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The Ability of *Mycobacterium avium* subsp. *paratuberculosis* To Enter Bovine Epithelial Cells Is Influenced by Preexposure to a Hyperosmolar Environment and Intracellular Passage in Bovine Mammary Epithelial Cells

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***Mycobacterium avium* subsp. *paratuberculosis* is the cause of Johne's disease in cattle and other ruminants. *M. avium* subsp. *paratuberculosis* infection of the bovine host is not well understood; however, it is assumed that crossing the bovine intestinal mucosa is important in order for *M. avium* subsp. *paratuberculosis* to establish infection. To examine the ability of *M. avium* subsp. *paratuberculosis* to infect bovine epithelial cells in vitro, Madin-Darby bovine kidney (MDBK) epithelial cells were exposed to *M. avium* subsp. *paratuberculosis*. It was observed that bacteria can establish infection and replicate within MDBK cells. *M. avium* subsp. *paratuberculosis* also has been reported to infect mammary tissue and milk, and we showed that *M. avium* subsp. *paratuberculosis* infects bovine mammary epithelial cells (MAC-T cell line). Using polarized MAC-T cell monolayers, it was also determined that *M. avium* subsp. *paratuberculosis* crosses apical and basolateral surfaces with approximately the same degree of efficiency. Because *M. avium* subsp. *paratuberculosis* can be delivered to the naïve host by milk, it was investigated whether incubation of *M. avium* subsp. *paratuberculosis* with milk has an effect on invasion of MDBK cells. *M. avium* subsp. *paratuberculosis* exposed to milk entered epithelial cells with greater efficiency than *M. avium* subsp. *paratuberculosis* exposed to broth medium or water ($P < 0.01$). Growth of *M. avium* subsp. *paratuberculosis* within MAC-T cells also resulted in augmented ability to subsequently infect bovine MDBK cells ($P < 0.001$). Microarray analysis of intracellular *M. avium* subsp. *paratuberculosis* RNA indicates the increased transcription of genes which might be associated with an invasive phenotype.**

Mycobacterium avium subsp. *paratuberculosis* is the etiologic agent of Johne's disease in cattle and other ruminants. It is assumed that *M. avium* subsp. *paratuberculosis* infects the young calf by crossing the intestinal barrier. Previous work (3, 26) has indicated that the interaction of *M. avium* subsp. *paratuberculosis* with bovine epithelial cells is a complex process which might involve participation of several bacterial and host factors. For example, it has been reported that both in calves and in mice, challenge by the gastrointestinal route results in *M. avium* subsp. *paratuberculosis* infecting M cells in the Peyer's patches (23, 26). Recently, Secott and colleagues have suggested that the invasion of the intestinal mucosa by *M. avium* subsp. *paratuberculosis* is secondary to the binding to fibronectin (26). In addition, Bannantine and colleagues demonstrated a role for a 35-kDa *M. avium* subsp. *paratuberculosis* protein in the invasion of cultured bovine epithelial cells (5). The 35-kDa protein is exposed in the outer layer of *M. avium* subsp. *paratuberculosis* and has also been associated with *Mycobacterium avium* invasion of human intestinal cells (22).

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After *M. avium* subsp. *paratuberculosis* crosses the intestinal mucosa, the infection spreads to other organs, leading to the advanced stages of disease. Several studies have reported the presence of *M. avium* subsp. *paratuberculosis* in the mammary glands and in milk of symptomatic or asymptomatic animals (6, 28–30). This observation raises the possibility that the mammary gland could be a reservoir for *M. avium* subsp. *paratuberculosis*. The environment inside the mammary gland is believed to be hyperosmolar (milk) and hypoxic (28). In addition, *M. avium* subsp. *paratuberculosis* organisms infecting mammary epithelial cells might suffer the influence of the intracellular environment. In fact, *M. avium* subsp. *paratuberculosis* may remain in contact with either the intracellular or milk environment for periods of up to 24 h before being excreted. Previous studies using *Mycobacterium avium* subsp. *avium* have shown that the environment to which the bacterium is exposed in the host can influence the expression of genes associated with its ability to enter epithelial cells. Incubation of *M. avium* subsp. *avium* in low oxygen tension or increased osmolarity conditions significantly enhances its ability to enter intestinal epithelial cells (8). A previous study on interactions between *M. avium* subsp. *avium* and environmental amoebae has shown that infection of amoebae results in enhanced virulence (14). Similar findings were reported regarding the *M. avium* subsp.

paratuberculosis 35-kDa protein (5), which was shown to be expressed under anaerobic and hyperosmolar conditions (5).

It is reasonable to hypothesize that bacterial exposure to the complex conditions in the mammary gland or mammary gland epithelial cells may have an effect on virulence gene expression and, therefore, on the efficacy of *M. avium* subsp. *paratuberculosis* invasion of the intestinal mucosa.

The objectives of this work were to determine whether *M. avium* subsp. *paratuberculosis* has the ability to enter cultured bovine epithelial cells in vitro and whether this characteristic can be influenced by exposure to milk or the intracellular environment of mammary epithelial cells.

MATERIALS AND METHODS

Cell lines. A bovine epithelial cell line (Madin-Darby bovine kidney [MDBK]) was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained on Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum. A bovine mammary epithelial cell line (MAC-T) was kindly provided by Lewis Sheffield (Department of Dairy Science, University of Wisconsin). MAC-T cells were maintained on Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal bovine serum, 5 μg/ml insulin, and 1 μg/ml hydrocortisone.

Bacteria. *M. avium* subsp. *paratuberculosis* ATCC 19698, a bovine clinical isolate from an animal with Johne's disease, was purchased from ATCC. It was grown at 37°C on modified Middlebrook 7H11 agar supplemented with Mycobactin J (2 mg/liter) and oleic acid-albumin-dextrose-catalase (OADC) (10%, vol/vol) or in 7H9 broth with Mycobactin J (2 mg/liter) and OADC (10%, vol/vol). For the invasion assay, individual colonies were selected, and a bacterial suspension was prepared using Hanks' balanced salt solution (HBSS) to give turbidity equivalent to a 0.5 McFarland standard. The *M. avium* subsp. *paratuberculosis* suspension (5 ml) was then passed through a 30-gauge needle 10 times, and clumps in the final suspension were allowed to settle. The top 1 ml out of the 5-ml suspension containing dispersed bacteria was used for the invasion assay.

Invasion assay. The invasion assays were carried out using the methods described by Bermudez and Young and by Sangari et al. (10, 24). Briefly, 24-well tissue culture plates (Corning Costar, New York) were seeded with 10^4 MDBK or MAC-T cells, and monolayers were grown in an atmosphere of 5% CO_2 at 37°C until confluence. Before infection, the medium was replaced with fresh culture medium. Monolayers were then infected with approximately 2×10^6 bacteria (approximate ratio, 10 bacteria:1 cell) and incubated at 37°C in 5% CO_2 for up to 4 h, depending upon the experimental design. After incubation, monolayers were washed three times with HBSS and then treated with 200 μg/ml of amikacin for 2 h at 37°C to kill extracellular bacteria, as shown in the *M. avium* system (8, 10) and confirmed using *M. paratuberculosis* (data not shown). Following incubation, the supernatant was removed and cell monolayers were washed three times with HBSS. To lyse the cells, wells were treated with 0.5 ml Triton X-100 (0.1%) for 10 min. Subsequently, 0.5 ml 7H9 broth containing Mycobactin J (2 mg/liter) and OADC (10%, vol/vol) was added to each well, and cells were disrupted by vigorous pipetting. Lysates were collected, serially diluted, and plated onto 7H11 agar for colony count. The percentage of invasion was calculated as the fraction of the inoculated bacteria that was recovered from the cell lysate.

***M. avium* subsp. *paratuberculosis* survival in MDBK and MAC-T cells.** Confluent monolayers of bovine epithelial cells were incubated with approximately 10^6 *M. avium* subsp. *paratuberculosis* organisms for 2 h. After the incubation period, extracellular bacteria were killed by amikacin treatment (as described above), the supernatant was removed, and fresh culture medium was added to the monolayers. Infected cells were incubated for up to 4 days. At the end of the incubation period, monolayers were lysed, the lysate was diluted, and the number of intracellular bacteria was determined by plating cell lysates onto 7H11 agar.

Translocation assay. The translocation assay was performed using the Transwell 2-chamber culture system (Corning Costar, New York). Monolayers were established either on the top or bottom sides of the membrane by seeding it with 1×10^5 MAC-T cells. Some monolayers had their apical surface exposed to the medium in the bottom chamber, whereas others had their basolateral side exposed to the medium in the top chamber. The culture medium was changed daily, and the integrity of the monolayers was determined by the following methods: (i) microscopic observation, (ii) measuring the transepithelial resistance, and (iii) the trypan blue (0.25%) permeability assay (optical density at 580 nm), as previously described (9, 24). Trypan blue (0.25%) was added to the monolayer, and 3 h later the supernatant of the lower chamber was obtained for spectrometer reading. Controls included medium only (baseline). The top chamber was infected with 3×10^7 bacteria as described above. After 1, 2, 3, and 4 days infection, 600 μl of filtrate was collected from the bottom chamber, and fresh culture medium (600 μl) was replenished. The ability to translocate was calculated as the cumulative percentage of the initial inoculum recovered in the bottom chamber at each time point.

Passage of *M. avium* subsp. *paratuberculosis* in MAC-T cells. Confluent monolayers of MAC-T cells in cell culture flasks (Corning Costar, New York) were inoculated with approximately 10^7 *M. avium* subsp. *paratuberculosis* bacteria. After 2 h of contact time, extracellular bacteria were removed, followed by amikacin treatment as described previously, and new medium was added to the monolayer. Internalized bacteria were allowed to grow at 37°C for a total of 1 or 4 days. At the end of the incubation period, MAC-T cells were lysed by adding 0.5 ml Triton X-100 (0.1%) for 10 min. Lysates were then submitted to differential centrifugation as reported previously (8). The *M. avium* subsp. *paratuberculosis* cells were collected into 7H9 broth containing Mycobactin J (2 mg/liter) and OADC (10%, vol/vol). The bacteria were recovered by centrifugation at $3,500 \times g$ for 30 min at 4°C to maintain their phenotype and then used in the invasion assays.

Preexposure of *M. avium* subsp. *paratuberculosis* to milk and milk components. Approximately 10^7 *M. avium* subsp. *paratuberculosis* organisms were inoculated into 5 ml of raw milk (from a pool of several cows) containing polymyxin B (5.5 mg/liter), amphotericin (11 mg/liter), carbenicillin (25 mg/liter), and trimethoprim (2.5 mg/liter). Incubation was carried out at 37°C. After 24 h, *M. avium* subsp. *paratuberculosis* was recovered by centrifugation at $3,500 \times g$ for 30 min at 5°C. Subsequently, bacteria were resuspended in HBSS, quantified by both McFarland turbidity standard and colony count, and used for infection of MDBK cells. Sterile water and 7H9 broth treated in the same manner as milk were used as controls. To determine the role of milk components, casein and serum protein fractions were separated by centrifuging milk at $90,000 \times g$ for 2 h at 5°C (16). The casein fraction formed the pellet, while serum protein and the lactose fraction remained in the supernatant. The volumes of the casein fraction and serum-protein-plus-lactose fractions were brought to the level of the original volume of milk by adding water. The casein fraction with 0.9% NaCl was prepared by addition of NaCl to the casein fraction. The 4.8% lactose sample was prepared by dissolving lactose in water.

RNA isolation and DNA microarray. An *M. avium* subsp. *paratuberculosis* whole genome microarray, containing 70-mer oligonucleotides representing 98% of *M. avium* subsp. *paratuberculosis* coding sequences identified in the *M. avium* subsp. *paratuberculosis* K-10 genome, was provided by the National Animal Disease Center, Ames, Iowa. For RNA isolation, *M. avium* subsp. *paratuberculosis* bacteria were kept in contact with confluent monolayers of MAC-T cells grown in T75 flasks for 2 h. Extracellular bacteria were removed by washing, followed by amikacin treatment for 2 h. Intracellular bacteria were recovered after 24 h by lysing MAC-T cells with sterile water. Cell debris and nuclear fractions were removed by low-speed centrifugation at 500 rpm for 5 min at 4°C (20). The bacterial pellet was recovered from the supernatant after centrifugation at $3,000 \times g$ for 10 min at 4°C. Pelleted bacteria were stored at −70°C. The RNA extraction procedure used a combination of guanidine-thiocyanate-based buffer (TRIzol; Invitrogen, Carlsbad, CA) and rapid mechanical cell lysis, as described previously (18). Briefly, 1 ml TRIzol reagent was added to the bacterial pellet and gently mixed. The mixture was added to a (2-ml) screw-cap tube containing 0.4 ml glass beads (0.1 mm). Cell disruption was carried out in a bead beater using three beatings for 30 s each. The TRIzol solution was transferred to a heavy phase lock gel (Eppendorf, Hamburg, Germany) after centrifugation at 3,000 rpm for 90 s at 4°C. Subsequently, 300 μl of chloroform:isoamyl alcohol (24:1) was added to the heavy phase lock gel tube. This was followed by rapid mixing for 15 s. Mixing was continued periodically for 2 additional min, followed by centrifugation at 6,000 rpm for 10 min at 4°C. The aqueous layers were precipitated with cold isopropanol. Isopropanol was then carefully removed without disturbing the RNA pellet, and the final washing was done in 80% ethanol. The RNA was stored at −70°C until further use. RNA quality was determined spectrophotometrically (optical density at 260/280 nm). A ratio of ≥2.0 was considered significant. RNA was treated with DNase as per the manufacturer's instructions (QIAGEN, Valencia, CA). The RNA-DNA hybridization and microarray analysis were carried out at the Central Service Laboratory, Oregon State University, Corvallis, Oregon. RNA prepared from *M. avium* subsp. *paratuberculosis* bacteria incubated in 7H9 broth served as a control, whereas *M. avium* subsp. *paratuberculosis* bacteria incubated in MAC-T cells served as the treatment. For the DNA microarray, the Array 900 MDX (Gemisphere, Hatfield, PA) labeling and detection system was used as per instructions.

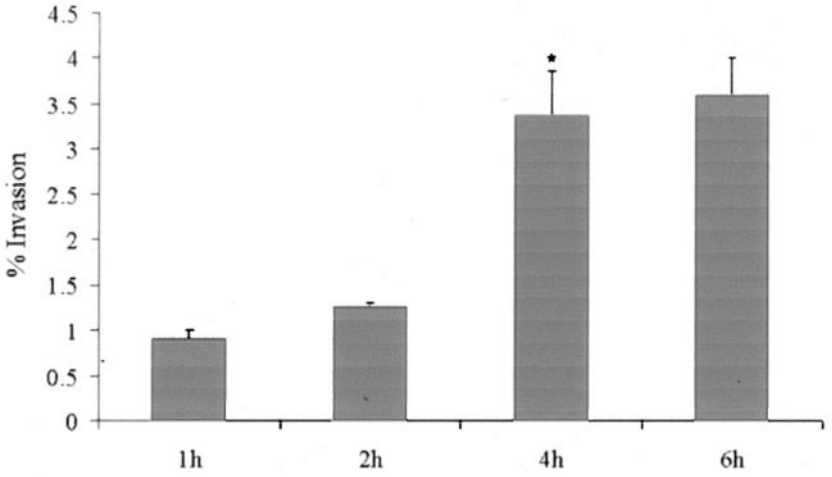

FIG. 1. Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis*. The percentage of invasion was defined as the fraction of inoculated bacteria that became internalized after the incubation period. Values represent the means of three experiments ± standard errors of the means (SEM). *, $P < 0.05$ compared with the percent invasion at 2 h.

Microarray analysis was carried out as follows. Briefly, RNA samples were reverse transcribed using deoxynucleoside triphosphates and a random primer. The cDNA was labeled at the 3′ end with a terminal deoxynucleotidyltransferase tailing reaction followed by ligation to the 3DNA capture sequence. The tagged cDNA was purified using a PCR purification kit (QIAGEN, Valencia, CA), and then successive hybridization of cDNA and 3DNA was carried out using the *M. avium* subsp. *paratuberculosis* genome microarray.

RT-PCR. cDNA amplification for genes that have been shown to be upregulated by DNA array was carried out as previously described (22). We used specific primers for five selected genes: MAP0482, MAP0706, MAP2450c, MAP2751, and MAP3305c. PCR amplification was carried out at 95°C for 3 min (1 cycle); 95°C for 3 s, 62°C for 30 s, and 72°C for 2 min in a linear range (35 cycles); and then 72°C for 10 min (1 cycle). Equivalent amounts of cDNA were used for the reverse transcriptase (RT)-PCR. The products obtained by PCR amplification were quantified using the Kodak EDEAS 290 system and the Kodak ID image analysis software (Eastman Kodak Company, Rochester, NY). The 16S cDNA was used as a control.

Transmission electron microscopy. Approximately 5×10^4 MAC-T cells were grown in T25 tissue culture flasks (Corning Costar, New York) until confluence was established and then inoculated with approximately 10^7 *M. avium* subsp. *paratuberculosis* bacteria. Incubation was carried out for 24 h under 5% CO_2 at 37°C. After the incubation period, MAC-T cells were treated with trypsin (0.5%) for 10 min and subsequently fixed in 0.1 M cacodylate buffer (pH 7.3) containing 3% glutaraldehyde and 2% paraformaldehyde. After fixing, cells were pelleted and surrounded by 1% agarose, cut into quarters, and processed as per the following schedule: 0.1 M cacodylate buffer (2×), 30 min at room temperature (RT); 1% osmium tetroxide in 0.1 M cacodylate buffer, 1 h at RT; 0.1 M cacodylate buffer (2×), 30 min at RT; acetone (10%, 30%, 50%, 70%, 80%, 95%, and 100%), all 10 min at RT; 3:1 acetone:resin, 30 min at RT; 1:1 acetone:resin, 30 min at RT; 1:3 acetone:resin, 30 min at RT; 100% resin, 1 h at 30°C; and 100% resin, 20 h at RT. Fresh resin was placed in polymerization capsules for 24 h at 60°C. The blocks were sectioned on an MT-5000 ultramicrotome (Sorvall, Newton, CO) using a diamond knife, and sections were placed on 300-mesh copper grids. Grids were stained with saturated uranyl acetate and lead citrate and viewed using a Zeiss 10A transmission electron microscope (Carl Zeiss, Thornwood, NY).

Statistical analysis. Each experiment was repeated at least three times, and significant differences were determined using Student's *t* test (unpaired) and analysis of variance. A *P* value of <0.05 was considered significant.

RESULTS

***M. avium* subsp. *paratuberculosis* invasion and survival in MDBK cells.** Invasion assays were carried out to examine the ability of *M. avium* subsp. *paratuberculosis* to enter bovine epithelial cells using MDBK cells as a model of intestinal mucosa. The results shown in Fig. 1 indicate that *M. avium* subsp. *paratuberculosis* can invade MDBK cells. The efficiencies of invasion at 1-h and 2-h time points were comparable; however, a significant increase ($P < 0.05$) in invasion was observed after 6 h of contact time.

The ability of *M. avium* subsp. *paratuberculosis* to survive inside MDBK cells was evaluated by incubating intracellular bacteria for 24 h or 96 h after invasion. The number of *M. avium* subsp. *paratuberculosis* bacteria increased at 96 h of infection compared with the inoculum taken at 2 h (Table 1), suggesting that *M. avium* subsp. *paratuberculosis* is able to survive inside MDBK cells.

Mammary gland as a reservoir for *M. avium* subsp. *paratuberculosis*. The results of *M. avium* subsp. *paratuberculosis* invasion of the MAC-T cell line are shown in Fig. 2A. *M. avium* subsp. *paratuberculosis* entered MAC-T cells, although the efficiency of invasion did not change during the first 2 h of incubation. However, invasion was greater at 4 h ($P < 0.05$) than at 2 h. *M. avium* subsp. *paratuberculosis* could be observed within vacuoles by using transmission electron microscopy (Fig. 2B). The survival experiments whose results are shown in Table 2 indicate that *M. avium* subsp. *paratuberculosis* is able to persist over time within MAC-T cells.

In the mammary gland, the apical surface of epithelial cells faces the alveolar lumen, whereas the basolateral side faces the

TABLE 1. Intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine epithelial cells (MDBK)

Time (h)[b]	Mean no. of intracellular bacteria ± SEM (CFU/ml)[a]
2	$2.36 \times 10^3 \pm 0.44 \times 10^3$
24	$2.81 \times 10^3 \pm 0.39 \times 10^3$
96	$3.26 \times 10^3 \pm 0.43 \times 10^3$

[a] Values represent means of three experiments ± SEM. A *P* value of >0.05 resulted for the comparison between the values for 24 h and 96 h and the value for 2 h.

[b] MDBK cells were exposed to *M. avium* subsp. *paratuberculosis* for 2 h. Extracellular bacteria were removed in all experiments after 2 h. Internalized bacteria were allowed to grow for 24 h or 96 h.

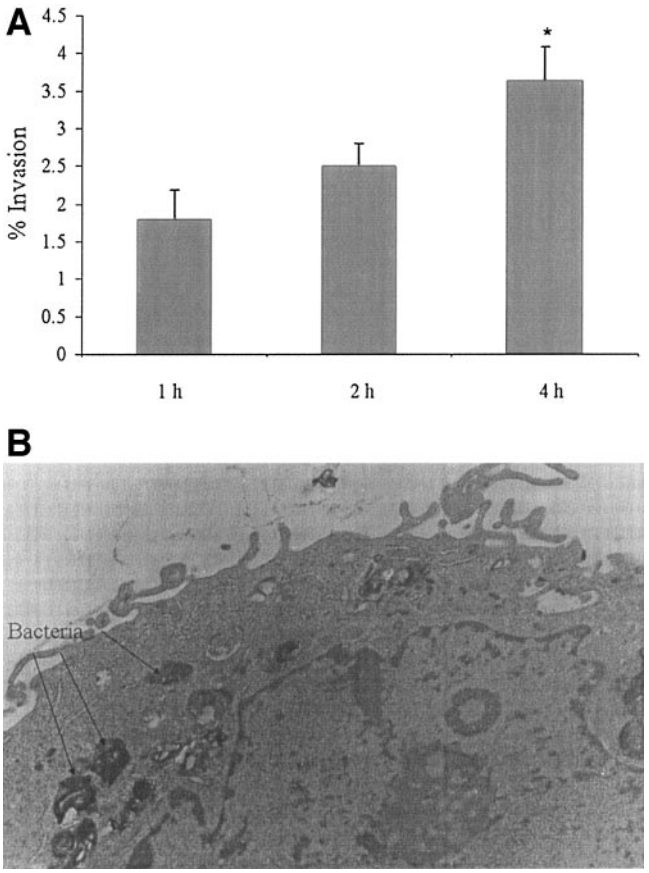

FIG. 2. (A) Invasion of bovine mammary epithelial cells (MAC-T) by *M. avium* subsp. *paratuberculosis*. The percent invasion was defined as the fraction of inoculated bacteria that became internalized after the incubation period. Values represent the means of three experiments ± SEM. *, $P \leq 0.05$ compared with the percent invasion at 2 h. (B) Representative transmission electron micrograph of mammary epithelial cells (MAC-T) after 24 h of infection by *M. avium* subsp. *paratuberculosis* bacteria (arrows). Bacterial cells can be seen within vacuoles. Magnification, ×10,000.

bloodstream. To investigate whether there was a preferential route for entry, invasion assays were performed using polarized cell monolayers. *M. avium* subsp. *paratuberculosis* crossed the MAC-T cells' polarized monolayers equally well from the apical and basolateral surfaces (Table 3). These results in vitro indicate that infection of mammary epithelial cells could potentially occur across both membrane surfaces.

TABLE 2. Intracellular survival of *M. avium* subsp. *paratuberculosis* in bovine mammary epithelial cells (MAC-T)

Time of infection (h)[b]	Mean no. of intracellular bacteria ± SEM (CFU/ml)[a]
2	$0.91 \times 10^4 \pm 0.25 \times 10^4$
24	$0.96 \times 10^4 \pm 0.28 \times 10^4$
96	$1.29 \times 10^4 \pm 0.39 \times 10^4$

[a] Values represent means of three experiments ± SEM.
[b] MAC-T cells were exposed to *M. avium* subsp. *paratuberculosis* for 2 h. Extracellular bacteria were removed in all experiments after 2 h. Internalized bacteria were allowed to grow for 24 h or 96 h.

TABLE 3. Translocation of *M. avium* subsp. *paratuberculosis* across polarized monolayers[b] of bovine mammary epithelial cells[c] (MAC-T)

Invasion surface	*M. avium* subsp. *paratuberculosis* inoculum recovered after designated incubation period (%)[a]			
	1 day	2 days	3 days	4 days
Apical	0.11 ± 0.02	0.25[d] ± 0.03	0.35[d] ± 0.04	0.49[d] ± 0.05
Basolateral	0.15 ± 0.02	0.26[e] ± 0.01	0.34[d] ± 0.02	0.47[d] ± 0.03

[a] Translocation percentage was defined as the percentage of *M. avium* subsp. *paratuberculosis* inoculum that was recovered from the bottom chamber of the Transwell apparatus. The results represent the means of three experiments within rows ± SEM.
[b] Monolayer integrity was verified by a trypan blue dye exclusion assay and by measuring transepithelial resistance.
[c] MAC-T cells were exposed to *M. avium* subsp. *paratuberculosis* for 1, 2, 3, or 4 days from either the apical or the basolateral surface.
[d] $P < 0.05$, cumulative percent translocation after 2, 3, and 4 days compared with that after 1 day within individual rows.
[e] $P < 0.01$, cumulative percent translocation after 2 days compared with that after 1 day within individual row.

Incubation with milk and efficiency of invasion. Since *M. avium* subsp. *paratuberculosis* in the mammary gland may be exposed to milk, we attempted to evaluate the effect of incubation of *M. avium* subsp. *paratuberculosis* in milk on the ability to enter bovine epithelial cells. *M. avium* subsp. *paratuberculosis* bacteria were exposed to milk (increased osmolarity conditions), 7H9 broth (iso-osmolar medium), or water (hypoosmolar medium) at 37°C for 24 h in the presence of antibiotics (polymyxin B [5.5 mg/liter], amphotericin [11 mg/liter], carbenicillin [25 mg/liter], and trimethoprim [2.5 mg/liter]). The antibiotics were used to prevent the growth of other microorganisms present in milk. After incubation, the ability to enter MDBK cells was evaluated. The efficiency of invasion was significantly greater when *M. avium* subsp. *paratuberculosis* was preincubated in milk than when *M. avium* subsp. *paratuberculosis* was exposed to other environments (Fig. 3A).

To determine whether a specific milk component was associated with increased invasion, *M. avium* subsp. *paratuberculosis* was exposed to different milk components prior to invasion of MDBK cells. Figure 3B shows that the efficiency of invasion of *M. avium* subsp. *paratuberculosis* after incubation in casein alone (low-osmolar medium) was lower than with milk ($P < 0.05$). However, differences were not significant when milk was compared to casein with 0.9% NaCl, lactose, or lactose plus serum protein (hyperosmolar conditions) (Fig. 3B).

Intracellular phenotype. Because *M. avium* subsp. *paratuberculosis* is encountered intracellularly during infection and is potentially eliminated from the mammary gland within detached epithelial cells, it is of paramount importance to determine whether *M. avium* subsp. *paratuberculosis* infection of mammary epithelial cells impacts its ability to enter bovine MDBK epithelial cells. MAC-T cells infected with *M. avium* subsp. *paratuberculosis* were maintained for 1 or 4 days in culture before they were lysed. Intracellular bacteria recovered after lysis were then used to infect MDBK cells. Results shown in Fig. 4 indicate that the efficiency of invasion by *M. avium* subsp. *paratuberculosis* collected from infected MAC-T cells was approximately 10-fold greater than that of *M. avium* subsp. *paratuberculosis* incubated in medium.

DNA microarray. To determine whether *M. avium* subsp. *paratuberculosis* genes are upregulated during the intracellular

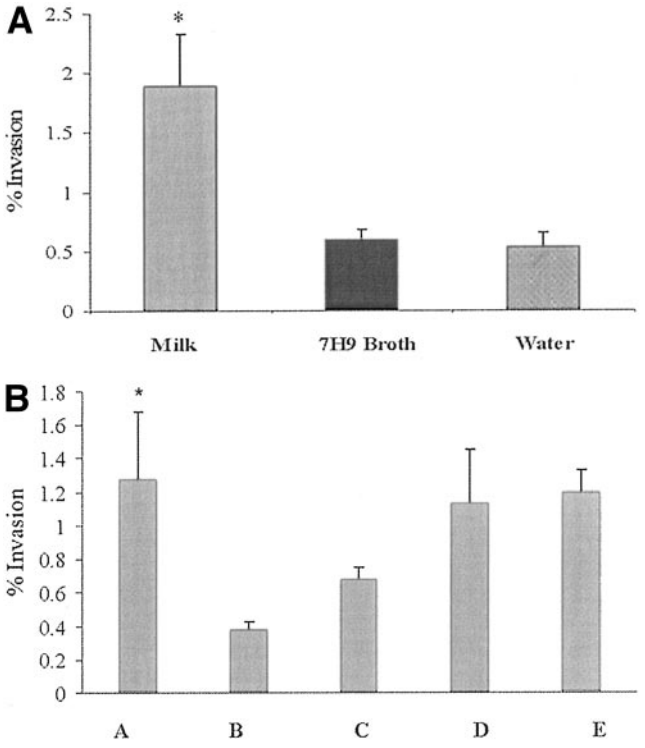

FIG. 3. (A) Ability of *M. avium* subsp. *paratuberculosis* to invade MDBK epithelial cells following exposure to three different environments: milk, 7H9 broth, or water for 24 h at 37°C. Values are the means of three experiments ± SEM. *, $P < 0.01$ compared with 7H9 broth or water. (B) Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis*. Prior to invasion, *M. avium* subsp. *paratuberculosis* bacteria were incubated in the presence of milk (column A), casein (column B), casein plus 0.9% NaCl (column C), serum protein plus lactose (column D), or lactose (4.8% solution) (column E) for 24 h at 37°C. Values represent the means of three experiments ± SEM. *, $P < 0.05$ for difference between the invasion percent for milk (column A) and casein (column B) casein plus NaCl (column C).

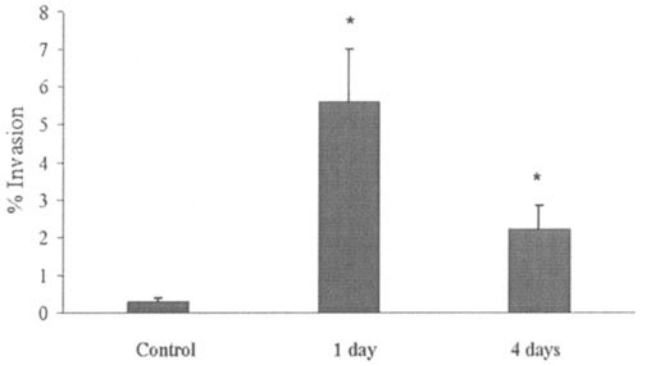

FIG. 4. Invasion of bovine epithelial cells (MDBK) by *M. avium* subsp. *paratuberculosis* after passage in MAC-T cells. *M. avium* subsp. *paratuberculosis* bacteria were incubated in MAC-T cells for 1 or 4 days and then used to infect MDBK epithelial cells. *M. avium* subsp. *paratuberculosis* incubated in medium was used as a control. Values represent the means of three experiments ± SEM. *, $P < 0.001$ compared with the invasion percent for the control.

stage in mammary epithelial cells, DNA microarray analysis was carried out. MAC-T cells were infected for 24 h and *M. avium* subsp. *paratuberculosis* RNA was obtained. The 24-h time point was chosen based on the greater invasion of MDBK cells. The results in Table 4 indicate that upregulation of a number of *M. avium* subsp. *paratuberculosis* genes occurs during infection of MAC-T cells.

M. avium subsp. *paratuberculosis* whole genome microarray analysis identified 20 genes that showed gene expression threefold or higher than control. The in silico analysis of these genes suggests a diverse array of regulatory, metabolic, and candidate virulence-associated factors. For example, genes MAP0482, MAP1695c, MAP3404, MAP1259, and MAP2652c encode transcription-regulatory proteins. Among other differentially expressed genes, MAP0392 belongs to an operon encoding an upstream transcription-regulatory protein. MAP0462, which encodes a urease alpha subunit, and MAP3404, encoding a biotin carboxyl bifunctional carrier protein, are essential genes for survival based on their homology with *Mycobacterium tuberculosis* (25). Among other differentially expressed genes are MAP2450c (encoding a probable ATP synthase), MAP0462 (encoding a tRNA synthetase), and MAP3224 (involved in secretion of β-lactamase). Finally, MAP2751, which has previously been shown to be present uniquely in *M. avium* subsp. *paratuberculosis* (4) and has no known function, also showed increased transcription within MAC-T cells. The expression of five of those genes was evaluated using RT-PCR and shown to correspond to the DNA array analysis (Table 4).

DISCUSSION

Mycobacterial invasion of intestinal epithelial cells is a complex event, requiring participation of several bacterial and host factors. However, *M. avium* subsp. *paratuberculosis* infection is difficult to study in large animal model systems. Tissue culture cell models have been shown to be useful to obtain insights into the host-pathogen interactions. For example, Madin-Darby canine kidney cells have been used by several laboratories to study bacterial pathogenesis (17, 20). In addition, a number of studies employed cultured epithelial cells to examine the interaction between the bacteria and host cells (8, 9, 14). Earlier work has established MDBK epithelial cells as a model of bovine intestinal mucosa (5). When confluent monolayers of MDBK cells were exposed to *M. avium* subsp. *paratuberculosis*, it was observed that *M. avium* subsp. *paratuberculosis* was capable of invading epithelial cells. Since epithelial cells are not phagocytic in nature, *M. avium* subsp. *paratuberculosis* invasion of MDBK cells indicates that the bacteria might trigger their own uptake, probably by inducing cytoskeleton reorganization. We did not observe significant replication of *M. avium* subsp. *paratuberculosis* inside cells after 4 days, which should be due to the long replication time (15), which might be even longer when inside epithelial cells.

Previous work has demonstrated the recovery of *M. avium* subsp. *paratuberculosis* from various sites in the body during advanced stages of infection. For instance, *M. avium* subsp. *paratuberculosis* has been isolated from milk, colostrum, and mammary lymph nodes from both asymptomatic and symptomatic cows (28–30), making it plausible that mammary epithelial cells could be a site of *M. avium* subsp. *paratuberculosis* infection. Our results demonstrated that *M. avium* subsp. *para-*

TABLE 4. DNA microarray profile showing differential expression of *M. avium* subsp. *paratuberculosis* genes

Gene	Fold increase in DNA array[a]	Function(s) or characteristic(s)	Fold increase in RT-PCR
MAP2450c	6.35	*atpC*; probable ATP synthase, *M. leprae*	7.1
MAP3305c	6.35	Conserved hypothetical protein, *M. leprae*	6.8
MAP0482	5.76	Putative transcription regulator, *Nocardia farcinica*	6.3
MAP2751	5.47	Unique to *M. avium* subsp. *paratuberculosis*	5.5
MAP0706	5.47	Probable cytoplasmic peptidase, *Listeria monocytogenes*	5.8
MAP0741c	4.91	Possible oxidoreductase	ND[b]
MAP3404	4.78	Biotin carboxyl bifunctional carrier protein; essential gene in *M. tuberculosis*	ND
MAP1259	4.49	Probable transcription regulatory protein in *M. tuberculosis*	ND
MAP2708c	4.33	Probable glutamine amidotransferase	ND
MAP3224	4.33	Possibly involved in secretion of β-lactamase in *M. tuberculosis*	ND
MAP1695c	4.25	Transcription factor mediated by hypoxic conditions in *M. tuberculosis*; essential gene in *M. tuberculosis*	ND
MAP2652c	4.02	Probable phosphate acetyl transferase	ND
MAP2524c	3.89	Oxidoreductase	ND
MAP0462	3.79	*ureC*; urease alpha subunit; essential gene in *M. tuberculosis*	ND
MAP0369	3.72	Probable nitrate reductase	ND
MAP1758c	3.56	*nrtC*; possible acyl coenzyme A dehydrogenase	ND
MAP3374	3.43	Probable F 420 biosynthesis protein	ND
MAP0392c	3.23	Probable bifunctional membrane associated penicillin binding protein	ND
MAP4310c	3.08	Possible acyl coenzyme A dehydrogenase	ND

[a] *M. avium* subsp. *paratuberculosis* bacteria were incubated intracellularly in MAC-T cells for 1 day prior to RNA microarray analysis. The expression was compared to that for bacteria grown in 7H9 broth.
[b] ND, not done.

tuberculosis infects MAC-T cells in vitro and that the infection is possible from both the apical and basolateral surfaces with comparable efficiency. Therefore, the implication of the observation is that infection in mammary gland tissue may potentially occur by either the systemic or the ascending route. Similar findings have been reported for other pathogens such as *Streptococcus dysgalactiae* and *Staphylococcus aureus* (1, 2). The results of transmission electron microscopy (Fig. 2B) confirmed that *M. avium* subsp. *paratuberculosis*, once inside MAC-T cells, is encountered within cytoplasmic vacuoles, similar to what has been described for *M. avium* subsp. *paratuberculosis* in macrophages (15) and other mycobacteria in epithelial cells (8). Furthermore, it was observed that *M. avium* subsp. *paratuberculosis* survived within mammary epithelial cells for several days in vitro. Collectively, these findings support the idea that the infection of the mammary gland can occur through the systemic route and that mammary gland epithelial cells may serve as a reservoir for *M. avium* subsp. *paratuberculosis* and a potential source of infection for young calves.

It is assumed that the intracellular environment in the mammary gland has high osmolarity, while the mammary gland milk is also a hyperosmolar fluid in nature. *M. avium* subsp. *paratuberculosis* incubated in milk prior to infecting MDBK epithelial cells became significantly more invasive than *M. avium* subsp. *paratuberculosis* that had been previously incubated in broth or water. The augmented ability to invade cells was then attributed to the hyperosmolar conditions of milk, a hypothesis that was strengthened by the observation that *M. avium* subsp. *paratuberculosis* incubated in four different hyperosmolar milk fractions acquired a similar phenotype. It appears that the environment with high osmolarity may serve as a trigger for expression of invasion-related determinants. In fact, osmolarity has been shown to be associated with the expression of virulence determinants in a number of bacteria, e.g., the *toxR* gene in *Vibrio cholerae* and *ompR* genes in *Salmonella* and *Shigella* (11, 13, 21). Previously, it was also shown that when *M. avium* was preincubated under high osmolarity conditions, a change in phenotype was induced, resulting in enhanced efficiency in entering human intestinal epithelial cells (8). These studies also showed that the invasive phenotype was likely to be related to the upregulation of genes involved in invasion, since incubation under high-osmolarity conditions in the presence of subinhibitory concentrations of amikacin, which inhibits protein synthesis, failed to result in expression of the invasive phenotype (8).

Prior incubation of *M. avium* subsp. *paratuberculosis* in MAC-T cells enhanced the efficiency of invasion of MDBK cells. DNA microarray analysis of *M. avium* subsp. *paratuberculosis* genes regulated during MAC-T cell infection showed that several genes had their expression altered. The upregulated *M. avium* subsp. *paratuberculosis* genes, for example, MAP0482, MAP1695c, MAP3404, MAP1259, MAP2652c, and MAP0392, encode proteins with transcription-regulatory functions. MAP0482 encodes a putative transcriptional regulator in *Nocadia farcinica*. The upstream gene MAP0483 encodes a transcription-regulatory protein in *M. tuberculosis*. Another transcription protein, encoded by MAP1695c, acts as a cochaperone in *M. tuberculosis* (19, 27). The upstream gene is for Hsp18, a stress protein induced by anoxia. Homology with *M. tuberculosis* suggests that the MAP1695c operon encodes a response regulator having an important role. MAP3404 belongs to an operon having an upstream sigma factor. MAP0392 encodes a probable bifunctional membrane-associated penicillin binding protein (PonA2, murine polymerase) under the control of the transcription-regulatory protein encoded by MAP0393. Studies with *M. smegmatis* and *M. tuberculosis* have suggested that transposon disruption of *ponA* resulted in a penicillin-binding-deficient mutant that was sensitive to beta-lactam antibiotics and grew slowly in culture (7, 12). The genes

identified may be associated with other functions, such as intracellular survival. Efficiency of invasion among intracellular bacteria peaks at 24 h, probably reflecting the fact that the mammary gland is emptied at least once a day. The function of the majority of the identified genes is unknown, and further studies are necessary to understand the role of the identified genes in intracellular survival.

In summary, we have examined the different conditions of *M. avium* subsp. *paratuberculosis* invasion and survival. A working model can be identified from the present results. Infection of the mammary gland and milk are observed in the majority of the infected cows. Therefore, it is plausible to hypothesize that *M. avium* subsp. *paratuberculosis* (within or outside of cells) fed to calves in milk is an organism with the ability to cross the intestinal barrier with efficiency, compared with organisms present in the water. Fecal material may be another important source of *M. avium* subsp. *paratuberculosis* expressing an invasive phenotype. Future work will address the role of genes upregulated within milk and mammary epithelial cells and will attempt to put together the 35-kDa protein, identified previously (5), and the present model of infection.

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