Isolation and identification of freshwater bacteria antagonistic to *Giardia intestinalis* cysts

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ABSTRACT

We have isolated three freshwater bacterial strains that demonstrate the ability to degrade *Giardia intestinalis* cysts. These strains have been identified by 16S rRNA sequencing and phylogenetic analysis as belonging to the *Flavobacterium columnare* clade of the *Cytophaga-Flavobacterium* group. While the cyst degradation mechanism is unclear, two different effects on the cysts were observed: non-viability and lysis. Cysts exposed to bacterial strains BR1 and SC1 were generally non-viable, but remained structurally intact. In contrast, cysts exposed to strain SR1 were clearly lysed. Increases in bacterial densities with a concomitant decrease in cyst viability suggest that these bacterial strains are capable of using the cysts to enhance their growth. We propose that the presence of bacterial strains such as SR1, BR1 and SC1 may play a role in reducing the viability of *G. intestinalis* cysts in natural waters.

Key words | cyst degradation, *Cytophaga-Flavobacterium*, *Giardia intestinalis*, microbial antagonism

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INTRODUCTION

Giardia intestinalis is a protozoan parasite that is recognized as one of the most common causes of waterborne disease in the United States (Lee *et al.* 2002). The infective cyst form of *Giardia* is transmitted to susceptible human and animal populations *via* water. *Giardia* has a number of mammalian reservoir hosts, and is considered a common biological contaminant of North American surface waters (Hibler 1988). LeChevallier *et al.* (1991) examined source waters of 66 surface water treatment plants for the occurrence of *Giardia* spp.; *Giardia* cysts were detected in 69 of 85 (81%) raw water samples. Despite the widespread occurrence of *Giardia* and the potential public health risk of disease resulting from contamination of drinking water supplies, little is known about the ecology of *Giardia* cysts in natural waters. Furthermore, there is evidence that a majority of *Giardia* cysts in the environment may be non-viable: LeChevallier *et al.* (1991) reported that of 618 *Giardia* cysts observed, only 12.8% showed viable type morphologies.

While previous research on cyst survival has primarily focused on physical and chemical factors (deRegnier *et al.* 1989), there has been growing interest in the role of microbial antagonism in the survival of *Giardia* cysts (Deng & Cliver 1992; Sattar *et al.* 1999). In one study, *G. intestinalis* cysts were incubated in test waters for 48 h at 20 °C. Cyst survival, as measured by percentage reduction in total number of cysts, was nearly 50% higher in reverse osmosis water and filter-sterilized river water compared with unfiltered river water. In a study on the survival of *G. intestinalis* cysts in mixed human and swine wastes, the lower the temperature of the mixed waste, the longer the cysts survived. Although these studies were unable to determine the individual causative agents responsible for decreased cyst survival, bacteria were certainly present and may have had a role in cyst die-off. In the present study, we examined the ability of heterotrophic bacterial isolates from a surface water source to degrade *G. intestinalis* cysts in the laboratory.

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MATERIALS AND METHODS

Isolation of environmental strains

Twenty-two distinct colony morphotypes were isolated on R2A (Difco, Detroit, Michigan) agar inoculated with water samples collected in July 1999 from Three Mile Creek in Kentucky. Samples were obtained from free-flowing areas, stagnant pools and from biofilm on rocks. Each isolate was purified by sub-culturing onto R2A agar. While all isolates grew on R2A agar, eight were unable to grow in R2A broth (Atlas 1997) without agar and made with either filter-sterilized (0.22 μm pore diameter) creek water or dH_2O. The eight fastidious isolates were grown in modified R2A broth prepared by adding dH_2O to dehydrated R2A agar medium, allowing the soluble components of the medium to dissolve at room temperature for one hour and filtering out the agar prior to sterilization by autoclaving. This modified R2A broth was used to grow all isolates for subsequent DNA extraction, viability and degradation assays.

Giardia cysts

Each isolate was initially screened for degradative potential against cysts of *G. intestinalis* strain CDC:0284:01. Due to poor survival of the CDC:0284:01 strain, *G. intestinalis* strain H3 cysts (Waterborne, Inc., New Orleans, Louisiana) were used for all subsequent degradation assays. Cysts were received in batches of 10^6 total cysts in 8 ml stock cyst suspension. Cysts were produced in Mongolian gerbils according to Belosevic *et al.* (1983), and harvested from feces using the sucrose flotation method of Roberts-Thomson *et al.* (1976) and Percoll density gradient centrifugation (Sauch 1984). Cysts were stored at 4 °C in phosphate-buffered saline and 0.01% Tween 20 at a final concentration of 10^5 per ml with an antibiotic cocktail (100 units penicillin and 0.1 mg ml^{-1} streptomycin) to inhibit bacterial growth. Cysts were used in degradation assays within 4 days of harvest.

Degradation assay

One ml aliquots of bacterial isolates in R2A broth were pelleted at 10,000 *g* for 2 minutes and washed twice with filter-sterilized creek water to remove residual nutrients. The final bacterial pellet was re-suspended in filter-sterilized creek water. Stock cyst suspension was pelleted at 2,000 *g* for 5 minutes and washed twice with 0.01% Tween 20. The final cyst pellet was re-suspended in filter-sterilized creek water. Cysts (10^5 ml^{-1}) and bacterial cells (10^4 ml^{-1}) were added together with filter-sterilized creek water in sterile 15 ml polypropylene conical tubes. Controls consisted of tubes containing cysts or bacteria in filter-sterilized creek water. Treatment and control tubes were run in triplicate. All tubes were incubated at 20 °C for 72 h and sampled at 12 h intervals for cyst viability and bacteria enumeration by serial dilution and plate count.

Viability

Cysts were stained with propidium iodide (PI) as described by Sauch *et al.* (1991). A stock solution of PI was prepared by dissolving 5 mg of PI in 50 ml of Dulbecco phosphate-buffered saline, pH 7. Fifty μl of cyst suspension of each sample was added to 15 μl of the stock solution, and incubated at room temperature for 10 minutes prior to examination by epifluoresence microscopy. The PI-stained samples were examined with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Oberkochen, Germany) equipped with a Plan-Neofluar objective (magnification, ×40). Excitation was carried out with a 50 W mercury bulb, band pass 450 to 490 nm exciter filter, 510 nm chromatic beam splitter, and long pass 520 nm barrier filter. Viability of cysts was determined by microscopic appearance and PI exclusion (Schupp & Erlandsen 1987*a*, *b*). Cysts were considered viable if they had both a bright, hyaline appearance (phase bright) and were non-fluorescent after staining with PI. Two haemocytometer chambers were counted for treatment and control tubes in triplicate at each time point for SR1 (n = 5,455), BR1 (n = 8,048) and SC1 (n = 9,295).

Extraction of DNA and PCR/sequencing

Preparation of genomic DNA from bacterial isolates was conducted either as described by Ausubel *et al.* (1995) or by using a commercial genomic DNA purification kit (Wizard, Promega, Madison, Wisconsin). The 16S ribosomal RNA gene

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was amplified with universal primers EC8 (AGAGTTTGATCCTGGCTCAG) and EC1492 (GGTTACCTTGTTACGACTT). PCR amplification was conducted with AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, California) and 25 cycles of 94 °C for 1 minute, 56 °C for 1 minute, and 72 °C for 1 minute. PCR products were analysed by electrophoresis in 1.5% agarose gels and purified with Microcon columns (Millipore, Bedford, Massachusetts). Alternatively, PCR products were gel-purified using a QIAquick gel extraction kit (QIAGEN, Valencia, California). Conserved primers (Lane *et al.* 1985) were used to fully sequence the 16S rDNA of the amplified fragments, using the Big Dye Kit (Applied Biosystems) chemistry following the manufacturer's instructions. Sequencing products were purified with Centri-Sep columns (Adelphia, New Jersey) and analysed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

Phylogenetic analysis of rDNA sequences

Closely related sequences were identified using the Advanced Blast Search program available from GenBank (Benson *et al.* 2000) and the SequenceMatch program from the Ribosomal Database Project (Maidak *et al.* 2001). For phylogenetic analysis the sequences were initially aligned using ClustalW version 1.4 (Thompson *et al.* 1994). Additional manual alignment was performed on the basis of conserved features of primary and secondary structures. Neighbour-joining, maximum-likelihood and maximum-parsimony analyses were conducted as implemented by PAUP * version 4.0b10 (Swofford 2000). *Cytophaga lytica* was used as the outgroup to root the tree. Random stepwise addition of taxa was used. Bootstrap resampling was performed and values reported represent percentages of 1,000 replications.

Data analysis

Statistical analyses were carried out using Excel (Microsoft Office 2000), Systat (Richmond, California) and Resampling Stats for Excel Version 2.0 (Resampling Stats Inc., Arlington, Virginia). Comparisons between treatment and control tubes were made using *t* tests with pairing on log-transformed viable cyst counts. Non-parametric methods were used to substantiate the comparisons. Statistical significance was assumed at a P value of <0.05.

Nucleotide sequence accession numbers

The 16S rDNA sequences of environmental isolates SR1, BR1 and SC1 are available from GenBank under accession numbers AY154889, AY154890 and AY154891, respectively.

RESULTS AND DISCUSSION

Twenty-two isolates were initially incubated overnight with cysts in filter-sterilized creek water to screen for their effect on cyst viability. Eleven isolates appeared to have an effect on the viability of the cysts as determined by microscopic appearance and PI exclusion (data not shown). Three isolates, SR1, BR1 and SC1, demonstrated the greatest effect and were tested in more depth.

Figure 1a–c shows the results of incubating strains SR1, BR1 and SC1 with *G. intestinalis* cysts. Cyst viability decreased and the test bacteria density increased in all tubes in which the cysts and test bacteria were incubated together. The number of bacteria in bacteria-only controls declined over time for SR1 (range, 5.6×10^4 CFU ml^{-1} to 8×10^3 CFU ml^{-1}), BR1 (range, 1.7×10^4 CFU ml^{-1} to 2×10^3 CFU ml^{-1}) and SC1 (range, 2×10^5 CFU ml^{-1} to 4.1×10^4 CFU ml^{-1}). In comparison, the concentration of SR1, BR1 and SC1 bacteria in tubes containing both bacteria and cysts increased ($P < 0.05$). The cyst die-off was most pronounced with SR1 and BR1; at 72 h no viable cysts remained in these tubes. With all three bacteria strains tested, the difference between the number of viable cysts in tubes containing cysts with bacteria versus control tubes containing only cysts was statistically significant ($P < 0.05$).

Phylogenetic analysis of 16S rDNA indicates that strains SR1, BR1 and SC1 fall within the *Flavobacterium columnare* clade of the *Cytophaga-Flavobacterium* group (Figure 2). Strains SR1, BR1 and SC1 each possess the signature sequences of the *Cytophaga-Flavobacterium* group, including a C residue at position 328 (Woese 1987). A search of the GenBank database shows that SR1, BR1 and SC1 have 98%, 97% and 98% sequence identity, respectively, to *Cytophaga* sp. str. Type 0092, a Gram negative, aerobic, filamentous microorganism previously found in activated sludge mixed liquors from several wastewater treatment facilities (Bradford *et al.* 1996).

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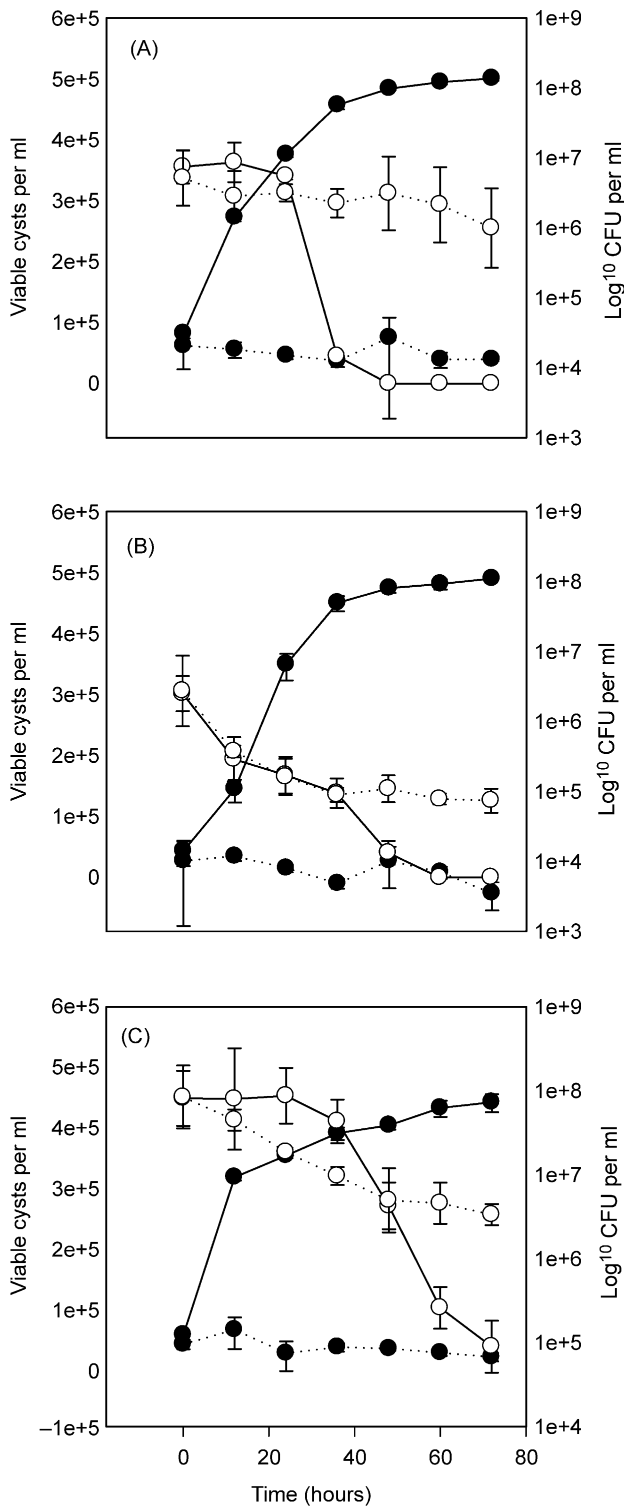

Figure 1 | Cyst degradation with corresponding cell growth of bacterial strains for microcosms containing cysts with bacteria (cysts —○—, bacteria —●—), cysts alone (- - -○- - -) and bacteria alone (- - -●- - -) in filter-sterilized river water incubated at 20 °C for 72 h. (A) strain SR1, (B) strain BR1, (C) strain SC1.

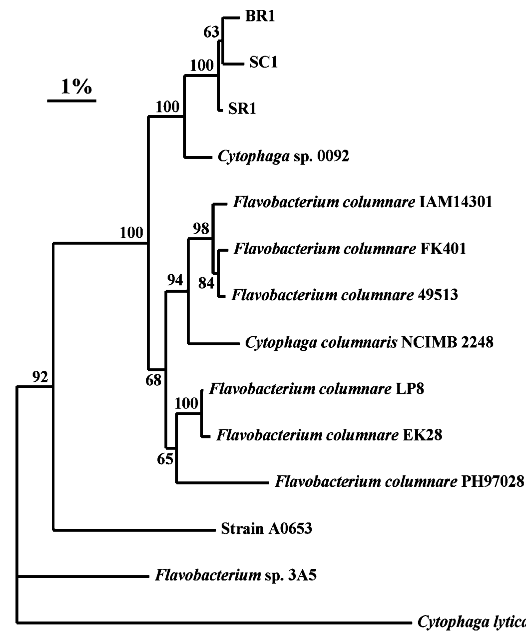

Figure 2 | Neighbour-joining tree for sequences of SR1, BR1 and SC1 with sequences of closely related strains, and *Cytophaga lytica* used as an outgroup. Bootstrap values are percentages of 1,000 replications. The scale bar corresponds to 0.01 estimated nucleotide substitutions per sequence position. The *Cytophaga-Flavobacterium* spp. (accession numbers) are as follows: BR1 (AY154890), SC1 (AY154891), SR1 (AY154889), *Cytophaga* sp. 0092 (X85210), *F. columnare* IAM14301 (AB010951), *F. columnare* FK401 (AB010952), *F. columnare* 49513 (AB023660), *C. columnaris* NCIMB2248 (CYT16SRR06), *F. columnare* LP8 (AB015480), *F. columnare* EK28 (AB016515), *F. columnare* PH97028 (AB015481), CFB strain A0653 (AF236016), *Flavobacterium* sp. 3A5 (AF368756.1), and *C. lytica* (M62796).

Members of the *Cytophaga-Flavobacterium* group are not only important constituents of wastewater, but are among the most frequently identified heterotrophic bacteria in surface waters, both marine and freshwater (Kirchman 2002), and they are a dominant and stable component of river and stream ecosystems (Manz *et al.* 1999). This group of bacteria exhibit a wide range of phenotypic diversity, and their ability to degrade complex biopolymers suggests an important role in the cycling of nutrients in the environment (Reichenbach 1992).

The ability of *G. intestinalis* cysts to survive for extended periods outside a suitable host is in part dependent on the highly protective nature of the cyst wall. The cyst wall consists of an inner double membrane and an outer filamentous portion arranged in a tightly packed meshwork (Erlandsen *et al.* 1996, 1989). Isolated cyst wall material contains carbohydrate and protein, and the carbohydrate portion is composed of a novel polysaccharide,

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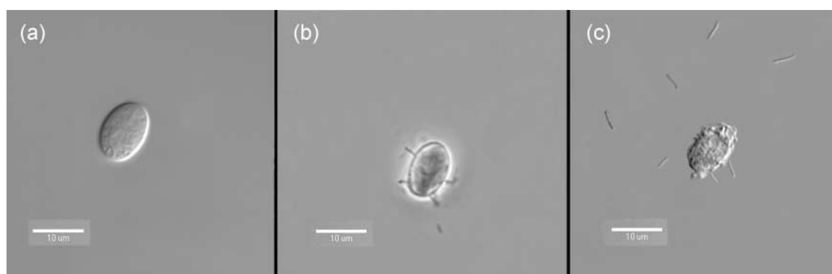

Figure 3 | Photomicrograph showing the appearance of the external surface of the *G. intestinalis* cyst wall. (a) *G. intestinalis* untreated control cyst (1,000X); (b) *G. intestinalis* cyst exposed to strain SRI (1,000X); (c) *G. intestinalis* cyst after incubation with strain SR1 at 20°C for 19 h (1,000X). Note that the integrity of the meshwork of tightly packed filaments has been compromised. Scale bar indicates 10 μm.

β(1-3)-*N*-acetyl-D-galactopyranosamine (Gerwig *et al.* 2002). The highly protective nature of the cyst wall is due to strong GalNAc polysaccharide interchain interactions and the potential covalent linkage between the protein and the polymers (Gerwig *et al.* 2002). In the current study, two different cyst degradation effects were observed: non-viability and lysis. Cysts incubated with strains BR1 or SC1 were non-viable as measured by PI, but not generally lysed (data not shown). In contrast, strain SR1 had a dramatic effect on the physical appearance of the cysts. Intact, viable cysts were either reduced to shrunken non-viable particles or completely lysed, with only debris remaining (Figure 3). It is unclear, however, what mechanism these three bacterial strains use to kill the cysts or the identity of the exact growth substrates they are obtaining from the cysts.

Cytophaga spp. are important predatory agents in fresh and marine waters where they are known to cause lysis of many genera of cyanobacteria (Daft *et al.* 1975; Imai *et al.* 1993; Rashidan & Bird 2001). These non-obligate predator bacteria do not require the presence of prey cells for survival and can survive on alternative food sources in the absence of prey. Moreover, the isolation of a *Flavobacterium* strain capable of killing *G. intestinalis* cysts (Rodgers *et al.* 2003) was recently reported, and this bacterial strain has been proposed to contribute to the common observation that most *Giardia* cysts recovered from natural waters are non-viable.

CONCLUSIONS

Much work remains to be done to identify the factors controlling the survival of *Giardia* cysts in natural waters. In this study, 50% of the isolated heterotrophic bacteria tested reduced cyst viability, and three strains belonging to the *Cytophaga-Flavobacterium* group were shown to rapidly kill the cysts. Further research is required, however, to elucidate the mechanism that strains SR1, BR1 and SC1 use to kill *G. intestinalis* cysts.

This study systematically investigated the effect of individual bacterial strains as potential stressors on *Giardia* cysts under laboratory-controlled conditions. Each of the bacterial strains was evaluated alone, shedding no light on possible additive or antagonistic effects between bacterial strains. Furthermore, laboratory-controlled systems are unable to accurately mimic all parameters of natural water sources. Characterizing the survival of cysts in natural waters is important and, in future experiments, cysts and bacterial strains could be placed in membrane diffusion chambers and submerged in natural waters. The advantage of this system over a model laboratory system is that a continuous exchange of water and solutes come in contact with the cysts and bacteria enabling the system to be responsive to physical, chemical and biological changes that may occur in the natural aquatic system.

The present study supports other published reports suggesting that naturally occurring bacteria play a significant role in the environmental fate of *Giardia* cysts. Cysts may encounter different populations of bacteria depending on whether they are suspended in the water column or attached to biofilms on submerged surfaces. Whatever the environmental microniche, we propose that bacteria such as strains SR1, BR1 and SC1 play a significant role in cyst survival in natural waters.

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