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Infection of *Tetrahymena pyriformis* by *Legionella longbeachae* and Other *Legionella* Species Found in Potting Mixes

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All *Legionella longbeachae* strains, both serogroups of *L. bozemanii*, and three strains of *L. anisa* reproducibly infected washed *Tetrahymena pyriformis* at 30°C. *L. pneumophila* serogroup 1 strains infected *T. pyriformis* less reproducibly than did *L. longbeachae*. Low-level concentrations of nutrients in cocultures inhibited infection. Four *L. micdadei* strains and *L. anisa* ATCC 35292 failed to infect *T. pyriformis*.

In Australia, *Legionella longbeachae* serogroup 1 strains have been a common cause of legionellosis since 1987. This species is also prevalent in composted plant material and composted pine bark used in potting mixes in Australia (5, 8, 9). *L. pneumophila* infection of washed cells of the ciliate *Tetrahymena pyriformis* was described in 1984 (4). In a subsequent report (3), it was suggested that the ability to multiply intracellularly in *T. pyriformis* represents one major virulence factor. In this study, the Tucker 1 strain of *L. longbeachae* (now serogroup 2) and an *L. anisa* strain from water did not infect *T. pyriformis* used directly from Proteose Peptone (PP) medium. It was not known if these strains were attenuated since wild-type strains were unavailable for study. However, a wild-type strain of *L. anisa* implicated in a Pontiac fever outbreak also failed to infect *T. pyriformis* in 1990 (2). Since environmental and human wild-type strains of *L. longbeachae* and other *Legionella* species are plentiful in Australia, we examined these for the ability to infect *T. pyriformis*. We also determined if low-level concentrations of PP in cocultures or laboratory subculturing of legionellae prevented the infection of *T. pyriformis.*

Bacteria. Legionellae were used directly from isolation plates or stored at −70°C in glycerol broth after one or two subcultures on buffered charcoal yeast extract agar with α-ketoglutarate and 1% bovine serum albumin (ABCYE-α). Those tested are listed in Table 1. Our American Type Culture Collection (ATCC) strains had been subcultured more than 10 times. One potting mix strain of *L. longbeachae* serogroup 1 and a human isolate of *L. pneumophila* serogroup 1 were subcultured 18 times on ABCYE-α every 4 days. *Legionella* suspensions were made from a 4-day growth on ABCYE-α by passing a straight Nichrome wire vertically through a colony to the agar surface and suspending the adherent growth in 10 ml of sterile water. They contained approximately 10^6 CFU of legionellae per ml and were used immediately. Determinations of the actual CFU of suspensions were made by spreading duplicate 50-μl volumes of 10-fold dilutions in sterile water on ABCYE-α plates and counting colonies, after incubation at 35°C for 4 to 7 days, with a dissecting microscope.

***Tetrahymena* culture.** A *T. pyriformis* culture, obtained from the University of New South Wales, Randwick, Australia, was grown at 25°C in 25% PY broth in 20 mM buffered Neff's amoeba saline (BNAS) containing 0.12 g of NaCl per liter (6). PY broth contained 20 g of Difco no. 3 PP and 1 g of yeast extract dissolved in 1 liter of distilled water. Cells from 50-ml 3- to 7-day *Tetrahymena* cultures were harvested by gravity filtration through an 8-μm-pore-size cellulose acetate filter, washed three times with BNAS, and suspended in BNAS. Cells were counted microscopically by the method of Fields et al. (4).

Cocultures. As our *Tetrahymena* strain did not survive incubation at 35°C and osmotic shock lysed some cells when they were washed with water, we incubated cocultures at 30°C and used BNAS for washing cells and in cocultures unless otherwise stated. Washed *Tetrahymena* cells and 400 μl of a 10^{-2} dilution of *Legionella* suspension were added to BNAS (final volume, 4 ml) to give 10^4 *Tetrahymena* cells per ml and approximately 10^3 CFU of legionellae per ml. The *Tetrahymena*-to-*Legionella* (T/L) ratio was calculated retrospectively. Cocultures were incubated for 5 to 12 days. Microscopy was used to determine the viability of ciliates daily. The *Legionella* CFU counts in cocultures were determined by preparing 10-fold dilutions of coculture to 10^{-4} in HCl-KCl buffer (1) and immediately spreading 50 μl in duplicate on ABCYE-α medium containing vancomycin and polymyxin B (9) when the majority of ciliates died or on day 12 if cells did not die. The CFU of ATCC strains, Tucker 1, and several wild-type *L. longbeachae* strains were counted at daily intervals by the same method.

L. longbeachae. All *L. longbeachae* isolates, including both ATCC strains and Tucker 1, infected *Tetrahymena* cells reproducibly, destroying them rapidly and multiplying to populations of 10^5 to 10^6 CFU/ml in 5 days. Detailed studies (Fig. 1) showed 10^4-fold increases for a potting mix strain of *L. longbeachae* serogroup 1 and serogroup 2 strains ATCC 33484 and Tucker 1. The increases in the *L. longbeachae* populations in cocultures were proportional to the T/L ratios. The mean increases in population in 5 days were 4.8×10^2-fold at T/L ratios of ≤10, 5.5×10^3-fold at ratios of 11 to 29, and 1.4×10^4-fold at ratios of ≥30. There was considerable overlap in the range of population increases at each ratio. Tested at T/L ratios of 6 and 9, respectively, the population of the subcultured strain of *L. longbeachae* increased 4.4×10^2-fold and that of the control increased 2×10^2-fold in 5 days.

Other *Legionella* spp. Both potting mix strains of *L. bozemanii* and three of four *L. anisa* strains infected *T. pyriformis* and showed 10^3-fold population increases to 10^6 CFU/ml in 3 to 5 days (data not shown). *L. anisa* ATCC 35292 and four *L. micdadei* strains failed to infect *T. pyriformis*. ATCC 35292 was tested three times, each human strain of *L. micdadei* was tested seven times, the environmental strain was tested three times, and ATCC 33218 was tested twice. One human strain of *L.*

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TABLE 1. Sources and identities of *Legionella* strains tested in *T. pyriformis* cocultures

Species	Strain, source, or origin[a]	No. of strains tested
L. longbeachae serogroup 1	Human	6
	Potting mix	10
	ATCC 33462	1
L. longbeachae serogroup 2	Potting mix	1
	ATCC 33484	1
	Tucker 1 (CDC)	1
L. pneumophila serogroup 1	Human	2
	Water	1
	ATCC 33152	1
	RI-243A (CDC)	1
L. bozemanii serogroup 1	Potting mix	1
L. bozemanii serogroup 2	Potting mix	1
L. anisa	Human	1
	Water (New Zealand)	1
	Potting mix	1
	ATCC 35292	1
L. micdadei	Human	2
	Potting mix	1
	ATCC 33218	1

[a] CDC, Centers for Disease Control and Prevention.

pneumophila infected *T. pyriformis* in six of eight tests, increasing 10^2- to 10^4-fold in 4 to 7 days, but the remaining three strains, including ATCC 33152, infected *T. pyriformis* in only 50% of tests, with a mean increase of 4×10^2-fold at T/L ratios of <30 (data not shown). This poor reproducibility was investigated, but the cause was not determined. At a T/L ratio of 10, the populations of the control *L. pneumophila* strain stored at −70°C and the subcultured strain increased 1.25×10^2- and 75-fold, respectively, in 4 days. RI-243A did not infect *T. pyriformis* in five tests. The viability of legionellae was unaffected by subculturing on ABCYE-α agar, BNAS, or 0.25× Ringer's solution with 2.25 g of NaCl per liter (Oxoid, Basingstoke, England), which was used in some controls and tests (see below).

Effects of nutrients and salt solutions. The inhibitory effects of nutrients on infection were determined by testing 0.2, 0.5, 1, 2, 5, and 20 g of PP per liter in BNAS in 4-ml cocultures. Each concentration was tested with two separate inocula of a potting mix *L. longbeachae* strain, 15 and 3.2×10^2 CFU/ml, and with 10^4 BNAS-washed *Tetrahymena* cells per ml. At a concentration of ≥0.5 g/liter, PP prevented the infection of *T. pyriformis*. At 0.2 g of PP per liter, complete inhibition occurred with 15 CFU of *L. longbeachae* per ml, but with the higher inoculum, the CFU count rose 2×10^2-fold in 4 days and 10^4-fold in duplicates without PP, indicating partial inhibition of multiplication. PP also inhibited infection by *L. pneumophila*. Legionellae disappeared from all cocultures containing PP in which infection did not occur. When 0.25× Ringer's solution replaced BNAS in the *Tetrahymena* medium, washes, and cocultures, the inhibitory effect of PP (≥2 g/liter) was abolished. *T. pyriformis* multiplied normally in 25% PY broth in 0.25× Ringer's. Coculture experiments using washed live and heat-killed (68°C, 30 min) *Escherichia coli* cells in BNAS showed that particulate food also inhibited the infection of *T. pyriformis* but permitted the growth of ciliates. Inhibition was also abolished by 0.25× Ringer's solution. Starvation conditions and 2.25 g of NaCl per liter apparently rendered the ciliates more susceptible to infection.

Our results conflict with previous reports (2, 3) in that *L. longbeachae* and some *L. anisa* strains reproducibly infected *T. pyriformis*, *L. pneumophila* gave variable results, and *L. micdadei* failed to infect. Although PP carryover to cocultures could have interfered with the infection of *T. pyriformis* in reported investigations (2, 3), discrepant results could also be due to *T. pyriformis* strain differences. The poor reproducibility of *L. pneumophila* results suggested that laboratory attenuation was not an important factor. We tested another strain of *T. pyriformis* in 1991. It also died rapidly at 35°C but was infected at 30°C by all wild-type and ATCC strains of *L. longbeachae* tested (7a). Host specificity, as described for amoebae (7, 10), could explain the inability of some *Legionella* spp. to infect different *T. pyriformis* strains but could not explain the poor reproducibility of infection by *L. pneumophila* strains.

Our finding that some *Legionella* isolates from humans did not infect *T. pyriformis* highlighted the lack of relationship between human and *Tetrahymena* infections previously noted (3). Investigators using *T. pyriformis* to study invasion and intracellular multiplication of *Legionella* spp. should be aware that strain differences, the presence of food, and other ill-defined factors influence the susceptibility of this protozoan to infection.

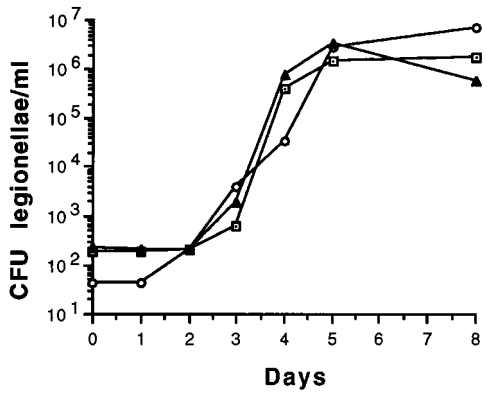

FIG. 1. Mean daily CFU counts of a wild-type *L. longbeachae* serogroup 1 strain from a potting mix (⊡), *L. longbeachae* serogroup 2 strain ATCC 33484 (▲), and Tucker 1 (○) in cocultures with *T. pyriformis* at 30°C. The variation between duplicate readings was 0.5 $\log_{10}$ or less.

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