencing a shift in the dominant species causing infections, from *S. flexneri* to *S. sonnei*. From 2001 to 2008 the prevalence of *S. flexneri* in Bangladesh decreased from 65.7% to 47%, while the prevalence of *S. sonnei* increased from 7.2% to 25% (4). During this time, Bangladesh underwent significant improvements in the nutritional status of children, health care, and water sanitation (5, 6). Other countries, such as China, Vietnam, and Brazil, have experienced a similar trend (7–9).

The reason for the rising dominance of *S. sonnei* in areas where the *S. flexneri* infection rate is decreasing is unclear. One hypothesis is that *S. sonnei* can use amoebae

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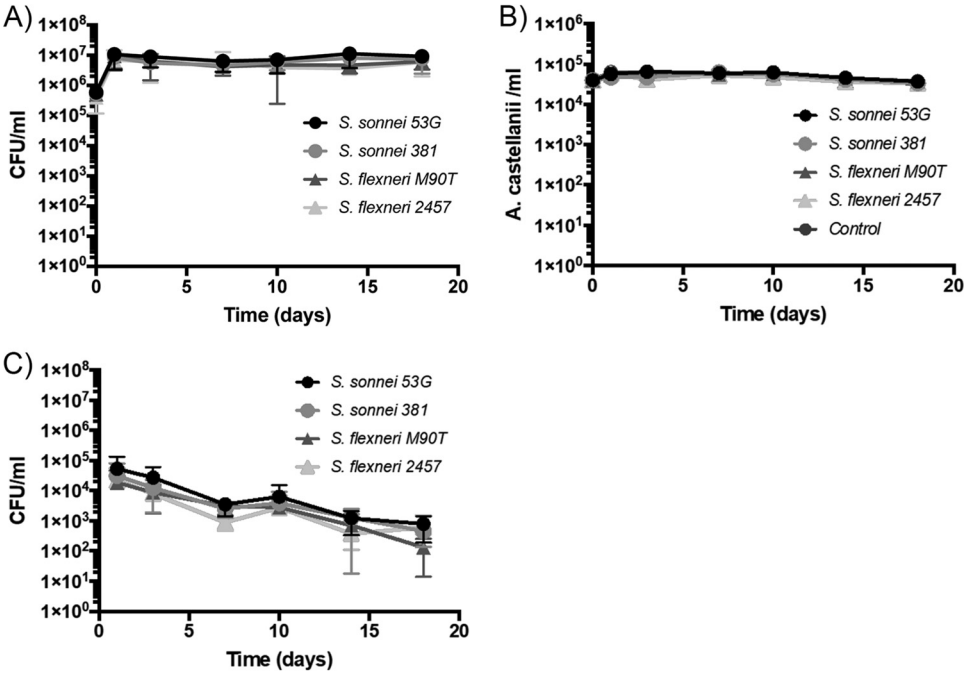

FIG 1 All *Shigella* strains survive extended coculture with *A. castellanii*. *Shigella* strains were cocultured with *A. castellanii* for 18 days in low-nutrient medium supplemented with heat-killed *E. coli* cells. At the indicated time points, the following measurements were made: (A) total bacteria determined by colony counts; (B) number of amoebae quantified by microscopy; (C) number of gentamicin-protected intracellular bacteria determined by colony counts.

as environmental hosts to protect it from water sanitation measures that are implemented in transitional countries (10). Amoebae are free-living organisms found in a variety of water sources, such as swimming pools and lakes, as well as in soil and dust. Importantly, they have even been found in chlorinated public water sources in developed countries (11). They are able to tolerate harsh and changing conditions, making them a good host for a variety of bacteria (12). *Legionella pneumophila* is the most well-known bacterium known to utilize amoebae as protective hosts, but *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, and *Vibrio cholerae* have also been shown to survive intracellularly in amoebae (13–15, 16). However, some bacteria which were initially described as surviving in amoebae have later been shown to grow extracellularly, potentially through saprophytic growth on dead amoebae or amoeba waste (17–20).

Previous work has suggested that *S. sonnei* can survive in amoebae for extended periods of time. *S. sonnei*, *Shigella dysenteriae*, and *S. flexneri* were all found to be phagocytosed by *Acanthamoebae castellanii*; however, only *S. sonnei* and *S. dysenteriae* appeared to survive and replicate in the cytosol (21).

Here, we explore the hypothesis that amoebae can act as an environmental reservoir for *S. sonnei*. Although *S. sonnei* is phagocytosed by amoebae, we found no evidence that *S. sonnei* is able to survive and replicate in the cytosol of *A. castellanii*.

RESULTS

***Shigella* cells survive extended coculture with amoebae.** Consistent with previous research, we observed that strains of *S. sonnei* and *S. flexneri* were able to survive in coculture with *A. castellanii* over 18 days at 22°C. We used two *S. flexneri* serotypes (strain M90T, serotype 5a, and strain 2457T, serotype 2a) and two *S. sonnei* isolates (the commonly used 53G strain and a recent clinical isolate, H140860381, here referred to as 381). All strains remained culturable at 10^6 to 10^7 CFU/ml over the 18 days (Fig. 1A). The amoebae were also maintained at ca. 5×10^4 cells/ml throughout this time period (Fig. 1B). These data indicate that *Shigella* species can survive extended coculture in the

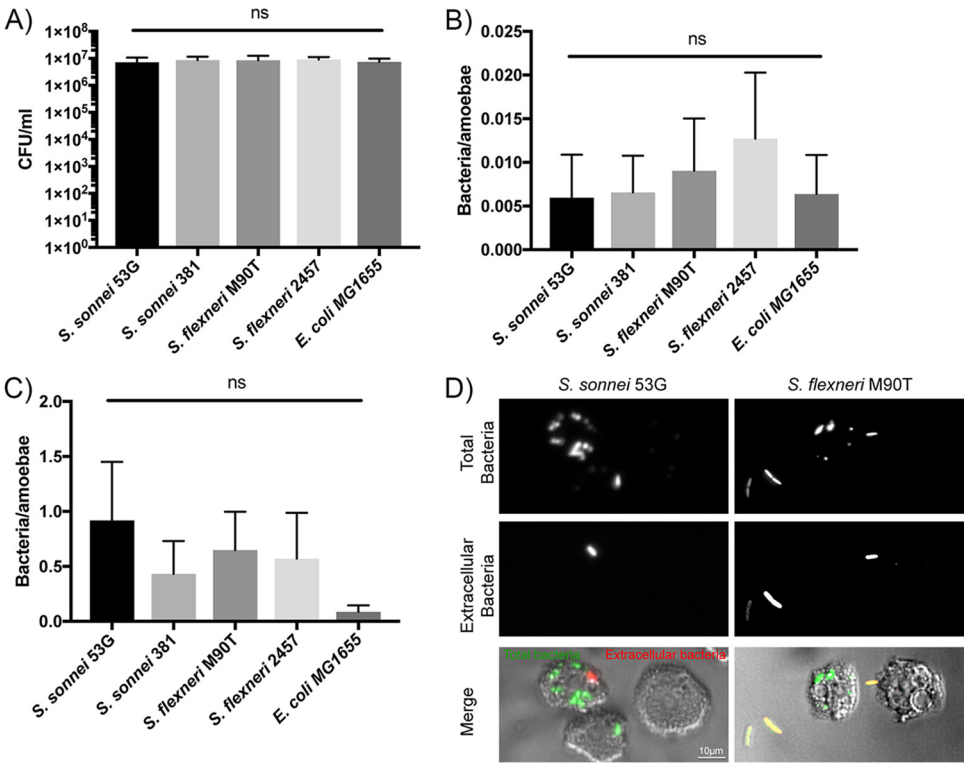

FIG 2 *Shigella* and *E. coli* show similar levels of amoeba association and phagocytosis at 22°C. *A. castellanii* was incubated with the indicated bacterial strains for 1 h, washed, and then either (A) followed by determination of cell-associated bacteria or (B and C) treated with gentamicin for 1 h to kill extracellular bacteria to determine the number of phagocytosed bacteria. (A and B) Experiments were conducted in low-nutrient medium or (C) high-nutrient medium. One-way analysis of variance (ANOVA) indicated no statistically significant differences between any bacterial strains. Mean and a standard deviation of 5 repeats are shown. (D) *S. sonnei* 53G and *S. flexneri* M90T strains expressing GFP were incubated with *A. castellanii* and washed, and extracellular bacteria were detected with specific antibodies prior to visualization. Individual fluorescence channels for total and extracellular bacteria are shown in the top panels. The bottom panel represents merged transmitted-light images (to visualize the amoebae) and fluorescence images (green, total bacteria; red, extracellular bacteria).

presence of amoebae but give no information as to whether the bacteria are residing within amoebae and potentially utilizing the amoebae as an environmental reservoir.

We determined the intracellular bacterial numbers by taking samples at the indicated time points, treating with gentamicin to kill extracellular bacteria, and lysing the amoebae prior to CFU determination (Fig. 1C). This analysis revealed that all bacterial strains could be recovered intracellularly at all time points. However, fewer intracellular bacteria were recovered at the later time points. No difference was observed in intracellular bacterial numbers between the *S. flexneri* and *S. sonnei* strains at any time point.

The intracellular bacteria observed in this assay could be recently phagocytosed bacteria that had not yet been degraded, or bacteria that had established an intracellular niche and were surviving and/or replicating. We sought to examine these possibilities further.

All *Shigella* strains are phagocytosed by *A. castellanii*. To determine the efficiency of phagocytosis of *Shigella* strains, amoebae incubated in low-nutrient medium (Page's modified Neff's amoeba saline [PAS]) at 22°C were allowed to phagocytose bacteria for 1 h, which was followed by 1 h gentamicin treatment to kill extracellular bacteria. The numbers of total bacteria (prior to gentamicin treatment) were similar for all strains and indicated that, on average, 5×10^6 CFU/ml, or approximately 50 bacteria/amoeba, were present (Fig. 2A). Following gentamicin treatment, recoverable CFU decreased by 4 log, indicating that the majority of the bacteria were extracellular or were rapidly degraded by the amoebae during the gentamicin incubation (Fig. 2B). There was no significant difference in the numbers of amoeba-associated bacteria or

phagocytosed bacteria between nonpathogenic *Escherichia coli* strain MG1655 and the *Shigella* strains, or between the *Shigella* species.

We repeated the experiments in high-nutrient medium (peptone-yeast-glucose medium [PYG]) to increase the rate of phagocytosis by amoebae. As anticipated, significantly higher numbers of phagocytosed bacteria could be enumerated than in low-nutrient medium (Fig. 2C). On average, there were 0.5 intracellular bacteria/amoeba. However, we again saw no difference between the phagocytosis rates for *S. flexneri* or *S. sonnei* strains. All *Shigella* strains showed a small, nonsignificant trend of increased phagocytosis by amoebae compared to that for nonpathogenic *E. coli*. Microscopic analysis of differentially stained bacteria confirmed the presence of intracellular bacteria for both *S. sonnei* and *S. flexneri* (Fig. 2D). Extracellular bacteria can be seen adhered to the plastic, rather than to the amoebae, suggesting that the amoebae efficiently phagocytose all bacteria contacted.

***Shigella* does not survive intracellularly in *A. castellanii*.** We tested the intracellular survival of *Shigella* by CFU determination at 1 h, 4 h, and 20 h. Cell counts for all strains decreased over this time frame similarly to those of the negative control of nonpathogenic *E. coli*, indicating they were efficiently digested by *A. castellanii*. The same trend of reduced intracellular numbers over time was observed in both high- (Fig. 3A) and low-nutrient media (Fig. 3B), with a 2-log decrease between 1 h and 20 h. Due to the low number of phagocytosed bacteria in low-nutrient medium, by 20 h all strains were below the limit of detection, unlike in high-nutrient medium, where approximately 1,000 bacteria/sample were still recoverable.

To confirm that the amoebae were capable of facilitating intracellular growth, *L. pneumophila* was used as a positive control. These experiments were conducted at 30°C, as this is the optimal temperature for *L. pneumophila* growth (Fig. 3C). As expected, the numbers of culturable wild-type *L. pneumophila* cells increased over the 20 h of incubation, whereas cell counts for all *Shigella* strains again decreased, in line with those of nonpathogenic *E. coli*.

To investigate the intracellular fate of bacteria, we observed the association of bacteria with amoebae stained with neutral red (Fig. 3D). Neutral red preferentially accumulates in lysosomes, due to their relative acidity (22, 23). *S. flexneri*, *S. sonnei*, and *E. coli* were all observed to have neutral red structures accumulating around the intracellular bacteria and to be infiltrated with neutral red, suggesting they were being digested by the amoebae (24). This provides a visual confirmation of the intracellular killing observed in the preceding assays by bacterial enumeration.

The T3SS does not alter *Shigella* interaction with amoebae. Considering the importance of the type 3 secretion system (T3SS) for *Shigella* virulence, the intracellular survival assays were repeated at 37°C, the temperature at which the T3SS is active and effector proteins are translocated (25). *Shigella* bacteria induce different T3SS-dependent outcomes depending on the cell type infected; in epithelial cells, vacuolar escape and intracellular replication, and in macrophages, vacuolar escape and pyroptosis.

If the T3SS facilitated intracellular survival within amoebae, we would expect to see increased intracellular bacterial counts at 4 h and 20 h during incubation at 37°C. Instead, we saw a decrease in viable intracellular bacteria numbers, similar to those at 22°C and 30°C, suggesting that an active T3SS could not facilitate intracellular survival in amoebae (Fig. 4A). To further investigate the involvement of the T3SS, the intracellular survival of T3SS mutants was determined. Again, no difference in intracellular survival between wild-type and T3SS mutants was found (Fig. 4B), indicating that the T3SS was not altering the interaction of *Shigella* with amoebae.

It was previously reported that *S. flexneri* used its T3SS to kill amoebae (21, 26). Having not seen an effect of the T3SS (Fig. 4A and B), or significant amoeba death upon long-term exposure to *S. flexneri* (Fig. 1C), we decided to investigate amoeba cell death further by using a propidium iodide (PI) assay to measure the membrane integrity of the amoebae (Fig. 4C). At all temperatures analyzed, there were no significant differ-

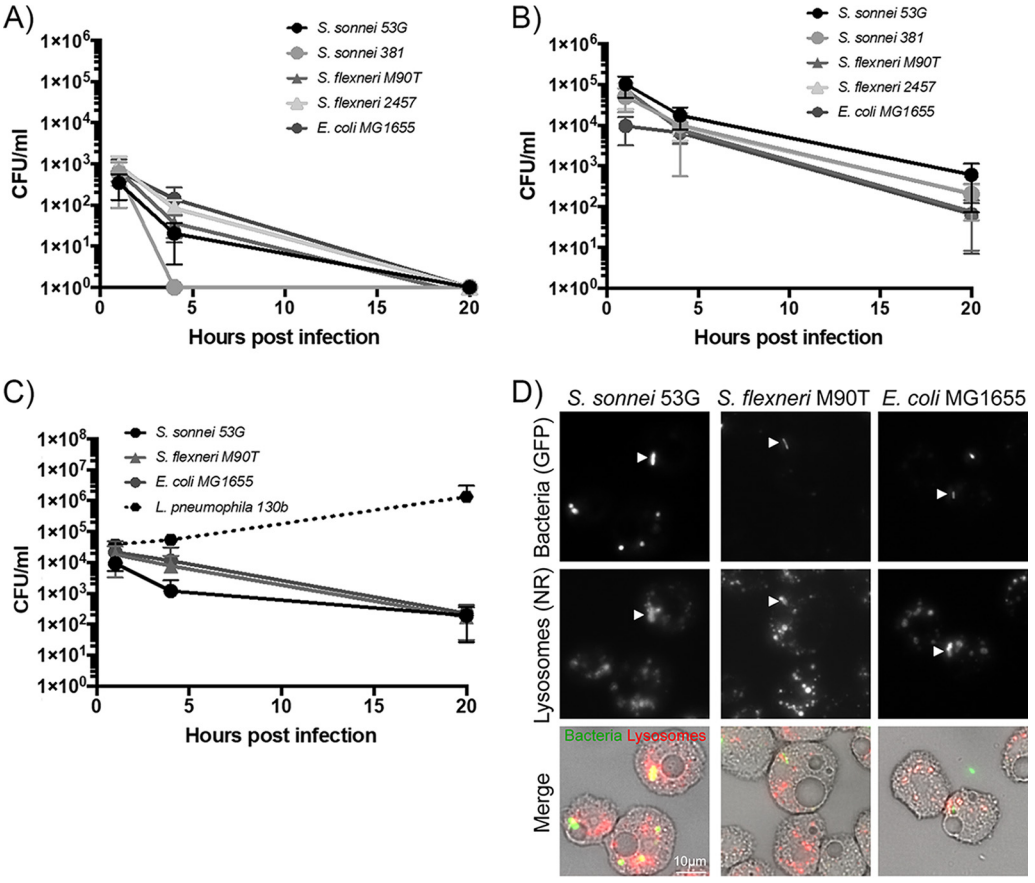

FIG 3 *Shigella* cells are degraded by amoebae while *Legionella pneumophila* cells are able to replicate within amoebae. (A to C) The indicated bacterial strains were incubated with *A. castellanii* for 1 h and then washed and treated with gentamicin for 1 h to kill extracellular bacteria, and the number of bacteria able to survive amoebae degradation was determined after 1 h, 4 h, and 20 h of gentamicin treatment. Experiments were conducted in low-nutrient medium at 22°C (A), high-nutrient medium at 22°C (B), and high-nutrient medium at 30°C (C). Mean and a standard deviation of 3 to 5 repeats are shown. (D) Amoebae stained with neutral red (NR), which preferentially accumulates in lysosomes, were imaged immediately following phagocytosis of the indicated GFP-expressing bacteria. Individual fluorescence channels for bacteria and lysosomes are shown in the top panels. The bottom panel represents merged transmitted-light images (to visualize the amoebae) and fluorescence images (green, bacteria; red, NR lysosomes). Arrows indicate bacteria that are being infiltrated with neutral red dye, indicating lysosomal digestion by the amoebae.

ences in PI levels between amoebae infected with the negative controls (*E. coli* MG1655 and *Shigella* T3SS mutants) and any of the wild-type *Shigella* strains. Therefore, in our assays neither *S. flexneri* or *S. sonnei* were able to induce cell death in amoebae.

***S. sonnei* is not released by amoebae.** *V. cholerae* was recently shown to resist intracellular killing by *A. castellanii*, and at low frequency it can be released intact by the amoebae (16). While we have seen no evidence of *Shigella* resisting intracellular killing, we questioned whether a small number of bacteria were being released from the amoebae. Following killing of extracellular bacteria with gentamicin treatment, fresh medium with no gentamicin was added to cells, and the cell supernatant was harvested after 4 h (Fig. 4D). Low numbers of *Shigella* were recovered from the supernatant, and these could potentially be a source of *Shigella* for infection. However, there was no significant difference in bacterial release between *S. sonnei* and *S. flexneri* strains, and indeed, no significantly increased numbers of bacteria were released compared to those for nonpathogenic *E. coli*, indicating this is not a *Shigella*- or *S. sonnei*-specific mechanism for dispersal.

Intracellular *Shigella* bacteria are not more infectious. Amoebae have been proposed to act as "training grounds" for intracellular pathogens, adapting them to an intracellular lifestyle (27). While this is considered a long-term adaptation, we ques-

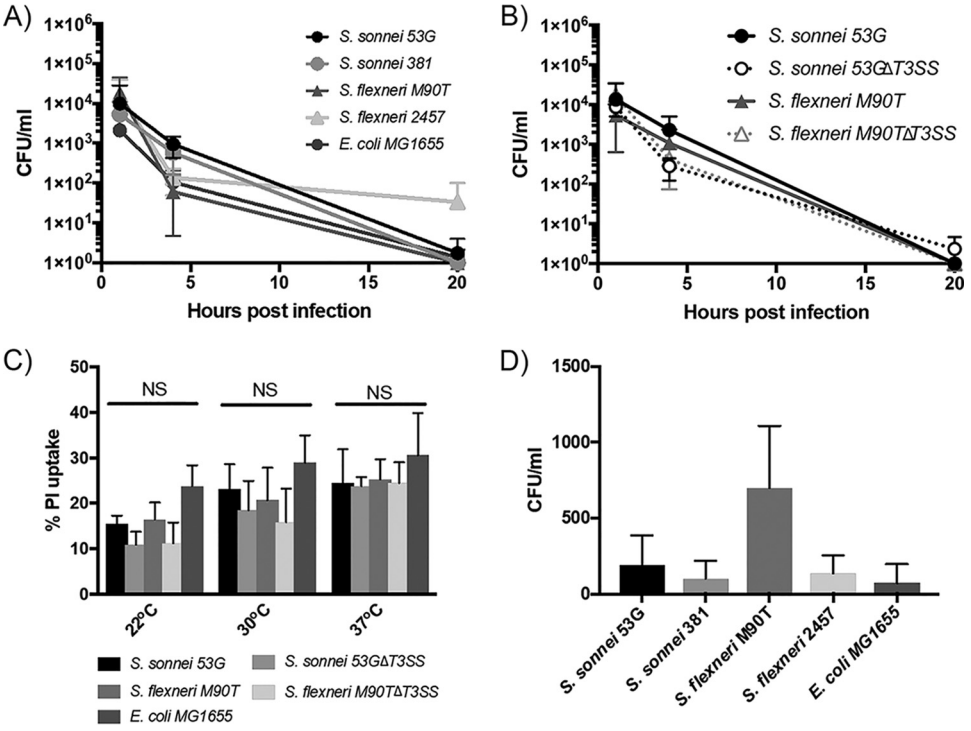

FIG 4 The T3SS does not enhance *Shigella* survival in amoebae. (A) Following phagocytosis, the number of bacteria able to survive degradation by amoebae was determined after 1 h, 4 h, and 20 h of gentamicin treatment during incubation at 37°C to activate the T3SS. (B) The intracellular survival was also determined for *S. flexneri* M90T and *S. sonnei* 53G T3SS mutants and compared to those of parental strains. (C) Propidium iodide uptake was used to measure membrane integrity in amoebae exposed to the indicated strains for 3 h. All values are a percentage of the maximum PI uptake calculated for amoebae exposed to 0.25% sodium deoxycholate for 10 min. (D) The indicated bacterial strains were incubated with *A. castellanii* for 1 h and then washed and treated with gentamicin for 1 h to kill extracellular bacteria. Fresh medium without gentamicin was then added for a further 3 h, after which time the supernatant was harvested and the number of released bacteria determined by colony counting. For all assays, two-way ANOVA indicated no significant differences between any strains at any time point. Mean and a standard deviation of 3 repeats are shown.

tioned whether it facilitated short-term infectivity as well. We therefore tested whether bacteria harvested from amoebae were more proficient at invading or replicating within mammalian cells. We found the bacteria harvested from amoebae were less able to invade and survive in mammalian cells (Fig. 5A and B). These findings support the previous conclusion that *Shigella* bacteria are being degraded by the amoebae, rather than adapting and surviving.

DISCUSSION

The frequency of *S. sonnei* isolation directly correlates with per capita gross domestic product (GDP) (28). The underlying reason(s) for this association is not understood, although a number of hypotheses have been proposed, one of which is that *S. sonnei* uses amoebae as a protective host (10). We show here that *S. sonnei* has no survival advantage in amoebae compared to *S. flexneri*, or indeed, compared to nonpathogenic *E. coli*. Both *Shigella* species were able to survive in long term coculture assays in low-nutrient medium suggesting that, like *Listeria monocytogenes*, *Shigella* species can utilize amoeba debris for nutritional requirements (17). However, this offers no explanation for why *S. flexneri*, but not *S. sonnei*, levels of infection are reduced in areas where living conditions and water sanitation are improved.

Having disproven one hypothesis explaining the emergence of *S. sonnei*, it remains to experimentally test additional hypotheses. One popular suggestion is that exposure to unsanitized water in developing countries can result in *Plesiomonas shigelloides* infection and hence in natural immunity against *S. sonnei*. *P. shigelloides* serotype O17

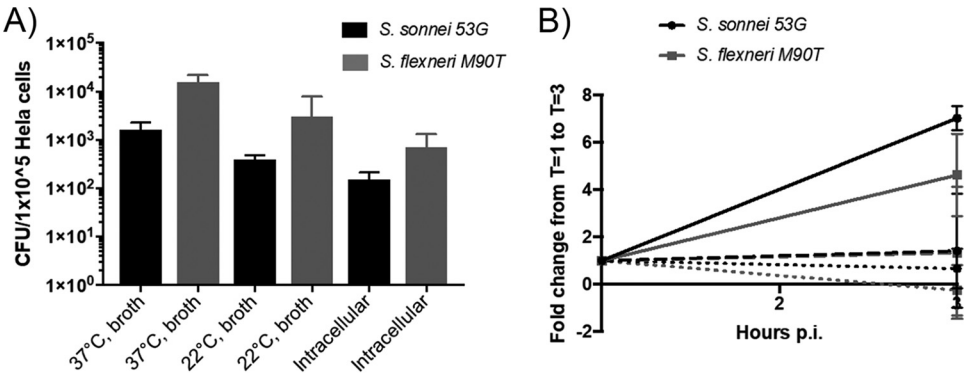

FIG 5 Intracellular bacteria are not hyperinfectious for epithelial cells. (A) Bacteria harvested from amoebae following 1 h of gentamicin treatment were used to directly infect epithelial cells, in parallel with standard log-phase bacteria grown in TSB at 37°C or 22°C. Following a 30 min infection and 1 h of gentamicin treatment, intracellular bacteria were released, and invasive bacteria was enumerated by colony counting. (B) The intracellular survival of these bacteria was measured after 3 h of gentamicin treatment. The fold change from 1 h to 3 h postinfection was calculated. Solid lines indicate bacteria grown in TSB at 37°C, dashed lines indicate bacteria grown in TSB at 22°C, and dotted lines indicate bacteria harvested from amoebae.

has a lipopolysaccharide O-antigen identical to that of *S. sonnei* (10). People living in areas with good water sanitation would therefore have reduced exposure to *P. shigelloides*, and hence reduced cross-protection against *S. sonnei*. This hypothesis is difficult to prove without widespread serological data from countries with high *S. flexneri* versus high *S. sonnei* infection rates. However, it also suggests additional differences regarding the transmission of *S. sonnei*, as the reduced exposure to *P. shigellelloides* and *S. flexneri* through improved water quality does not extend to *S. sonnei*. This could be explained by the suggestion that *S. sonnei* is spread directly from person to person or that *S. sonnei* has an increased ability to acquire antibiotic resistance. Acquisition of antimicrobial resistance has clearly aided the spread and establishment of particular *S. sonnei* isolates (29). However, epidemiological data indicate that *S. flexneri* and *S. sonnei* isolates have similar resistance profiles (30, 31), suggesting antibiotic resistance alone does not explain the altered transmission.

S. sonnei possesses multiple antibacterial mechanisms. The majority of clinical isolates produce colicins (29, 32, 33), which are effective against a narrow phylogenetic range of bacteria. While *S. flexneri* cells are also reported to produce bacteriocins (34), there are few studies indicating the prevalence or identity of bacteriocins in *S. flexneri* clinical isolates. *S. sonnei* has also recently been shown to have a functional type 6 secretion system (T6SS), which provides a niche-specific competitive advantage for *S. sonnei* over *S. flexneri* (35). Therefore, the success of *S. sonnei* may be explained by a combination of these factors potentially altering colonization dynamics and facilitating person-to-person spread.

We have demonstrated that amoebae are not a protective host for *S. sonnei* and that alternative explanations for the rising rates of *S. sonnei* infection in transitional countries require further investigation. Now that it is well established that *S. sonnei* possesses unique pathogenic traits (35–37), considerable work is required to understand the differences in virulence and transmission of *S. sonnei* in comparison to those of *S. flexneri*.

MATERIALS AND METHODS

Bacterial strains and growth. Isolates of *Shigella* (Table 1) were plated on trypticase soya agar (TSA) + 0.01% Congo red to identify those with a large virulence plasmid (LVP) (38). Colonies were inoculated in trypticase soya broth (TSB) and incubated overnight at 37°C and 200 rpm. The overnight culture was diluted 1:100 in TSB and incubated until an optical density at 600 nm (OD_{600}) of 0.5 was reached. Bacteria were washed in phosphate-buffered saline (PBS), resuspended in the appropriate medium, and added to cells.

Legionella cells were plated on buffered charcoal-yeast extract (CYE) agar plates for 3 days at 37°C. Bacterial colonies were diluted to OD_{600} = 0.1 in ACES [*N*-(2-acetamido)-2-aminoethanesulfonic acid] yeast extract (AYE) broth and incubated for 21 h at 37°C and 200 rpm.

TABLE 1 Bacterial strains

Strain	Details	Source and/or reference
S. sonnei 53G	Clinical isolate	36
S. sonnei 381	Clinical isolate H140860381	C. Jenkins, PHE[b]
S. flexneri M90T	Serotype 5a	37
S. flexneri 2457T	Serotype 2a	38
S. flexneri M90TΔT3SS	*mxiD* replaced with *aphA-3*, conferring kanamycin resistance	39
S. sonnei 53GΔT3SS	*mxiD* replaced with *aphA-3*, conferring kanamycin resistance	This study
S. sonnei 53G/GFP	Expresses GFP[a] from pUltraGFP-GM	This study, 42
S. flexneri M90T/GFP	Expresses GFP from pUltraGFP-GM	This study
L. pneumophila 130b	Serotype O1; clinical isolate	ATCC BAA-74 (41)

[a]GFP, green fluorescent protein.
[b]PHE, Public Health England.

Cell culture. *Acanthamoeba castellanii* cells (a kind gift from C. Buchrieser, Institut Pasteur) were cultured in ATCC 712 peptone-yeast-glucose medium [PYG] medium 2% protease peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM $MgSO_4 \cdot 47H_2O$, 0.4 mM $CaCl_2$, 0.05 mM $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2.5 mM KH_2PO_4, 2.5 mM $Na_2HPO_4 \cdot 7H_2O$, and 0.01% Na citrate$\cdot 2H_2O$) in tissue culture flasks at 22°C. During infections, amoebae were seeded in PAS medium (2 mM $NaCl_2$, 16 μM $MgSO_4 \cdot 7H_2O$, 26 μM CaCl2$\cdot 2H_2O$, 1 mM Na2HPO4, and 1 mM KH_2PO_4) or PYG.

HeLa cells were maintained and seeded in Dulbecco's modified Eagle medium (DMEM), 1,000 mg/ml glucose, supplemented with 10% fetal bovine serum (FBS) and 5% Glutamax and incubated at 37°C and 5% CO_2.

Mutagenesis. The T3SS mutant was created by deletion of *mxiD* using lambda red recombination (39, 40). A DNA fragment was created by amplifying the kanamycin gene from pKD4 (with primer pair 5′-tgtgtaggctggagctgcttc-3′ and 5′-catatgaatatcctccttag-3′), 500 bp upstream of *mxiD* (with primer pair 5′-tagggataacagggtaatcagtggtgctctagtagagc-3′ and 5′-gaagcagctccagcctacacaggtaacaatacaatcaagag-3′) and 500 bp downstream of *mxiD* (with primer pair 5′-ctaaggaggatattcatatgggaagacgaaaaatcattgg-3′ and 5′-tagggataacagggtaatcgacttgttcagcagtaagatc-3′), followed by overlapping PCR (I-SceI sites are underlined). This fragment was cloned into pGEM T-Easy and transformed into *S. sonnei* 53G alongside pACBSR. *mxiD* was then replaced with the kanamycin resistance gene with the help of lambda red recombinase and I-SceI induced from pACBSR.

Amoeba coculture. *A. castellanii* was seeded at 10^6 cells/flask in T75 tissue culture flasks in PAS medium. Amoebae were infected with *Shigella* at a multiplicity of infection (MOI) of 10. Every other day 10^7 heat-killed (100°C for 20 min) *E. coli* DH5α was added to flasks to prevent amoebae starvation. At time points indicated, 1 ml of culture was removed. Ten microliters was used to count amoebae on a hemocytometer. Twenty microliters was used to perform serial dilutions to calculate coculture CFU. Remaining sample was washed with PAS and treated with 150 μg/ml gentamicin for 1 h. Amoebae were washed again with PAS and lysed with 0.25% sodium deoxycholate. Serial dilutions were performed to calculate intracellular CFU.

Infection of amoebae. *A. castellanii* was seeded at 1×10^5/ml in 24-well plates and infected with an MOI of 100. To synchronize infection, plates were centrifuged at 600 $\times$ *g* for 10 min and then incubated for 50 min at 22°C or 37°C. Cells were washed with PAS, and 150 μg/ml gentamicin in PAS/PYG was then added to cells for 1 h. If required, medium was replaced with 20 μg/ml gentamicin in PAS/PYG for a further 3 h or 19 h. At the time points indicated, cells were washed with PAS and lysed with 0.25% sodium deoxycholate for 10 min. Serial dilutions were carried out and plated to calculate intracellular CFU. Experiments using *L. pneumophila* were performed at 30°C in PYG, and amoebae were infected with *L. pneumophila* at an MOI of 1. To enumerate intracellular CFU in these experiments, amoebae were washed in PAS, detached from the well, and lysed by vortexing for 10 s. Serial dilutions were performed and plated on appropriate agar. All other experimental steps were as above.

Fluorescence microscopy. Amoebae seeded on 4-well μ-slides (Ibidi) were infected as above with GFP (green fluorescent protein)-expressing *S. sonnei* 53G and GFP-expressing *S. flexneri* M90T. Amoebae were then washed with low-fluorescence (LF) medium (41) and placed on ice, and extracellular bacteria were detected with sera against *S. sonnei* (Remel agglutinating sera) or *S. flexneri* 5a (Public Health England [PHE]) in 2% BSA in LF medium for 30 min. Amoebae were washed with ice-cold LF medium, followed by anti-rabbit Cy3 (Jackson ImmunoResearch) in 2% BSA in PAS for 30 min. Amoebae were washed with LF medium before being overlaid with agarose and immediately imaged on a Zeiss Axio Observer inverted microscope.

For neutral red staining, amoebae were incubated in 125 μM neutral red in LF medium for 20 min at room temperature (RT). Amoebae were washed in LF medium before addition of bacteria harboring GFP and centrifuged briefly (2 min, 1,000 $\times$ *g*). Amoebae were overlaid with agarose and immediately imaged on a Zeiss Axio Observer inverted microscope.

PI assay. *A. castellanii* was seeded as described previously in 24-well plates. Immediately prior to infection, medium was replaced with 5 μM propidium iodide in PAS. Infection was carried out as described, and cells were incubated at 22°C, 30°C, or 37°C. At 1 h postinfection, gentamicin was added directly to wells to a final concentration of 150 μg/ml for 3 h. For 100% membrane permeabilization control, sodium deoxycholate was added to wells at a final concentration of 0.25% for 10 min. Fluorescence was measured at 530/620 nm on a FLUOstar Omega microplate reader (BMG Labtech).

HeLa cell infection. HeLa cells were seeded in 96-well plates at 1×10^5 cells/ml 24 h prior to infection. *A. castellanii* was seeded at 10^7 cells/flask in T75 flasks in PAS medium, infected with *Shigella* at an MOI of 100, and incubated at 22°C. After 24 h, amoebae were detached, centrifuged at $500 \times g$ for 5 min, and resuspended in 150 μg/ml gentamicin in PAS for 1 h. Amoebae were then washed with PAS and lysed with 0.25% sodium deoxycholate. Released bacteria were centrifuged, washed, and resuspended in DMEM. Broth-cultured *Shigella* cells were prepared as described above and resuspended in DMEM. Prior to infection, medium was replaced with serum-free DMEM, and HeLa cells then infected at an MOI of 100 with *Shigella* cells released from amoebae or grown in broth at 37°C or 22°C. Cells were centrifuged at $600 \times g$ for 10 min and incubated for 30 min at 37°C and 5% CO_2. Medium was replaced with 150 μg/ml gentamicin in supplemented DMEM and incubated for a further 1 h or 3 h. At these time points, cells were washed with PBS and lysed with 0.5% Triton X-100. Serial dilutions were performed and plated to calculate intracellular CFU.

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