INFECTION AND IMMUNITY, Dec. 1994, p. 5641–5646
0019-9567/94/$04.00+0

Vol. 62, No. 12

# Bovine Mammary Epithelial Cell Invasion by *Streptococcus uberis*

K. R. MATTHEWS,[1]* R. A. ALMEIDA,[2] AND S. P. OLIVER[2]

*Agricultural Research Service, U.S. Department of Agriculture,[1] and Department of Animal Science, Institute of Agriculture,[2] The University of Tennessee, Knoxville, Tennessee 37901-1071*

Received 27 May 1994/Returned for modification 15 July 1994/Accepted 10 September 1994

***Streptococcus uberis* commonly causes bovine mastitis; however, the pathogenesis of this infection is poorly understood. In this study, the ability of *S. uberis* to invade mammary epithelial cells in culture was investigated. Two strains of *S. uberis* isolated from bovine mammary secretions were capable of invading bovine mammary epithelial cells in vitro at different levels, suggesting strain differences in invasiveness. Invasion required microfilaments but not microtubular cytoskeletal elements. No morphological changes in epithelial cells were observed for up to 24 h postinfection, suggesting no cellular injury. Strains of *S. uberis* evaluated were genetically distinct and differed phenotypically in expression of potential virulence factors. Whether a single factor or combination of factors was responsible for differences in invasiveness was not addressed in this study. These data provide a foundation for a better understanding of the processes used by *S. uberis* to invade epithelial cells. Epithelial cell invasion may be a potentially important mechanism in the pathogenesis of *S. uberis* mastitis.**

*Streptococcus uberis* is an environmental pathogen responsible for a high proportion of cases of clinical and subclinical mastitis in lactating cows and is the predominant organism isolated from mammary glands during the nonlactating period (3, 4, 18). Udder infection with *S. uberis* is of major importance since it is poorly controlled by existing measures such as teat dipping and antibiotic dry cow therapy (4). Lack of success in controlling mastitis caused by *S. uberis* is associated with inadequate information on pathogenesis of the infection (3, 4, 10).

The influence of *S. uberis* virulence factors on host defense mechanisms and mammary gland physiology is poorly defined and has not received adequate research attention. Potential virulence factors associated with *S. uberis* include hyaluronidase (20), hyaluronic acid capsule (2, 16), and uberis factor, believed to be similar to CAMP factor of *Streptococcus agalactiae* (21). Recently, a culture filtrate of *S. uberis* was shown to activate bovine plasminogen, similar to the streptokinase activity of other streptococcal species (15). Hyaluronidase was isolated from all *S. uberis* strains evaluated (20). Recently, Matthews et al. (17) reported on the influence of specific bacterial virulence factors on proliferation of a bovine mammary epithelial cell line (MAC-T). Both hyaluronidase and hyaluronic acid capsule significantly decreased mammary epithelial cell proliferation, which may be of paramount importance during the periparturient period when mammary glands are undergoing marked physiological changes. The aforementioned virulence factors may be involved in adherence and/or penetration of stromal tissue by *S. uberis* (20).

Adherence of bacteria to host cells has been suggested as a prerequisite for colonization and establishment of infection. Thomas et al. (24) reported on use of explant cultures of bovine mammary tissue to investigate the hypothesis that adhesion to epithelium may be the first stage of pathogenesis. Although they did not find evidence to support this hypothesis, they determined that *S. uberis* bound to endothelium coated with fibrin (24), suggesting the interaction of this organism with extracellular membrane proteins. This conclusion is supported by a recent study in our laboratory which demonstrated that strains of *S. uberis* adhered to collagen, fibronectin, and laminin (1).

In addition, studies in our laboratory utilizing several in vitro adherence assays provided evidence for adherence of *S. uberis* to mammary epithelial cells (1). Interestingly, during investigation of adherence, *S. uberis* was observed in membrane-bound vacuoles within the cytoplasm of epithelial cells. Recent studies have shown that pathogens previously regarded as noninvasive (enteropathogenic *Escherichia coli* and *Edwardsiella* species) are capable of invading epithelial cells (5, 13). The ability of *S. uberis* in vivo to invade bovine mammary epithelial cells could result in protection from host defense mechanisms and the action of most antimicrobial agents (6). Lack of success in controlling *S. uberis* mastitis may be related to the ability of the organism to invade mammary epithelial cells. Invasion of bovine mammary secretory epithelial cells by bacteria may affect secretory cell function, and this may be related to decreased milk production associated with mastitis. In this study, the ability of *S. uberis* to invade a bovine mammary epithelial cell line was investigated.

## MATERIALS AND METHODS

**Bacteria.** *S. uberis* UT101 and *S. uberis* UT102 isolated from bovine mammary secretions from individual cows in the same herd were used. Strains were isolated from mammary glands with subclinical mastitis, subsequently becoming clinical. Isolates were identified to species level by the API Rapid Strep System (Analytab Products Inc., Plainview, N.Y.). Strains were genetically distinct on the basis of PCR-based DNA fingerprinting (14). Isolates had been previously evaluated for several factors related to virulence (1, 2, 16, 17). Strains were stored in 10% skim milk at −80°C. Isolates were passaged more than three times prior to use in this study.

For all assays, bacteria were routinely grown on Trypticase soy agar supplemented with 5% defibrinated sheep blood for

* Corresponding author. Present address: 105 Terrill Hall, The University of Vermont, Burlington, VT 05405. Phone: (802) 656-4343. Fax: (802) 656-4343.

16 h at 37°C. On the day of experimentation, bacteria were centrifuged and washed in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand Island, N.Y.) and resuspended in the same volume of DMEM. The concentration and purity of each inoculum were determined by quantitative culture on blood agar plates.

**Cell cultures.** The MAC-T cells were grown as described by Huynh et al. (11). Briefly, MAC-T cells were grown on plastic tissue culture flasks in growth medium (DMEM containing 10% fetal bovine serum [HyClone, Logan, Utah], 5 μg of insulin per ml, and 1 μg of hydrocortisone [Sigma Chemical Co., St. Louis, Mo.] per ml). Cells were cultured in 25-$cm^2$ flasks at 37°C in 5% $CO_2$–95% air. Prior to passage, cells were washed with calcium-free Dulbecco's phosphate-buffered saline (DPBS; Gibco). Cells were released from plastic by rinsing with trypsin solution (0.5% trypsin in DPBS) for approximately 30 s, trypsin was aspirated, and flasks were incubated at 37°C until cells were released (10 min). Trypsinization was halted by addition of growth medium plus fetal bovine serum.

**Invasion assay.** The invasion model used standard in vitro invasion methodology involving incubation of bacteria with epithelial cells followed by selective killing of extracellular bacteria by penicillin G and gentamicin and release of internalized bacteria by lysis of mammalian cells with detergent (22). MAC-T cells grown to confluence in 24-well plates were inoculated with approximately $2 \times 10^7$ CFU of *S. uberis* UT101 or *S. uberis* UT102. Cultures were incubated for 2, 4, 6, and 8 h at 37°C in 5% $CO_2$–95% air. At 2-h intervals, medium was removed, nonadherent bacteria were removed by washing with DPBS, and growth medium containing 2,000 U of penicillin G per ml and 2 mg of gentamicin per ml was added to monolayers for 30 min to kill any remaining extracellular bacteria. Medium was removed, and monolayers were washed twice with DPBS. Internalized bacteria were released by incubating infected monolayers with 1 ml of a 1.0% (vol/vol) solution of Triton X-100 in DPBS for 20 min at 37°C. The lysate was agitated to break up streptococcal chains, serially diluted, and plated on Trypticase soy agar plates supplemented with 5% defibrinated sheep blood. The assay was carried out in duplicate, and three replicate cultures were collected at each sample time. Data from each experiment were analyzed by Student's *t* test (23).

**Invasion inhibition assays.** Assays for determining effects of microtubule and microfilament inhibitors on invasion were performed by a modification of the invasion assay described above. MAC-T cell monolayers (24-well plates) were incubated for 1 h either with or without colchicine (Sigma), cytochalasin B (Sigma), or cytochalasin D (Sigma). The MAC-T cell monolayers were then inoculated with either *S. uberis* UT101 or *S. uberis* UT102 and incubated for 2 h at 37°C in 5% $CO_2$–95% balance air. The remainder of the protocol was identical to the invasion assay. The assay was carried out in duplicate, and three replicate cultures were collected at each sample time. Statistical analysis was performed as described above.

**Electron microscopy.** *S. uberis* UT101 and *S. uberis* UT102 ($5 \times 10^7$ CFU/ml) prepared as described above were added to monolayers in 35-mm-diameter petri dishes. At 2, 4, 6, 8, and 24 h, cultures (extracellular bacteria were not killed as described for invasion assays) were prepared for electron microscopy. Following incubation, cultures were washed three times with 0.1 M cacodylate buffer and fixed at 37°C by adding 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Samples were then washed three times in 0.1 M cacodylate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 15 min at room temperature. Fixed cultures were washed three times in 0.1 M cacodylate buffer, dehydrated through a graded series of ethanol, and embedded in epoxy resins. Ultrathin sections were examined by transmission electron microscopy (TEM) using a Philips EM 100 electron microscope. For scanning electron microscopy, epithelial cells were cultured on 12-mm-diameter glass coverslips. Coverslips with epithelial cells were processed as described for TEM, but after dehydration in ethanol, samples were dried at critical point. An ETEC Autoscan (ETEC Corporation, Hayward, Calif.) was used for observation.

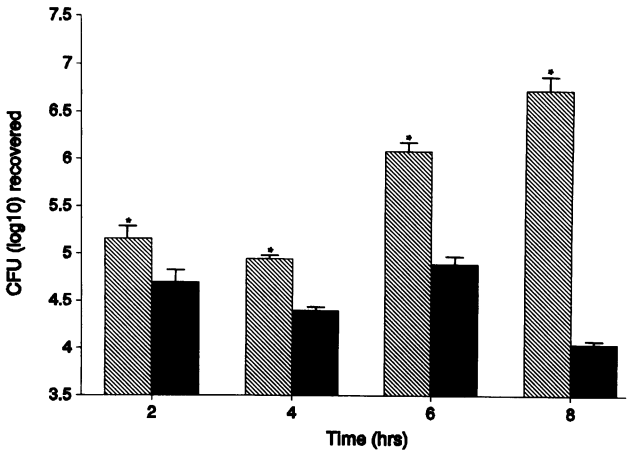


FIG. 1. Recovery of *S. uberis* UT101 (striped bars) and *S. uberis* UT102 (solid bars) intracellularly from MAC-T cells over 8 h. Error bars indicate standard errors of the means. *, $P < 0.05$.

## RESULTS

The invasion model used in this study demonstrated the ability of *S. uberis* to enter bovine mammary epithelial cells in cultured monolayers. The assay allows quantification of intracellular bacteria after extracellular bacteria are killed. *S. uberis* UT101 was significantly more invasive (Fig. 1) than *S. uberis* UT102 at all time points measured. After 4 h of incubation, the number of recoverable intracellular *S. uberis* UT101 organisms increased at each sampling, with the greatest number of recoverable intracellular organisms at 8 h. The number of recoverable intracellular *S. uberis* UT102 organisms fluctuated slightly over the 8-h sample period.

Scanning electron microscopy of infected MAC-T cells showed that *S. uberis* attached to epithelial cells in vitro (Fig. 2). Attachment appeared to be tenacious, since after a rigorous fixation process, numerous bacteria remained attached to epithelial cell surfaces.

To document that *S. uberis* was entering MAC-T cells in vitro, TEM was performed on glutaraldehyde-fixed monolayers at 2, 4, 6, 8 and 24 h of incubation. By 2 h, *S. uberis* UT101 was in close proximity to epithelial cell microvilli (Fig. 3A). Pedestal formation at the interfacing region was detected where intimate attachment of *S. uberis* UT101 to the epithelial cell membrane occurred (Fig. 3B). Figure 3C shows effacement of some microvilli and early invagination of the epithelial cell at the site of bacterial attachment. The intracellular location of *S. uberis* UT101 was confirmed by the presence of bacteria in membrane-bound vacuoles within the cytoplasm of epithelial cells (Fig. 3D). At 8 h, epithelial cells often had vacuoles containing several bacteria, suggesting some intracellular replication; however, most cells contained one or two *S. uberis*

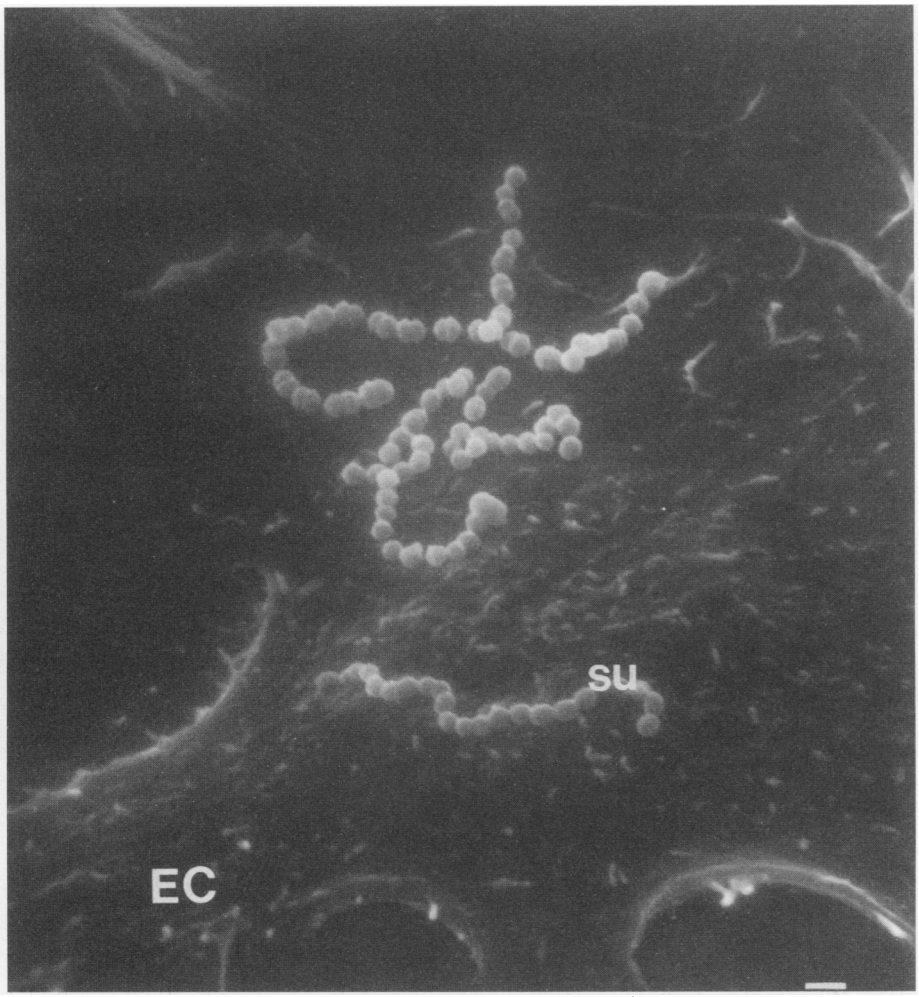


FIG. 2. Electron micrograph of *S. uberis* UT101 (su) adhering to a MAC-T cell (2-h coculture). The epithelial cell (EC) has >100 organisms adhering to it. Bar = 1 μm.

organisms per vacuole, with few bacteria free in the cytoplasm (data not shown). After prolonged (24-h) infection, many epithelial cells were completely filled with *S. uberis*. Bacteria were never observed within the epithelial cell nucleus (Fig. 4). Most bacteria appeared to be free within the cytoplasm of the epithelial cell, some undergoing division (Fig. 4). No morphological changes in epithelial cells suggestive of cell injury were observed for up to 24 h. Data were similar for *S. uberis* UT102.

The effects of microtubule and microfilament inhibitors in the invasion assay are shown in Table 1. Cytochalasins B and D, microfilament inhibitors, significantly inhibited entry of *S. uberis* into MAC-T cell monolayers at three different concentrations. Colchicine, which inhibits eukaryotic microtubule formation, did not influence *S. uberis* invasion of MAC-T cells. These data suggest an active role for MAC-T cell microfilaments in the internalization of *S. uberis*.

## DISCUSSION

Results of this study demonstrate the ability of *S. uberis* to invade bovine mammary epithelial cells in vitro. Using an invasion model that employed standard in vitro invasion methodology (12) and TEM, we defined some of the characteristics important for entry of *S. uberis* into eukaryotic cells. Entry of *S. uberis* into MAC-T cells was inhibited by cytochalasin B and cytochalsin D, as shown previously for other pathogens (9, 22). These results suggest that microfilaments are necessary for epithelial cell internalization of *S. uberis*.

Out of necessity, the first step in invasion by nonmotile bacteria must include attachment to host cells by random contact (7). Scanning electron microscopy of infected MAC-T cells confirmed that *S. uberis* attached to epithelial cell surfaces and cellular structures. A previous study demonstrated that both strains of *S. uberis* adhered readily to MAC-T cells (1). Reports on other organisms have shown that most invasive bacterial species do attach to eukaryotic cells prior to invasion (7, 8). However, it has not been determined whether attachment by *S. uberis* is a prerequisite for invasion.

Significantly more *S. uberis* UT101 than *S. uberis* UT102 organisms were recovered intracellularly at each sampling time (Fig. 1). The number of *S. uberis* UT101 organisms recovered intracellularly increased also from 2 h to 8 h, which may have been due to an increase in the number of extracellular bacteria. However, the two strains evaluated had similar growth curves (bacteria cultured in cell culture growth medium alone) over the 8-h experiment, with bacterial numbers increasing approx-

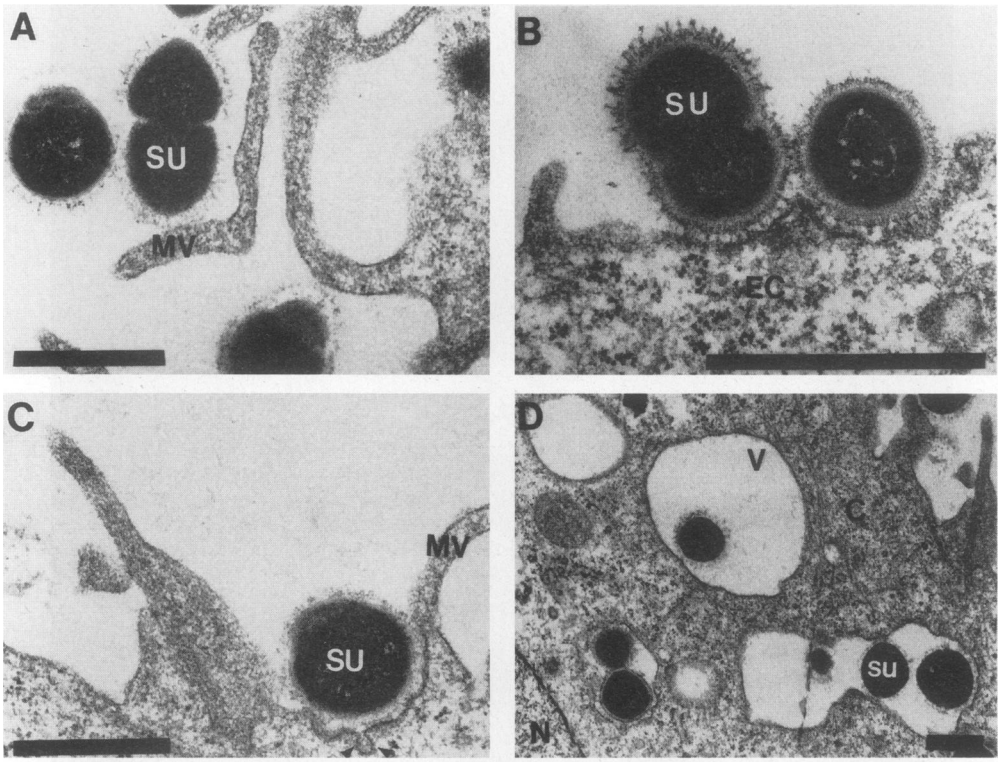


FIG. 3. Electron micrographs of *S. uberis* UT101 entering cultured bovine mammary epithelial cells. By 2 h, *S. uberis* (SU) organisms were in close proximity to epithelial cell microvilli (MV). Effacement of some microvilli with attachment of the organism and early invagination is shown (panels B and C). By 2 h, *S. uberis* was visualized deep in the cytoplasm (C) within membrane-bound vacuoles (V; panel D). N, nucleus. Bar = 1 μm.

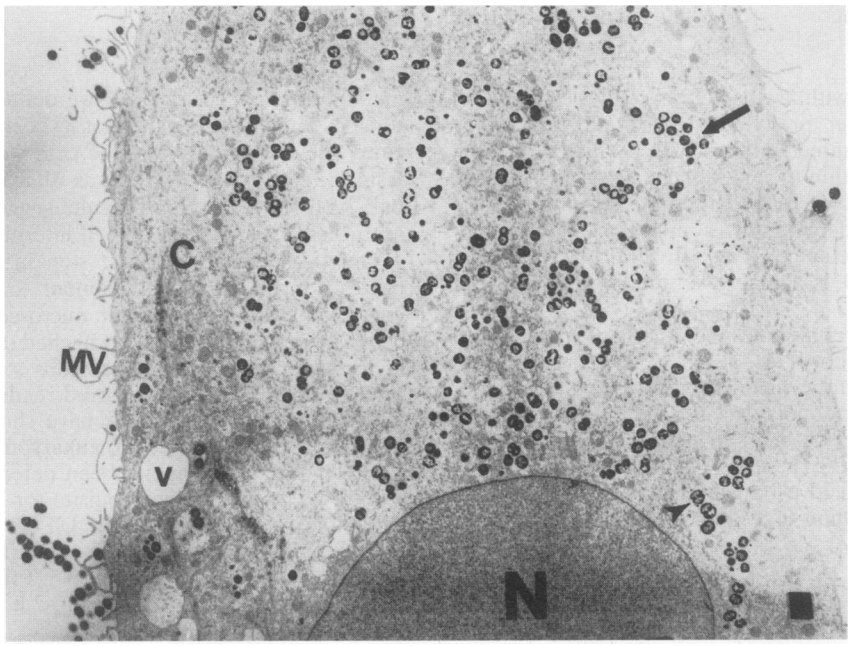


FIG. 4. Electron micrograph of internalized *S. uberis* UT101 following prolonged (24-h) infection of epithelial cells. Most bacteria (arrow) were free in the cytoplasm (C), and many appeared to be replicating (arrowhead). No degeneration of the epithelial cell as a consequence of internalization of the organism was observed. N, nucleus; MV, microvilli; V, vacuole. Bar = 1 μm.

TABLE 1. Effects of microfilament and microtubule inhibitors on *S. uberis* invasion of bovine mammary epithelial cells

| Inhibitor | Concn (μg/ml) | CFU invading (% of control)[a] | |
|---|---|---|---|
| | | UT101 | UT102 |
| Cytochalasin B | 0.0 | 100.0[a] | 100.0[a] |
| | 0.1 | 31.7[b] | 9.6[b] |
| | 1.0 | 15.4[b] | 0.7[b] |
| | 10.0 | 0.6[b] | 0.0[b] |
| Cytochalasin D | 0.0 | 100.0[a] | 100.0[a] |
| | 0.1 | 4.5[b] | 0.8[b] |
| | 1.0 | 0.2[b] | 0.0[b] |
| | 10.0 | 0.0[b] | 0.0[b] |
| Colchicine | 0.0 | 100.0 | 100.0 |
| | 10.0 | 110.0 | 120.0 |
| | 20.0 | 127.0 | 136.0 |
| | 40.0 | 135.0 | 128.0 |

[a] Means with different superscripts within a column and for an inhibitor differ ($P < 0.01$).

imately 1 log unit. Possible reasons why strain UT101 was more invasive include differences between strains in expression of specific components critical for adherence or invasion and ability of the organism to survive intracellularly. Each strain was evaluated for several virulence factors or traits, including hyaluronidase production, capsule expression, uberis factor (determined by CAMP reaction), adherence to extracellular matrix proteins (collagen, fibronectin, and laminin), and presence of M protein (1, 14, 16). Differences in the aforementioned properties between strains were evident; however, whether a single factor or trait or a combination of these is responsible for differences in invasiveness has not yet been determined. In this study, this issue was not addressed since our objective was to determine if *S. uberis* was capable of entering bovine mammary epithelial cells.

Microfilaments were necessary for epithelial cell internalization of *S. uberis*; however, microtubules apparently were not required for invasion (Table 1). Microtubules are key components of the mammalian cytoskeleton that maintain the cellular architecture (21). Results of the present study are consistent with reports on *Salmonella choleraesuis*, *Shigella flexneri*, and *Yersinia enterocolitica* (6–8, 12). However, some organisms such as enteropathogenic *E. coli* require both microfilaments and microtubules for entry into eukaryotic cells (6). The microtubule inhibitor used in this study, colchicine, enhanced invasion by *S. uberis* at all concentrations evaluated. Similar results have been reported (19, 22), with no clear explanation for this observation.

TEM of MAC-T cells containing *S. uberis* for up to 8 h revealed that most organisms remained within vacuoles and did not escape into the cytoplasm. Some vacuoles contained multiple organisms, suggesting vacuole coalescence or intracellular replication. In contrast, by 24 h, numerous bacteria were free in the cytoplasm of the epithelial cell, and many appeared to be replicating (Fig. 4). Electron microscopy also showed the absence of epithelial cell degeneration as a consequence of internalization of the organism.

Results of this study suggest that *S. uberis* is capable of entering mammary epithelial cells in vitro. These data provide a foundation for a better understanding of the processes used by *S. uberis* to invade epithelial cells. Intracellular location may protect the organism from host defense mechanisms and antibiotics. Experiments are in progress to determine specific components critical for adherence and invasion by *S. uberis*. Epithelial cell invasion may be a potential mechanism in the pathogenesis of *S. uberis* mastitis.

## ACKNOWLEDGMENTS

We express our appreciation to Arlene Stewart for excellent word processing assistance and to Jeff Turner for providing the MAC-T cell line.

This investigation was supported by the Tennessee Agricultural Experiment Station and by The University of Tennessee, College of Veterinary Medicine, Center of Excellence Research Program in Livestock Diseases and Human Health.

## REFERENCES

1. **Almeida, R. A., D. Luther, S. J. Kumar, J. D. Turner, and S. P. Oliver.** 1993. Adherence of encapsulated and nonencapsulated strains of *Streptococcus uberis* to bovine mammary epithelial cells and extracellular matrix proteins. J. Dairy Sci. **76**(Suppl. 1):259.
2. **Almeida, R. A., and S. P. Oliver.** 1993. Antiphagocytic effect of the capsule of *Streptococcus uberis*. J. Vet. Med. **40**:707–714.
3. **Bramley, A. J.** 1984. *Streptococcus uberis* udder infection—a major barrier to reducing mastitis incidence. Br. Vet. J. **140**:328–335.
4. **Bramley, A. J., and F. H. Dodd.** 1984. Reviews of the progress of dairy science. Mastitis control—progress and prospects. J. Dairy Res. **49**:369–373.
5. **Donnenberg, M. S., A. Donohue Rolfe, and G. T. Keusch.** 1990. A comparison of HEp-2 cell invasion by enteropathogenic and enteroinvasive *Escherichia coli*. FEMS Microbiol. Lett. **57**:83–86.
6. **Falkow, S.** 1991. Bacterial entry into eukaryotic cells. Cell **65**:1099–1102.
7. **Finlay, B. B.** 1990. Cell adhesion and invasion mechanisms in microbial pathogenesis. Curr. Opin. Cell Biol. **2**:815–820.
8. **Finlay, B. B., and S. Falkow.** 1989. Common themes in microbial pathogenicity. Microbiol. Rev. **53**:210–230.
9. **Gibson, R. L., M. K. Lee, C. Soderland, E. Y. Chi, and C. E. Rubens.** 1993. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion. Infect. Immun. **61**:478–485.
10. **Hill, A. W.** 1988. Pathogenicity of two strains of *Streptococcus uberis* infused into lactating and non-lactating bovine mammary glands. Res. Vet. Sci. **45**:400–404.
11. **Huynh, H. T., G. Robitaille, and J. D. Turner.** 1991. Establishment of bovine mammary epithelial cells (MAC-T): an in vitro model for bovine lactation. Exp. Cell Res. **197**:191–199.
12. **Isberg, R. R., and S. Falkow.** 1985. A single genetic locus encoded by *Yersinia pseudotuberculosis* permits invasion of cultured animal cells by *Escherichia coli* K-12. Nature (London) **317**:262–264.
13. **Janda, J. M., S. L. Abbott, and L. S. Oshino.** 1991. Penetration and replication of *Edwardsiella* spp. in HEp-2 cells. Infect. Immun. **59**:154–161.
14. **Jayarao, B. M., B. J. Bassam, G. Caetano-Anollès, P. M. Gresshoff, and S. P. Oliver.** 1992. Subtyping of *Streptococcus uberis* by DNA amplification fingerprinting. J. Clin. Microbiol. **30**:1347–1350.
15. **Leigh, J. A.** 1993. Activation of bovine plasminogen by *Streptococcus uberis*. FEMS Microbiol. Lett. **114**:67–71.
16. **Matthews, K. R., S. P. Oliver, B. M. Jayarao, A. J. Guidry, E. F. Erbe, and W. P. Wergin.** 1994. Encapsulation of *Streptococcus uberis*: influence of storage and cultural conditions. Vet. Microbiol. **39**:361–367.
17. **Matthews, K. R., J. J. Rejman, J. D. Turner, and S. P. Oliver.** 1992. Proliferation of a bovine mammary epithelial cell line in the presence of bacterial virulence factors. J. Dairy Sci. **75**(Suppl. 1):258.
18. **Oliver, S. P.** 1988. Frequency of isolation of environmental mastitis-causing pathogens and incidence of new intramammary infections during the nonlactating period. Am. J. Vet. Res. **49**:1789–1793.
19. **Rubens, C. E., S. Smith, M. Hulse, E. Y. Chi, and G. Van Belle.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect. Immun. **60**:5157–5163.
20. **Schaufuss, P., R. Sting, W. Schaeg, and H. Blobel.** 1989. Isolation and characterization of hyaluronidase from *Streptococcus uberis*. Zentralbl. Bakteriol. **A271**:46–53.
21. **Skalka, B., and J. Smola.** 1981. Lethal effect of CAMP-factor and

uberis-factor—a new finding about diffusible exosubstances of *Streptococcus agalactiae* and *Streptococcus uberis*. Zentralbl. Bakteriol. Hyg. **A249:**190–194.

22. **Sreenivasan, P. K., D. H. Meyer, and P. M. Fives-Taylor.** 1993. Requirements for invasion of epithelial cells by *Actinobacillus actinomycetemitans*. Infect. Immun. **61:**1239–1245.
23. **Steel, R. G. D., and J. H. Torrie.** 1980. Principles and procedures of statistics: a biometrical approach, 2nd ed. McGraw-Hill Book Company, New York.
24. **Thomas, L. H., J. A. Leigh, A. P. Bland, and R. S. Cook.** 1992. Adherence and colonization by bacterial pathogens in explant cultures of bovine mammary tissue. Vet. Res. Commun. **16:**87–96.