Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* Enhances Invasion

JEFFREY D. CIRILLO, STANLEY FALKOW, AND LUCY S. TOMPKINS*

Department of Microbiology and Immunology, Stanford University
School of Medicine, Stanford, California 94305

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Legionella pneumophila is considered to be a facultative intracellular parasite. Therefore, the ability of these bacteria to enter, i.e., invade, eukaryotic cells is expected to be a key pathogenic determinant. We compared the invasive ability of bacteria grown under standard laboratory conditions with that of bacteria grown in *Acanthamoeba castellanii*, one of the protozoan species that serves as a natural host for *L. pneumophila* in the environment. Amoeba-grown *L. pneumophila* cells were found to be at least 100-fold more invasive for epithelial cells and 10-fold more invasive for macrophages and *A. castellanii* than were *L. pneumophila* cells grown on agar. Comparison of agar- and amoeba-grown *L. pneumophila* cells by light and electron microscopy demonstrated dramatic differences in the morphology and structure of the bacteria. Analyses of protein expression in the two strains of bacteria suggest that these phenotypic differences may be due to the expression of new proteins in amoeba-grown *L. pneumophila* cells. In addition, the amoeba-grown bacteria were found to enter macrophages via coiling phagocytosis at a higher frequency than agar-grown bacteria did. Replication of *L. pneumophila* in protozoans present in domestic water supplies may be necessary to produce bacteria that are competent to enter mammalian cells and produce human disease.

Legionella pneumophila is the causative agent of the potentially lethal pneumonia commonly known as Legionnaires' disease (13, 31). It is estimated that 6.1 cases of *Legionella* infection occur for every 100,000 adults annually in the United States alone (28). Often the mortality rate of Legionnaires' disease is greater than 25% (14, 27). *L. pneumophila* is thought to replicate intracellularly in humans (6, 47) and has been demonstrated to grow extracellularly only under laboratory conditions (7). The cell types that *L. pneumophila* has been shown to enter and replicate within include epithelial cells (5, 37), macrophages (20, 26), fibroblasts (37, 48), and a number of protozoan species (11, 35, 40, 43). However, in human infections (47) as well as the guinea pig model of *L. pneumophila* pneumonitis (6), the majority of bacteria are seen intracellularly in macrophages.

Although entry into host cells is likely to be a critical step in *Legionella* pathogenesis, little is understood concerning the mechanisms involved. The initial studies on the *Legionella* entry mechanisms were carried out with macrophages *in vitro* (19). This work led to the observation that entry may occur through a complement-mediated mechanism involving complement receptors (38). In addition, phagocytosis was shown to occur through a novel mechanism termed "coiling phagocytosis" (19). The possibility that other cell types, particularly epithelial cells, are involved in *Legionella* pathogenesis has been suggested recently (24, 33). The mechanism of entry into epithelial cells has not been studied, although they have been shown to express complement receptors (12, 32).

Previous studies on *Legionella* invasion have focused on bacteria cultured in laboratory media (19, 38). However, protozoans present in domestic water supplies have been shown to provide an environmental reservoir for *Legionella* species (1, 18, 40). In addition, epidemiological studies have suggested that human infections occur by inhalation of aero-

sols from these contaminated sources (2, 3, 18). Furthermore, it has been amply shown that multiple passage of fresh clinical isolates of *L. pneumophila* on agar causes a loss of virulence as measured by the ability to replicate in tissue culture cells and cause pneumonitis in guinea pigs (4, 8, 30). This evidence may indicate that there is an important link between Legionnaires' disease and protozoans present in domestic water supplies.

These observations led us to examine the effects upon invasion of growth of *L. pneumophila* in one of its environmental hosts, *Acanthamoeba castellanii* (18, 40). Previous studies in our laboratory have shown that *Legionella* cells replicate efficiently within *A. castellanii* (35). In the current study, we examined *L. pneumophila* cells grown on laboratory media (BCYE grown [BG]) and in the amoebae (amoeba grown [AG]) for their ability to enter a number of cell types, including epithelial cells (HEp-2), macrophages (RAW 264.7 and THP-1), and amoebae (*A. castellanii*). The ultrastructure, morphology, and protein expression of AG and BG bacteria were compared. These results further support a role for protozoans in the production of Legionnaires' disease and have significant implications for studies on *Legionella* pathogenesis.

MATERIALS AND METHODS

Strains and growth conditions. A spontaneous streptomycin-resistant mutant of *L. pneumophila* serogroup 1 (130b) (9), shown to be virulent in guinea pigs and amoebae (34), was used for all procedures. *L. pneumophila* cells were either grown on BCYE agar (BG) in the standard manner (7) or harvested immediately after growth in amoebae (AG) as follows. First, a large-scale invasion assay was carried out in a 75-cm² tissue culture flask (Falcon) at 37°C. In this assay a monolayer of approximately 10⁷ amoebae was infected with *L. pneumophila* at a multiplicity of infection of 100 for 30 min. Then the monolayer was washed once with 10 ml of A.c. buffer (35) and incubated for 2 h in 10 ml of A.c. buffer containing 100 µg of gentamicin per ml. It was then washed as before and incubated

* Corresponding author. Phone: (415) 723-6384. Fax: (415) 725-5671.

in 25 ml of A.c. buffer at 37°C with 5% CO₂ until the complete amoeba monolayer was destroyed (~72 to 104 h). The resulting culture was harvested and centrifuged for 10 min at 350 × g to pellet the bacteria and amoebae. The pellet was suspended in 1 ml of distilled water for 10 min and passed through a 27-gauge syringe three times to lyse remaining amoebae as described previously (35). To remove any remaining amoebae or amoebic cysts, we centrifuged this preparation for 1 min at 150 × g and transferred the supernatant to a new tube. The resulting bacterial suspension, containing approximately 10⁷ to 10⁸ bacteria, was found to be free of intact amoebae by light microscopy.

L. pneumophila cells that were grown on BCYE agar but had come in contact with amoebae (AG/30) were prepared by carrying out an invasion in a 75-cm² flask as described above but lysing the amoebae by incubation with water and passage through a syringe immediately after the 30-min invasion. BCYE-passaged AG bacteria (AG/B) were prepared by harvesting AG bacteria as described above, plating them on BCYE agar, and harvesting AG/B bacteria that grew as a lawn on this plate. *Escherichia coli* K-12 strain HB101 (*ara-14 leuB6 proA2 lacY1 glnV44 galK2 recA13 rpsL20 xyl-5 ml-1 thi-1 hsdS20*) (Promega) grown in Lennox broth or agar (GIBCO BRL) was used as a noninvasive control.

A. castellanii ATCC 30234 was grown to confluence at 23°C in 75-cm² tissue culture flasks containing PYG broth (35). Amoebae were harvested before use by rapping the flask sharply to bring them into suspension, and the number of viable cells was determined as described previously (35).

Cell lines and culture conditions. HEP-2 cells (ATCC CCL23), established from a human epidermoid carcinoma, were grown in RPMI 1640 plus 5% fetal calf serum (GIBCO). RAW 264.7 cells (39), a mouse macrophage cell line, were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (GIBCO). THP-1 cells (ATCC TIB202), a human monocytic cell line, were grown in RPMI 1640 plus 10% fetal calf serum.

Invasion and adhesion assays. Invasion assays were carried out in a similar fashion for all cell lines and amoebae used. For THP-1 cells, however, the assays were carried out in suspension. This required that the cells be pelleted by centrifugation at 100 × g for 1 min before each change of solution. The other cells were seeded in 24-well tissue culture dishes (Falcon) at a concentration of 2.5 × 10⁵ cells per well and allowed to adhere overnight at 37°C for tissue culture cells and 23°C for amoebae. In addition, the amoebae were washed and incubated in A.c. buffer (35) for 1 h at 37°C with 5% CO₂ before use. The BG *Legionella* cells were suspended and diluted in the same medium as the cells that were to be infected. After addition of the bacteria at a multiplicity of infection of 100, they were spun onto monolayers at 300 × g and allowed to interact with the cells for 30 min (amoebae, RAW 264.7 cells, and THP-1 cells) or 90 min (HEP-2 cells). The cells were then washed with phosphate-buffered saline (PBS) and incubated in the appropriate culture medium plus 100 µg of gentamicin per ml for 2 h. After antibiotic treatment the cells were washed with PBS and then with water and incubated for 10 min in 1 ml of water. The cells were then passed through a 27-gauge needle four times to ensure breakage of the cells. The number of intracellular bacteria was determined by plating for CFU on BCYE (*L. pneumophila*) or Lennox agar (*E. coli*). Adhesion was tested in the same manner as invasion, except that the wells were not treated with gentamicin before lysis and plating.

Microscopic techniques. Electron microscopy was used to examine entry of *L. pneumophila* into THP-1 cells. The procedure for preparation of samples before fixation was essen-

tially as described previously (19). The samples were then fixed in 2% glutaraldehyde and stained with 1% OsO₄ for 2 h and 0.5% uranyl acetate overnight at 4°C.

HEP-2 cells to be used for light microscopy were seeded in 24-well dishes as described above except that the wells had glass coverslips. These coverslips were then prepared for light microscopy by fixation in methanol and staining by a modification of the technique used by Giménez (15). In this procedure the coverslips are incubated in hot (50°C) 0.4% carbol basic fuchsin for 10 min, 0.8% malachite green for 5 to 10 s, and 0.004% methylene blue for 60 to 90 s, being washed with water between each step. Bacteria were examined for motility by light microscopy without fixation or staining.

Samples were prepared for fluorescence microscopy essentially as described previously (25), except that propidium iodide was excluded during the secondary-antibody incubation and the primary antibody used was raised against either AG or BG bacteria. Primary antibodies were used at a dilution of 1:500, and the secondary fluorescein isothiocyanate-conjugated sheep anti-rabbit antibody (Sigma) was used at a dilution of 1:3,000. Antibodies against each strain were produced in New Zealand White rabbits by immunization with 10⁸ formalin-killed bacteria once every 7 days for 3 weeks. The resulting antibody titers were greater than 1:20,000 by enzyme-linked immunosorbent assay (10).

[³⁵S]methionine labeling of *Legionella* proteins. *L. pneumophila* cells were first allowed to invade the amoebae under standard conditions. After gentamicin treatment the flask was washed once with 5 ml of A.c. buffer and incubated for various periods in 25 ml of A.c. buffer at 37°C and with 5% CO₂. The amoebae, some of which contained bacteria, were then harvested from the flask, pelleted at 185 × g for 10 min, and suspended in 1 ml of A.c. buffer plus 60 µCi of [³⁵S]methionine (Amersham) at 37°C for 2 h. Membrane fractions of each strain of bacteria were prepared for polyacrylamide gel electrophoresis (PAGE) by extraction of this suspension with Sarkosyl as follows. Approximately 10⁸ bacteria, either AG or BG, were suspended in 1 ml of 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4)–2% Sarkosyl–1 mM EDTA for 1 h at 37°C. After this incubation the insoluble fraction was pelleted by centrifugation at 13,000 × g for 2 min. After this extraction was repeated twice, the pellet was dissolved in 100 µl of PBS and aliquots were compared by PAGE (41).

RESULTS

Invasion of *L. pneumophila* cells grown in *A. castellanii*. *L. pneumophila* cells that had been grown within amoebae were tested for their ability to invade HEP-2, RAW 264.7, THP-1, and *A. castellanii* cells. The results of invasion assays with AG and BG bacteria are shown in Fig. 1. Entry into all three cell lines increased for AG bacteria more than with BG bacteria. The most dramatic increases in invasion were seen in HEP-2 cells, in which invasion of the AG bacteria was nearly 1,000-fold higher than that of BG bacteria. Increases in invasion of the AG bacteria compared with the BG bacteria were observed with RAW 264.7 and THP-1 cells, although background levels of uptake of the noninvasive bacterial control, *E. coli* HB101, were also higher. The increases in invasion of the AG bacteria into RAW 264.7 and *A. castellanii* cells were similar, approximately 10- to 100-fold greater than with BG bacteria. In the human macrophage cell line THP-1, however, the AG bacteria invaded to an approximately 1,000-fold greater extent than did BG bacteria. As shown in Fig. 1, when AG bacteria were subsequently passed on BCYE agar (AG/B), their frequency of

FIG. 1. Comparison of HEp-2 (A), *A. castellanii* (B), RAW 264.7 (C), and THP-1 (D) cell invasion by AG *L. pneumophila* (AG), *E. coli* HB101, BG *L. pneumophila* (BG), BG *L. pneumophila* grown in amoebae for 30 min (AG/30), and AG *L. pneumophila* grown on BCYE agar (AG/B). All assays were done in triplicate, and the error bars represent the standard deviation of the results.

invasion returned to approximately that of the BG bacteria. The variability in the levels of invasion by AG/B bacteria in the different cell lines was within the range of that seen with different cultures of BG bacteria. To rule out the possibility that the increased capacity of the AG bacteria to invade was caused by an increase in adherence to cultured cells, we measured the levels of adherence of AG and BG bacteria to HEp-2 cells and found no significant difference (data not shown). The observed differences in invasion could also be attributed to increased gentamicin resistance of the AG bacteria. However, the AG and BG bacteria were found to be equally sensitive to gentamicin (data not shown).

We found that bacteria harvested from amoebae immediately following entry and BG bacteria were equally invasive for HEp-2 cells. The capacity of *L. pneumophila* cells grown in *A. castellanii* to invade HEp-2 cells increased gradually over 5 days (Fig. 2). Invasiveness of the population normally reached a peak at 3 to 4 days and decreased afterward. In separate experiments, we observed variability in the day of maximal invasiveness, reaching a peak between days 3 and 4. The number of viable bacteria present in the amoebae culture increased for the first 24 h and remained constant until day 5, when an approximately 10-fold decrease was observed. For 2 days after this time point there was little change in the bacterial numbers and invasiveness of the culture, although both continued to gradually decline (data not shown).

Characterization of invasive *L. pneumophila*. We examined AG and BG bacteria for motility by light microscopy and found that neither growth condition produced motile bacteria. However, several differences between AG and BG bacteria were observed. Giménez stains of BG and AG bacteria in contact

FIG. 2. Level of invasion of AG *L. pneumophila* cells into HEp-2 cells, measured each day for 5 days. All assays were done in triplicate, and the error bars represent the standard deviation of the results. The day zero time point was taken at 2.5 h, after killing of extracellular bacteria with gentamicin.

with HEp-2 cells are shown in Fig. 3A to D. By using this staining technique, it was found that BG bacteria stained very faintly in comparison with AG bacteria. BG bacteria were also consistently found as single or a few bacteria within the cell, whereas AG bacteria were usually found as aggregates of a few to very many bacteria. In addition, AG bacteria appeared to be shorter and to have a larger diameter than BG bacteria.

To determine whether the morphologic changes were due to artifacts of the staining technique, we labeled the bacteria with polyclonal sera directed against the BG and AG bacteria and examined them by fluorescence microscopy. Using this technique, we found that the outline of the bacterial cells was clearly visible and allowed fairly accurate determination of the dimensions of the bacteria produced under these growth conditions. The fluorescence microscopy studies confirmed the results obtained with the Giménez stains (Fig. 3E and F). Electron microscopy of thin sections of the BG and AG bacteria confirmed these changes in size and shape and demonstrated further morphological differences (Fig. 4).

In addition to the morphologic changes in the bacteria after growth in amoebae, we observed that AG bacteria contained large, electron-transparent vesicles that filled the majority of the cytoplasmic space (Fig. 4C and D). These vesicles were not seen very frequently in the BG bacteria; when present, they were much smaller (Fig. 4A and B). The cytoplasm of AG bacteria appeared to be more dense than that of BG bacteria, and the cell wall of AG bacteria was more clearly defined and appeared thicker.

Characterization of membrane proteins in invasive *L. pneumophila*. To examine changes in bacterial protein expression during growth in amoebae, we harvested *L. pneumophila* cells from amoebae at various times from immediately after entry to 28 h thereafter. Bacterial proteins expressed at these times were labeled for 2 h with [³⁵S]methionine. Under the conditions of these experiments, no labeling of amoeba proteins with [³⁵S]methionine was observed (data not shown). Thus, use of cyclohexamide to inhibit eukaryotic protein synthesis was not necessary. AG bacteria were extracted with Sarkosyl, and the membrane protein profiles were compared with those of BG bacterial membrane proteins. During the early stages of growth in amoebae, two proteins appeared to be transiently expressed at very high levels (Fig. 5, 0 to 4 h). Potentially, these proteins were required to overcome environmental stresses exerted on the bacteria during this time but were no longer

FIG. 3. (A to D) Giménez stains of BG (A and C) and AG (B and D) bacteria invading HEP-2 cells. Small arrowheads indicate faintly staining BG bacteria. AG bacteria stain bright red and are usually seen in large clumps. (E and F) Fluorescence microscopy of BG (E) and AG (F) bacteria demonstrates differences in cell width and length. Bar in panel D, 10 μ m.

required once the bacteria has established the proper growth environment. A number of newly expressed or induced proteins were observed at the later times during growth in the amoebae. The most prominently induced proteins observed under these growth conditions are indicated by arrows in Fig. 5. The apparent molecular masses of these proteins were estimated to be 20, 24, 70, 75, and 150 kDa. In addition to these proteins, a number of proteins appeared to be down regulated or displayed low-level transient expression.

AG bacteria enter macrophages via coiling phagocytosis. In previous studies of phagocytosis in *L. pneumophila*, human monocytic cell lines were used to demonstrate coiling phagocytosis (19). In those studies, coiling phagocytosis was described as asymmetrical coil of filopodia around the bacterium, resulting in uptake. When we examined entry of the BG bacteria into THP-1 cells by electron microscopy, we observed entry events that were consistent with the description of coiling phagocytosis (Fig. 6B and D). In addition, we observed that approximately 50% of the entry events were via standard or symmetrical phagocytosis (Fig. 6A and C). In contrast, AG *L. pneumophila* cells entered THP-1 cells almost exclusively by coiling phagocytosis. At least 50 entry events of AG bacteria were examined, and only one of these appeared to occur through a standard phagocytic event.

DISCUSSION

We found that AG-grown *L. pneumophila* expressed an increased capacity to enter tissue culture cells compared with that of BG-grown bacteria. Although the level of invasion of AG bacteria was variable and dependent on the intrinsic phagocytic capabilities of the eukaryotic cell type, it was always significantly higher than that of BG bacteria. These results suggest that *L. pneumophila* cells that have been grown intra-

cellularly in protozoans may be more infectious. The greatest increase in invasion of the AG bacteria compared with that of the BG bacteria was observed for entry into epithelial cells. Since epithelial cells are not professional phagocytes, they have lower levels of background nonspecific uptake of bacteria. Epithelial cells are not commonly used as an in vitro model for *Legionella* pathogenesis since the majority of the bacteria are observed in macrophages during mammalian infection (6, 22, 46, 47). However, several studies have indicated that *Legionella* cells may enter and replicate within epithelial cells in vitro (5, 37). Furthermore, the ability of *Legionella* cells to enter and survive within epithelial cells has been suggested as an important mechanism in establishing lung infections (33). Even though the capability of AG bacteria to enter professional phagocytic cells was not as dramatic, a significant increase in invasion was noted. Thus, there may be an in vivo correlation with the results obtained in vitro.

Characterization of AG bacteria demonstrated a number of phenotypic differences that may be responsible for this phenomenon. Invasive *L. pneumophila* cells appeared to form large aggregates, possibly because of differences in hydrophobicity or interbacterium adherence. This phenotype is reminiscent of the localized adherence seen with enteropathogenic *E. coli* cells expressing the bundle-forming pilus (16, 44). We examined AG and BG bacteria for the presence of pili by electron microscopy. Although pili were observed by electron microscopy, they were not very abundant under either growth condition (data not shown). Therefore, it is unlikely that pilus expression could account for the observed results.

Another clear difference in the AG and BG *Legionella* strains is seen in their staining characteristics when the Giménez technique was used. The enhanced staining of the AG bacteria may be caused by the relative impermeability of the *L. pneumophila* lipid layer. A similar phenomenon is observed in

FIG. 4. Structure of BG (A and B) and AG (C and D) bacteria by electron microscopy. Bars, 0.1 μ m.

FIG. 5. Incorporation of [35 S]methionine into *L. pneumophila* proteins during growth on BCYE agar (lane B) or after various periods of growth within *A. castellanii* cells (0 to 28 h). Positions of protein size standards are shown at the left. Arrows indicate positions of new proteins expressed in *L. pneumophila* after growth in *A. castellanii*.

mycobacteria, where acid-fast staining, in which carbol fuchsin is the active stain, is attributed to cell wall lipids (17). In this regard, we cite the observation that *L. micdadei* is acid fast in tissue but loses acid-fast staining after growth on agar (36, 45), which indicates a correlation between fuchsin staining and intracellular growth. AG bacteria also contained large vesicles in their cytoplasm. Although the role of these vesicles in *L. pneumophila* is not clear from these experiments, previous studies with other bacteria have shown similar vesicles to be involved in energy storage in the form of glycogen or poly- β -hydroxybutyrate (42). Possibly, after exiting the eukaryotic cell, the bacteria must have sufficient energy stored to allow survival for an extended period in water environments in the absence of nutrients.

AG bacteria were shorter and wider than BG bacteria, as demonstrated by fluorescence microscopy and electron microscopy. The shorter average length of the AG bacteria may be a result of a higher replication rate in amoebae rather than any direct effect on the phenotype. This possibility is supported by the fact that the generation time of BG bacteria is approximately 3 h whereas the generation time of AG bacteria is approximately 1 h (35). If the growth rate is taken as an indication of the preferred growth environment for *L. pneumophila*, the higher replication rate in amoebae may reflect the status of protozoans as natural hosts.

The most likely explanation for the observed increase in invasion after growth in the amoebae is an increase in expression of genes involved in the entry event. To test this hypothesis, we used [35 S]methionine labeling of membrane proteins in AG bacteria to examine protein expression. When com-

FIG. 6. Examples of standard (A and C) and coiling (B and D) phagocytic events in *L. pneumophila*.

pared with BG bacteria, five new proteins were found to be expressed by AG bacteria, suggesting that the intracellular environment may directly or indirectly stimulate invasion-related protein expression. Although an increase in expression of various proteins is the simplest explanation for enhanced invasiveness, repression of proteins that inhibit invasion would result in the same phenotype. We did note that some proteins were no longer expressed in AG bacteria. Although we have shown that growth in amoebae leads to changes in protein expression that correlate with an increased capacity to invade eukaryotic cells, the nature of these proteins and their involvement remains to be determined.

When coiling phagocytosis was first observed in *Legionella* cells, it was noted that standard phagocytic events also occurred (19). Our electron-microscopic studies on the entry of the BG bacteria into THP-1 cells confirmed this observation. In addition, we found correlation between entry by coiling phagocytosis and invasiveness. In this regard, we observed that the BG bacteria entered THP-1 cells only half of the time via coiling phagocytosis, whereas the AG bacteria entered almost exclusively by coiling phagocytosis. It appears that growth of *L. pneumophila* in the amoebae resulted in an increased ability to enter by coiling phagocytosis. This observation raises the possibility that the proteins induced by intra-amoebic growth have a role in triggering this novel phagocytic event. We hypothesize that the frequencies of standard and coiling phagocytic events observed are the result of competition for host cell receptors involved in the standard bactericidal phagocytic mechanisms of the macrophage and coiling phagocytosis. A more detailed examination of changes in protein expression

and correlation with the frequencies of coiling phagocytosis under these growth conditions should allow us to determine the validity of these conclusions.

Environmental factors have been shown to have a dramatic effect on the ability of a number of invasive pathogens to enter eukaryotic cells. In the case of *Shigella* (29) and *Yersinia* (21) species, bacterial growth temperature has been shown to significantly affect frequencies of invasion. In addition, *Salmonella* species are fully invasive only when in the correct growth phase under low oxygen (23). By analogy, it is possible that *L. pneumophila* would require specific growth conditions to trigger expression of its invasive phenotype. Developing an understanding of the environmental conditions that give rise to invasive bacteria may not only be important in the understanding of the pathogenic mechanisms used but also enable us to prevent infections through modification of practices that promote these conditions. In addition to indicating the need for further investigation of the specific mechanisms of *L. pneumophila* entry, these observations suggest that the behaviors of AG and BG bacteria should be compared in virulence studies. If previous evidence indicating a correlation between the presence of protozoans in domestic water supplies and legionellosis is correct (2, 3, 18), the bacteria grown in protozoan hosts, such as *A. castellanii*, may be more representative of *L. pneumophila* strains that produce Legionnaires' disease via aerosols of potable water than BG bacteria are.

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