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# In Vitro Model of Adhesion and Invasion by *Bacillus piliformis*

CRAIG L. FRANKLIN,* DARRELL A. KINDEN, PRESTON L. STOGSDILL, AND LELA K. RILEY

*Department of Veterinary Pathology, College of Veterinary Medicine, University of Missouri, Columbia, Missouri 65211*

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**An in vitro model of *Bacillus piliformis* infection was developed to investigate the mechanisms of adhesion and internalization of this obligate intracellular bacterium. Adhesion and internalization events were examined by electron microscopic evaluation of infected Caco-2 cell monolayers. A few bacteria were identified in apical surface invaginations and in vacuoles subjacent to the apical surface, whereas the majority of bacteria were observed free within the cytoplasm, suggesting that *B. piliformis* entered epithelial cells via a phagocytic process and rapidly escaped the phagosome. To confirm that host cell phagocytosis was involved in entry of *B. piliformis* into mammalian cells, Intestine 407 cells were treated with the phagocytic inhibitor cytochalasin D, infected with *B. piliformis*, and evaluated for bacterial internalization by double-fluorescence labeling. The results showed decreased intracellular bacteria, suggesting that internalization was dependent on host cell microfilament function. To examine the role of *B. piliformis* in internalization, growth of live and Formalin-killed bacteria was compared. Dead bacteria were not internalized, suggesting that *B. piliformis* actively participates in internalization. *B. piliformis* appears to enter host cells by a bacterially directed phagocytic process. The in vitro system described should prove invaluable in further investigations of *B. piliformis* pathogenic mechanisms.**

Tyzzer's disease is an often fatal enterohepatic disease of numerous domestic, laboratory, and wild animal species (15). There is also suggestive evidence that the disease may occur in humans (12). The etiologic agent of Tyzzer's disease is *Bacillus piliformis,* a unique, unclassified, large, rod-shaped, motile bacterium (15). *B. piliformis* has not been grown on conventional media (38) but can be propagated in embryonated eggs or on several established mammalian cell lines (31, 36), suggesting that it is an obligate intracellular bacterium. The pathogenesis of *B. piliformis* infection is poorly defined and has been studied primarily by histologic observations of tissue from diseased animals. *B. piliformis* is thought to be spread via ingestion of spores shed in the feces of infected animals (15, 20). The initial site of infection appears to be the intestinal epithelium. Histologic evaluation of the lower small intestine, cecum, and large intestine of affected animals reveals extensive necrosis of intestinal epithelial cells, with packets of *B. piliformis* in adjacent viable enterocytes (15). *B. piliformis* is thought to enter and replicate within these enterocytes, destroy the host cell, and spread to adjacent enterocytes, with eventual dissemination to the liver and occasionally to the heart (15, 31).

An essential feature of disease pathogenesis by *B. piliformis* is bacterial invasion. The mechanisms by which *B. piliformis* attaches to and enters target host cells are unknown. The cellular tropism of this organism encompasses several epithelial cell types which are not normally phagocytic. Other obligate intracellular organisms, such as *Chlamydia* spp. (4, 43) and *Rickettsia prowazekii* (40), and several facultative intracellular bacteria, such as *Listeria monocytogenes* (13), *Salmonella* spp. (11), and *Shigella flexneri* (17, 18), have been shown to enter so called nonprofessional phagocytes by parasite-mediated endocytosis, in which both the organism and host play an active role in bacterial internalization.

To investigate the mechanisms of *B. piliformis* attachment and invasion, we developed an in vitro model of infection with a human colon carcinoma cell line, Caco-2, and a human embryonic intestinal epithelial cell line, Intestine 407. The Caco-2 line has particular applicability in that this cell line forms a polarized monolayer with a functional brush border, closely resembling that of the small intestine (28, 33). Our results suggest that *B. piliformis* invades intestinal epithelial cells via a bacterially directed host microfilament-dependent mechanism.

## MATERIALS AND METHODS

**Bacterial cultivation.** *B. piliformis* isolate R1 was obtained from a naturally infected rat and maintained in established mammalian cell lines of buffalo rat liver (BRL 3A [ATTC CRL 1442]; American Type Culture Collection, Rockville, Md.), Intestine 407 (ATTC CRCM 30), and Caco-2 (ATTC HTB 37) as previously described (31). Briefly, cell lines were grown in 5 to 10% $CO_2$ at 37°C in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 5 to 10% Serum Plus (Hazelton, Lenexa, Kans.) and 2 mM L-glutamine (Sigma). The medium used for propagation of Caco-2 cell lines also contained 1% nonessential amino acids (Sigma). Subconfluent monolayers were infected with *B. piliformis* and incubated at 37°C until approximately $10^5$ to $10^6$ bacteria per ml were present. Culture supernatant containing bacteria was used as the inoculum in experimental studies. To ensure maximum viability, the medium was changed 24 h prior to collection of the inoculum to remove nonviable organisms. Organisms were counted with a hemacytometer.

**Growth kinetics.** Caco-2 and Intestine 407 cells were grown in 24-well tissue culture plates to subconfluency and infected with approximately $10^7$ *B. piliformis* organisms. The

* Corresponding author.

medium was changed 6 h postinfection to remove organisms that had not attached to or entered mammalian cells. At 6, 18, 30, 42, 54, 66, and 114 h postinfection, monolayers from three wells were harvested by physical scraping and sonicated on ice three times for 30 s each to release intracellular *B. piliformis*. Cellular debris was pelleted by centrifugation at 200 × *g*. Bacteria in the resulting supernatant were quantitated with a hemacytometer viewed by phase-contrast microscopy. More than 95% of the total bacteria were obtained by this procedure.

**Electron microscopy.** To examine the penetration and intracellular growth of *B. piliformis* ultrastructurally, Caco-2 cells were grown to subconfluency on 12-mm Millicell-HA or Millicell-CM microporous membranes (Millipore, Bedford, Mass.) in 24-well tissue culture plates and infected with approximately $10^7$ organisms. At 24 and 48 h postinfection, the membranes were removed, washed three times in phosphate-buffered saline (PBS), and fixed with 2% paraformaldehyde–2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 1 h at 4°C. After a thorough rinsing with cacodylate buffer, the membranes were removed from the insert housing and postfixed with aqueous 1% osmium tetroxide for 1 to 2 h at 4°C. Specimens were stained en bloc with aqueous 1% uranyl acetate for 1 h, dehydrated in a graded ethanol series, and embedded in a mixture of Epon and Araldite epoxy resins. To minimize damage to cell monolayers, sections were made perpendicular to the monolayer-membrane axis, and samples were oriented so that the cutting edge of the knife passed through the cell monolayer prior to sectioning the membrane. Two-micrometer-thick sections were cut, stained with 1% toluidine blue, and examined by light microscopy. Areas where approximately 30% of the monolayer was infected with *B. piliformis* were selected for electron microscopic examination. Ultrathin sections were poststained with uranyl acetate and lead citrate and examined and photographed with a Hitachi H-600 transmission electron microscope.

**Double-fluorescence microscopy.** To differentiate attached bacteria from internalized bacteria, the double-fluorescence technique described by Detilleux et al. (8) was used. Briefly, Caco-2 and Intestine 407 cells were grown on coverslips in six-well tissue culture plates and infected with *B. piliformis* under the conditions described above. Coverslips were incubated for 15 min in PBS (pH 7.4) containing 5% powdered nonfat milk and 0.01% Tween 20 to prevent nonspecific binding of antibody. Coverslips were then incubated with rabbit anti-*B. piliformis* flagellum antiserum (22) diluted 1:100 in PBS–5% nonfat milk (blocking buffer) for 30 min at 27°C. Following three washes with PBS, the coverslips were fixed in methanol for 5 min at 4°C and air dried. Coverslips were then incubated with donkey anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories, West Grove, Pa.) diluted 1:50 in blocking buffer for 30 min. Because the primary antibody was added prior to fixation, it was unable to penetrate cells and therefore labeled only attached bacteria. After antibody labeling of the bacteria, coverslips were stained for 5 min with propidium iodide (25 μg/ml) to visualize attached and internalized bacteria. Coverslips were mounted with a semipermanent medium containing polyvinyl alcohol (19). Approximately 300 mammalian cells per coverslip were examined by epifluorescence microscopy with two filters, a blue 490-nm excitation filter (emission at ≥520 nm) to detect FITC-labeled bacteria and a green 545-nm excitation filter (emission at 590 nm) to detect propidium iodide-stained bacteria. The percentage of cells with attached *B. piliformis* or internalized packets of *B. piliformis* was then determined. Results are expressed as the mean percentage of affected cells from three coverslips per time point.

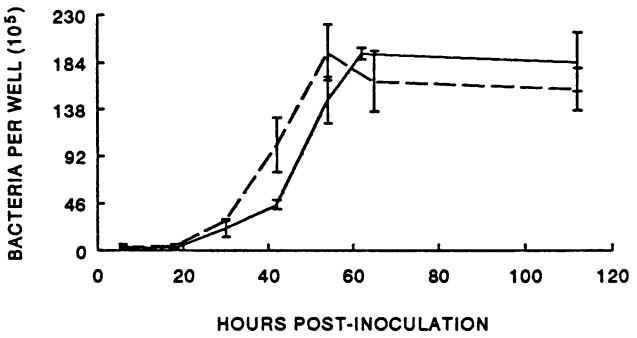


FIG. 1. Growth kinetics of *B. piliformis* on Caco-2 (– – –) and Intestine 407 (——) cells. Values are expressed as the mean number of bacteria ± the standard error from three wells per time period.

**Formalin-killed bacteria.** To evaluate the role of *B. piliformis* in host cell entry, approximately 3 × $10^7$ viable or Formalin-killed *B. piliformis* organisms were inoculated onto subconfluent Intestine 407 cell monolayers grown on coverslips. To this end, *B. piliformis* organisms from active cultures were collected and split into two fractions. One fraction was used immediately to infect tissue cultures as described above. Formaldehyde was added to the second fraction to a final percentage of 10%, and the pH was adjusted to approximately 7. After incubation at 27°C for 30 min, this fraction was centrifuged at 12,000 × *g* to pellet the bacteria. Bacterial pellets were washed in PBS, resuspended in fresh medium, and used to inoculate additional tissue cultures. Because Formalin-killed *B. piliformis* organisms are not motile, plates from both groups were centrifuged at 550 × *g* to ensure adequate contact between bacteria and cells. At 10, 24, and 36 h postinoculation, three coverslips were removed and processed for double-fluorescence microscopy staining as described above.

**Treatment of monolayers with cytochalasin D.** To evaluate the role of the host cell in *B. piliformis* entry, we examined the effects of cytochalasin D, a potent inhibitor of actin-dependent phagocytosis, on internalization of *B. piliformis*. Subconfluent monolayers of Intestine 407 cells grown on coverslips were incubated with medium containing 2.0 μg of cytochalasin D (Sigma) per ml. After 1 h, monolayers were infected with 3 × $10^7$ *B. piliformis* organisms in medium containing cytochalasin D (2.0 μg/ml). Analogous experiments with medium lacking cytochalasin D served as controls. At 10, 24, and 48 h postinoculation, three coverslips were removed and processed for double-fluorescence microscopy staining.

**Statistical analysis.** Differences between mean percentages of infected cells in the cytochalasin and viability studies were evaluated by analysis of variance with Fisher's least-significant-difference rule (35).

## RESULTS

**Growth characteristics and kinetics.** Growth of *B. piliformis* was similar on Caco-2 and Intestine 407 cells (Fig. 1). A lag period of approximately 20 h followed by a 40-h period of logarithmic growth and a stationary phase were evident. A generation time of approximately 6 h was calculated. The

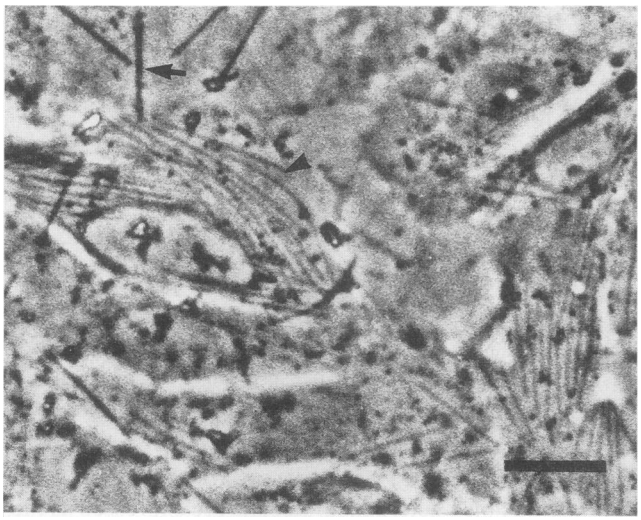

FIG. 2. Phase-contrast micrograph of Intestine 407 cells infected with *B. piliformis* for 48 h. Numerous attached (arrow) and intracellular packets (arrowhead) of organisms were present. Bar, 25 μm.

events observed by microscopic examination of infected tissue culture flasks correlated with these phases. During the lag phase, only a few *B. piliformis* organisms attached to epithelial cells were detected. These bacteria were usually not motile. As the lag phase waned, motile bacteria were occasionally found in the medium and attached to cells. Internalized packets of *B. piliformis* were first noted during early logarithmic growth (Fig. 2). The bacteria in several of these packets could be observed darting rapidly back and forth within the confines of the cell. Increased numbers of motile attached and extracellular bacteria were also evident. During the mid- to late logarithmic growth phase, the number of dead cells in the monolayer increased, as did the number of extracellular bacilli (motile and nonmotile). At the peak of logarithmic growth, the majority of the cells in the monolayer were dead. During the subsequent stationary phase, the majority of bacteria were nonmotile and were observed either adhered to cell debris or floating in the medium.

**Electron microscopy.** On ultrastructural examination of Caco-2 cell monolayers, polarization of host cells was confirmed by the presence of tight junctions and the formation of an apical brush border. Ultrastructural examination of Caco-2 cell monolayers infected with *B. piliformis* revealed a putative series of events which may occur in the life cycle of *B. piliformis* (Fig. 3). Occasional bacteria were nestled amid host cell microvilli, but direct attachment was not observed. A few (<1% of organisms observed) *B. piliformis* were present in invaginations of the apical microvillar cell surface (Fig. 3A). Bacteria were never observed adhering to the lateral or basal cell surfaces. An almost equally rare event was the occurrence of *B. piliformis* in membrane-bound vacuoles, usually subjacent to the microvillar surface (Fig. 3B). The majority of intracellular *B. piliformis* were located within the cytoplasm, with bacterial numbers ranging from 1 to 50 per cell (Fig. 3C). Occasionally, intracytoplasmic bacteria undergoing replication were evident (Fig. 3D). In addition to cytoplasmic bacteria, bacteria also were present in the nucleus (Fig. 3B and E). A few bacteria were actually observed bridging the nuclear membrane (Fig. 3E).

The morphology of lightly infected cells was comparable to that of uninfected cells, whereas heavily infected cells, i.e., cells with >20 bacteria per cell, exhibited marked vacuolation, suggesting deterioration (Fig. 3C and F). Conversely, even in heavily infected host cells, bacteria appeared to be undamaged. Cells containing abundant bacteria were also noted to be undergoing lysis (Fig. 3F).

These results suggest that *B. piliformis* entered host cells through the apical surface by a phagocytosis-like process, rapidly escaped the phagosome, and replicated within the cytoplasm. Eventual cell lysis occurred, resulting in release of bacteria.

**Double-fluorescence microscopy.** The double-fluorescence microscopy technique allowed differentiation of attached and internalized bacteria. Attached but not internalized bacteria fluoresced green as a result of FITC labeling, whereas intracellular *B. piliformis* and the mammalian cell nuclei fluoresced red as a result of intercalation of propidium iodide into both cellular and bacterial DNA. Peritrichous flagella were often identified on FITC-labeled bacteria. The tendency of Caco-2 cells to polarize and even pile up in culture made identification of intracytoplasmic bacteria difficult. Therefore, Intestine 407 cells were used for all double-fluorescence experiments. Numerous *B. piliformis* organisms were attached to cell monolayers by 10 h postinoculation. However, internalized bacteria were not usually evident until 24 h postinoculation.

**Formalin-killed bacteria.** Bacterial adhesion was evident with both live and Formalin-killed bacteria at all three times postinoculation (Fig. 4A). The mean percentage of cells with adherent bacteria was consistently lower on coverslips inoculated with killed bacteria than on coverslips inoculated with viable bacteria. The difference in mean percentages was statistically significant ($P < 0.05$) at 10 and 36 h. Internalized bacteria were not found in either preparation at 10 h but were evident in control (viable) preparations by 24 h, and the percentage of infected cells increased significantly over the subsequent 24 h (Fig. 4B). In contrast, no internalized bacteria were evident in studies with Formalin-killed bacteria. These results suggested that internalization of *B. piliformis* required a viable bacterium.

**Cytochalasin treatment.** To determine whether host cell microfilament-dependent pathways such as phagocytosis were involved in the internalization of *B. piliformis,* monolayers were treated with cytochalasin D, a compound which prevents polymerization of actin and in turn inhibits phagocytosis. Cytochalasin D treatment of monolayers had no effect on bacterial adhesion (Fig. 5A) but significantly decreased ($P < 0.05$) internalization of *B. piliformis* at 48 h postinoculation (Fig. 5B). These results suggested that *B. piliformis* entered host cells via a microfilament-dependent pathway.

It was difficult to determine what effects cytochalasin D had on the viability of this obligate intracellular bacterium. To this end, active cultures of *B. piliformis* were treated with 2 μg of cytochalasin D per ml. The bacteria in these cultures remained motile and active. After 2 h of incubation, 1-ml aliquots of cytochalasin D-treated bacterial cultures were transferred to uninfected monolayers. *B. piliformis* growth was established in 100% of the monolayers infected in this way, indicating that viable bacteria were present. Growth also resumed in the original cytochalasin D-treated culture after replacement of cytochalasin D-containing medium with fresh medium. These results indicated that cytochalasin D was not bacteriocidal.

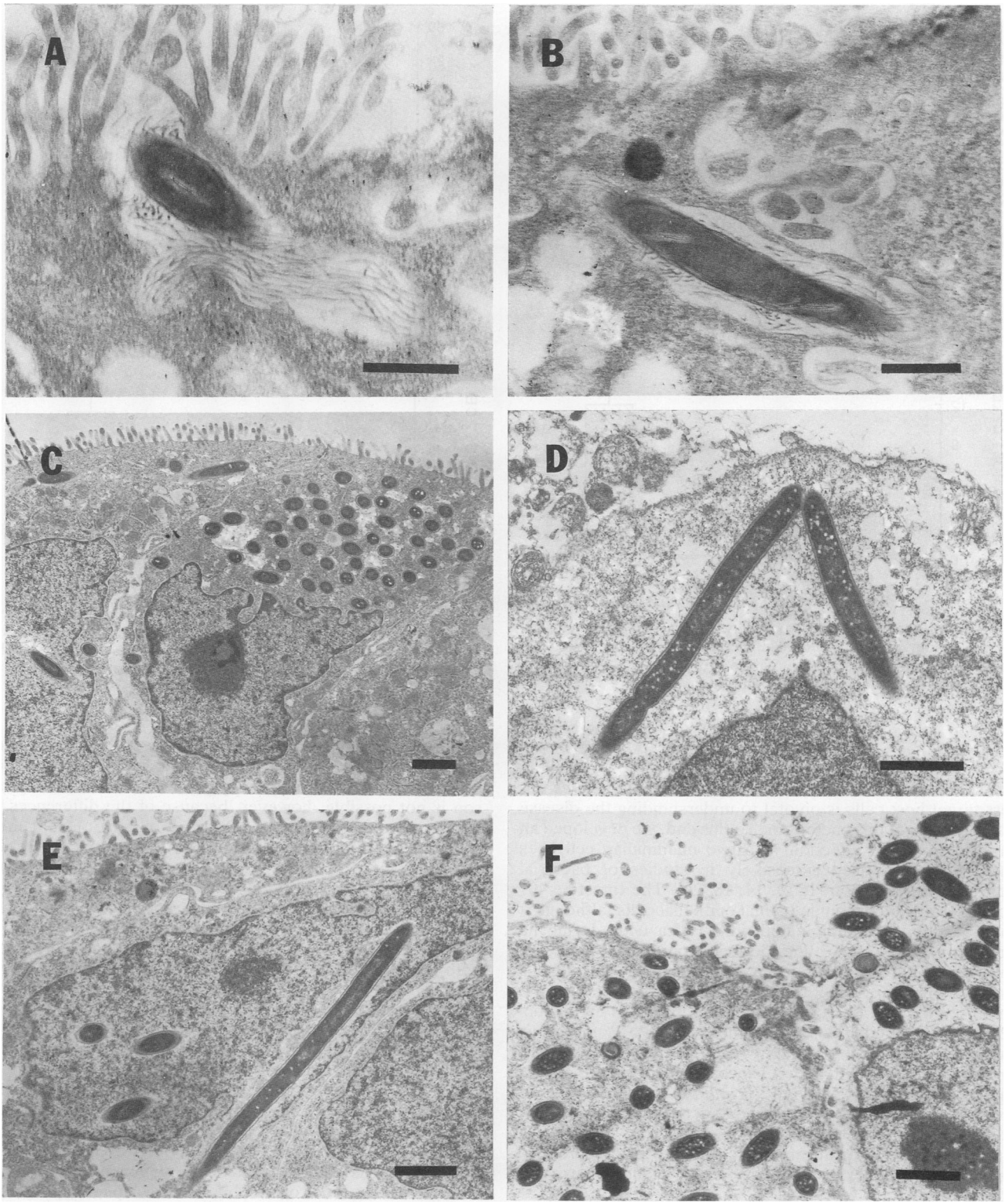


FIG. 3. Transmission electron micrographs of Caco-2 cell monolayers infected with *B. piliformis*. (A) Bacterium within a Caco-2 cell apical surface invagination. (B) Bacterium within a membrane-bound vacuole subjacent to the microvillar surface of a Caco-2 cell. (C) Two infected Caco-2 cells. The cell on the left contains a few intracytoplasmic bacteria and a single intranuclear bacterium, whereas the cell on the right contains abundant intracytoplasmic bacteria and is mildly vacuolated, suggesting deterioration. (D) Bacterium undergoing replication within the cytoplasm of an infected cell. (E) Infected Caco-2 cell containing four intranuclear bacteria, with one bacterium actually bridging the nuclear membrane. (F) Two heavily infected Caco-2 cells. The cell on the left is vacuolated and contains abundant bacteria, whereas the cell on the right appears to have lysed and is releasing bacteria. Bars: (A and B) 0.5 μm; (C through F) 1.0 μm.

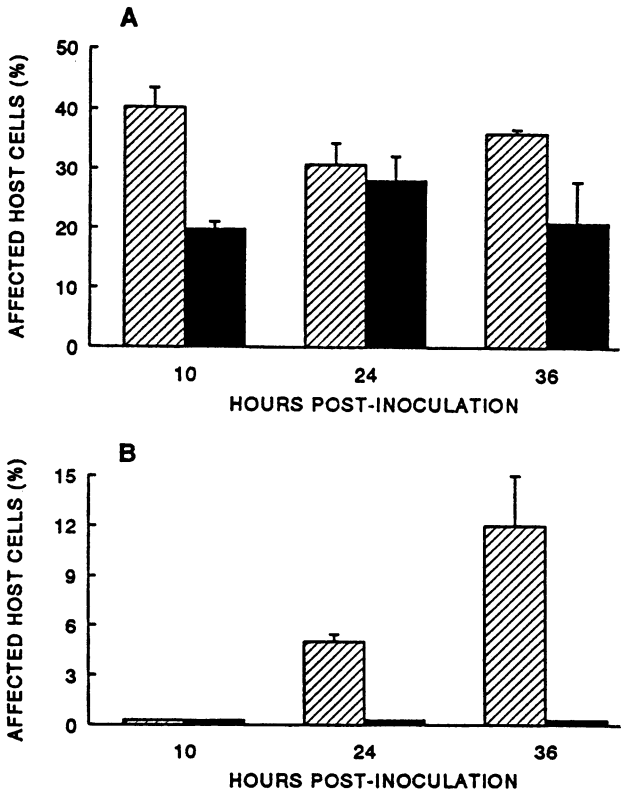


FIG. 4. Effect of bacterial viability on (A) adhesion and (B) internalization of *B. piliformis*. Hatched bars, viable bacteria; solid bars, Formalin-killed bacteria. Values are expressed as the mean percentage of affected cells plus standard error.

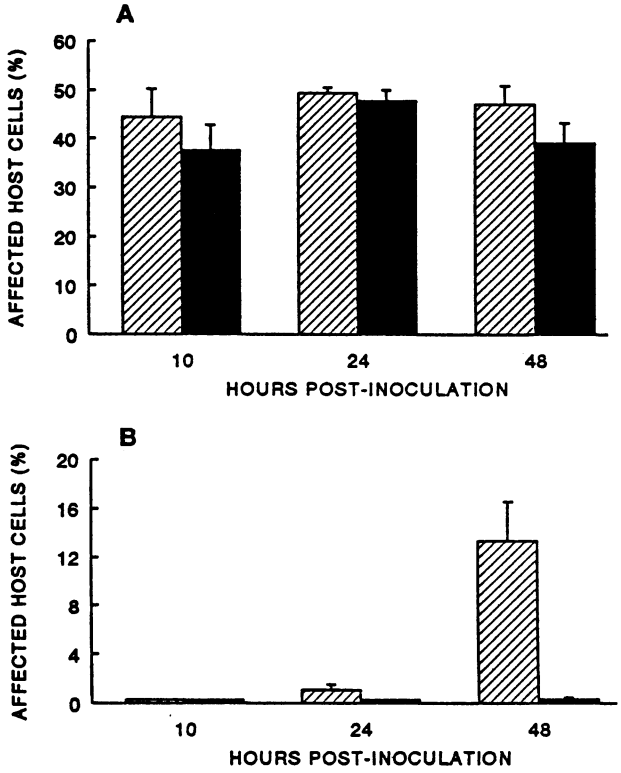


FIG. 5. Effect of cytochalasin D on (A) adhesion and (B) internalization of *B. piliformis*. Hatched bars, untreated control; solid bars, cytochalasin D-treated bacteria. Values are expressed as the mean percentage of affected cells plus standard error.

## DISCUSSION

Ascertaining the mechanisms by which *B. piliformis* enters target host cells is pivotal to understanding the disease pathogenesis of this bacterium. To this end, we developed an in vitro model of infection with two mammalian cell lines that have been used extensively in studies of bacterial adhesion and invasion (1, 10, 14, 18, 26). The Intestine 407 cell line originated from intestinal epithelial cells, although it may have been contaminated with HeLa cells during its derivation. The Caco-2 line, derived from cancerous colon tissue, is of particular importance because these cells form a polarized monolayer with a functional brush border and are therefore thought to closely mimic the intestinal epithelium (28, 33). The model closely resembles in vivo *B. piliformis* infection, since the initial site of bacterial invasion in animals appears to be the intestine. The pattern of growth in our model also mimics in vivo *B. piliformis* infection. For example, the characteristic appearance of *B. piliformis* in naturally affected animals is that of intracellular bacteria in a "pick-up sticks" arrangement (39). This pattern is also evident in a number of in vitro systems, including ours (31, 36).

*B. piliformis* theoretically replicates within the cytoplasm of epithelial cells, escapes these cells, and "swims" via its peritrichous flagella to another host cell. We investigated this putative life cycle in our in vitro model of infection. Initially, the growth kinetics of *B. piliformis* infection were investigated. Our findings are in many ways similar to those reported by Spencer et al. (36), who noted a period of inactivity for 24 h postinfection, followed by a period of rapid growth which corresponded to increasing numbers of motile bacteria and a stationary phase. A detailed comparison is not possible, however, because of the differences in bacterial isolate (rat origin versus rabbit origin), inoculum source (buffalo rat liver maintained versus primary chicken embryo liver cell, egg yolk sac, or infected mouse liver maintained), mammalian cell line (Intestine 407 and Caco-2 versus 3T3, a mouse fibroblast line), and enumeration technique (hemacytometer versus breed count).

As stated, the events observed in tissue culture correlated with the growth kinetics curve. The generation time calculated is unusually long for bacterial species. However, the generation time calculated for *B. piliformis* is not a simple doubling time, as several events in addition to replication are occurring during this time. Since *B. piliformis* is an obligate intracellular pathogen, it must travel from cell to cell to survive and replicate. Therefore, the generation time incorporates the time necessary to find a host cell, attach to and enter that cell, replicate within the cell, and escape the cell. In this way, *B. piliformis* resembles other obligate intracellular pathogens such as rickettsiae, which have relatively long generation times of 9 h (42). After escape, the progeny of the original bacterium must then survive transit to another host cell. It is likely that not all bacteria released can accomplish this end. Craigie (6) reported that *B. piliformis* lost appreciable infectivity within 1 h of removal from embryonated eggs. Similar bacterial attrition in our system would further lengthen the apparent generation time. Following logarithmic growth, an apparent stationary phase was

present. This latter phase is probably not a true stationary phase but rather a complete termination of growth due to lack of viable host cells needed for the proliferation of this obligate intracellular organism.

To further investigate the cellular events of *B. piliformis* infection, we examined cultures at early and mid-logarithmic growth phases by electron microscopy. During this time period, a spectrum of events were occurring, i.e., bacterial attachment, entry, and escape. A putative series of events was established from these observations and the frequency with which certain events occurred. The finding of bacteria within surface invaginations and vacuoles subjacent to the microvillar surface suggests that *B. piliformis* enters cells via a phagocytic process. Several obligate and facultative intracellular pathogens have been reported to enter cells in a similar manner. Because the majority of bacteria were found not within vacuoles but rather free within the cytoplasm, we hypothesized that *B. piliformis* rapidly escaped this phagocytic vacuole. In this way, *B. piliformis* may be similar to *Listeria monocytogenes* (13) and *Shigella flexneri* (18), which escape the phagosome shortly after entry, or rickettsiae, which actually escape the phagosome while being internalized (41). By contrast, this mode of entry differs from that of *Salmonella* (9), *Yersinia* (3), *Chlamydia* (24), and *Neisseria* (37) spp., which remain in a phagocytic vacuole throughout their intracellular life.

How *B. piliformis* escapes the phagocytic vacuole is unknown. Hemolysins which function in escape from the phagocytic vacuole (13) have been identified in *Listeria* (listeriolysin) (13) and *Shigella* (34) spp. Likewise, rickettsiae (41) and listeriae produce phospholipases which contribute to dissolution of the phagosome membrane and release into the cytoplasm. It is tempting to postulate that a similar toxin may be involved in *B. piliformis* escape, particularly as *B. piliformis* has been reported to produce a cytotoxin whose function has not yet been determined (32).

Following entry, *B. piliformis* replicated within the cytoplasm, in some cases reaching up to 50 bacteria per cell. In heavily infected cells, host cell deterioration was evident, and heavily infected cells that were apparently undergoing lysis were occasionally found. We hypothesized that this cellular lysis may be one method of *B. piliformis* escape, allowing dissemination to another host cell. The mechanism of host cell lysis is unknown. The accumulation of a certain critical number of organisms may physically disrupt cell functions, causing cell death in a manner similar to that by rickettsiae, for which cell lysis by physical disruption is believed to be the primary means of bacterial escape (41). Other mechanisms such as the aforementioned cytotoxin may also be involved in *B. piliformis* escape.

Alternative methods of cell-to-cell spread that do not cause host cell lysis may be utilized by *B. piliformis*. For instance, Tyzzer (39) and Okada et al. (25) observed what they interpreted to be *B. piliformis* spreading directly from cell to cell. This method of spread would be analogous to that utilized by *L. monocytogenes* (30) and *S. flexneri* (2), which have been shown to travel from cell to cell without exiting the intracellular environment. However, no conclusive evidence for this type of spread was seen in our model.

Several of the in vitro events found in our ultrastructure investigations have also been reported in cases of naturally occurring and experimentally induced Tyzzer's disease, corroborating our experimental model. Bacteria are invariably found free within the cytoplasm of infected cells (16, 27, 29), and replicating forms and intranuclear bacteria have been reported (27). The intranuclear location of the bacteria is an intriguing phenomenon that remains unexplained. This finding raises several questions. Why has *B. piliformis* entered the nucleus? What is the mechanism of this entry? Is this strictly a random event, or is there functional or nutritional significance? Does intranuclear infection augment bacterial pathogenesis? We are currently pursuing answers to some of these questions in our laboratory. To our knowledge, other intracellular pathogens are rarely, if ever, found in the nucleus.

To further define the mechanism of *B. piliformis* entry, we investigated the role of the host and the role of the bacterium in these events. Internalization of invasive bacteria often requires a metabolically active bacterium in a process called parasite-directed endocytosis (17, 21, 23, 40). Because the target cells of *B. piliformis* are not normally phagocytic, it is likely that *B. piliformis* participates in its internalization. To determine the role of the bacteria in *B. piliformis* internalization, we evaluated the attachment and internalization of viable and Formalin-killed bacteria. Formalin killing of *B. piliformis* completely abolished their ability to internalize. These results suggest that *B. piliformis* does indeed play an active role in its internalization.

The effects of Formalin killing on bacterial adhesion were not as definitive as those on bacterial internalization. Adhesion of killed bacteria was evident, but it was significantly lower than adhesion of viable bacteria at two time points. The reasons for this are unknown. Because these experiments were done with subconfluent monolayers of tissue culture cells, it is possible that bacteria were centrifuged onto areas of the plate containing no cells. Killed nonmotile bacteria unable to locate host cells would in turn be washed off in slide preparation. This problem would be further compounded if *B. piliformis* adhesion is mediated by specific receptors. These factors may have accounted for the discrepancy in the percentage of cells with live and killed adherent bacteria. Other possibilities include partial Formalin-associated alteration of bacterial adhesins or steric hindrance created by cross-linking of proteins adjacent to bacterial adhesins. If this were the case, however, one would expect inhibition of adhesion uniformly across all three time points. Further work to characterize the effects of different methods of killing *B. piliformis* on bacterial adhesion is under way in our laboratory.

To examine the role of the host in *B. piliformis* internalization, we evaluated the need for host microfilament-dependent functions on this internalization. Most invasive bacteria are thought to utilize host cell mechanisms of phagocytosis for entry. During phagocytosis, monomeric G actin polymerizes to form F actin, which functions in cell membrane movement and eventual engulfment of the bacterium. This polymerization can be prevented by cytochalasin D, a metabolite of the fungus *Metarrhizium anisopliae* (5, 7). The entry of several bacteria has been inhibited by the cytochalasins, suggesting that these bacteria require cell cytoskeletal function in order to invade (13, 18). To this end, we investigated the effects of cytochalasin D treatment of monolayers on bacterial internalization. Our results suggest that entry of *B. piliformis* occurs through interaction with the host cell cytoskeleton. These results correlate with the finding of bacteria in membrane-bound vacuoles, which may represent phagosomes.

In summary, *B. piliformis* is a unique obligate intracellular bacterium. It is relatively large compared with other intracellular bacteria, obtaining lengths of up to 40 μm (15). Peritrichous flagella confer rapid motility on the bacterium; this motility is evident both within and outside the host cell.

In this study, *B. piliformis* entered cultured cells by a microfilament-dependent parasite-directed endocytosis mechanism, similar to that of several other invasive pathogens. Once inside the host cell, *B. piliformis* rapidly escaped the phagosome and replicated within the cytoplasm in a manner similar to that of *Shigella* and *Rickettsia* spp. Its intracellular lifestyle differs from that of other intracellular pathogens in that *B. piliformis* exhibits rapid motility while in the cell and may invade the nucleus. The generation time of *B. piliformis* is markedly longer than that of most facultative invasive bacteria but resembles that of other obligate intracellular pathogens such as rickettsiae. The mechanism of *B. piliformis* escape from the cell is unknown. Our ultrastructural studies suggest that *B. piliformis* may damage the cell when a critical number of bacteria is reached. Subsequent release of bacteria and death of the cell, as occur with rickettsiae, may then occur. The results of these studies indicate that Caco-2 and Intestine 407 cells can be used as an in vitro model to investigate the molecular aspects of *B. piliformis* growth and survival within mammalian cells. Further studies are needed to elucidate the precise mechanisms of adhesion, invasion, and dissemination of this unique intracellular pathogen.

## ACKNOWLEDGMENTS

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