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MINIREVIEW

No Holes Barred: Invasion of the Intestinal Mucosa by *Mycobacterium avium* subsp. *paratuberculosis*

John P. Bannantine,[a] Luiz E. Bermudez[b]

National Animal Disease Center-USDA-ARS, Ames, Iowa, USA[a]; Departments of Biomedical Sciences and Microbiology, Oregon State University, Corvallis, Oregon, USA[b]

The infection biology of *Mycobacterium avium* subsp. *paratuberculosis* has recently crystallized, with added details surrounding intestinal invasion. The involvement of pathogen-derived effector proteins such as the major membrane protein, oxidoreductase, and fibronectin attachment proteins have been uncovered. Mutations constructed in this pathogen have also shed light on genes needed for invasion. The host cell types that are susceptible to invasion have been defined, along with their transcriptional response. Recent details have given a new appreciation for the dynamic interplay between the host and bacterium that occurs at the outset of infection. An initial look at the global expression pathways of the host has shown a circumvention of the cell communication pathway by *M. avium* subsp. *paratuberculosis*, which loosens the integrity of the tight junctions. We now know that *M. avium* subsp. *paratuberculosis* activates the epithelial layer and also actively recruits macrophages to the site of infection. These notable findings are summarized along with added mechanistic details of the early infection model. We conclude by proposing critical next steps to further elucidate the process of *M. avium* subsp. *paratuberculosis* invasion.

Mycobacterium avium subsp. *paratuberculosis* causes Johne's disease in cattle, sheep, goats, and other ruminant hosts. This disease takes a significant economic toll on livestock producers worldwide. And although many variables need to be accounted for, including prevalence, a more defined annual burden on the U.S. dairy industry is estimated in the millions of dollars of unrealized revenue (1). Much of the biology of *M. avium* subsp. *paratuberculosis* and Johne's disease has been reviewed elsewhere (2, 3). *M. avium* subsp. *paratuberculosis* infects young livestock through the gastrointestinal tract by ingestion of milk or fecal material containing significant levels of viable bacteria. The bacteria are shed in the animal's feces before clinical signs develop, making it difficult to remove the disease from infected herds. Sophisticated transmission studies have emerged with the development of molecular epidemiological tools showing that many different strains can circulate within a herd as well as between herds, but one dominant strain is consistently observed in those settings (4, 5). The disease course generally takes 2 to 5 years before clinical signs appear. Two hallmarks of advanced disease are the thick, inflexible intestinal sections observed from the strikingly inflamed epithelial cell layer (6, 7) and granuloma formation in the small intestine and regional lymph nodes (8). This is due to the massive influx of proinflammatory cells at the site of infection. Visible signs of advanced-stage disease include weight loss and decreased milk production, which factor into the economic toll on the dairy industry. However, the initial events of *M. avium* subsp. *paratuberculosis* interaction with the intestinal mucosa of cattle are the focus of this communication.

THE PROCESS OF INVASION

The intestinal epithelium acts as a primary barrier against pathogens through innate mechanisms such as the glycocalyx, tight junctions, and antimicrobial peptides. *M. avium* subsp. *paratuberculosis* is adept at circumventing these barriers and establishing a long, chronic infection in the host (9). Upon ingestion, *M. avium* subsp. *paratuberculosis* is ushered along the gastrointestinal tract until it reaches the mucosa lining the small intestine. At this point *M. avium* subsp. *paratuberculosis* has the ability to enter two cell types to initiate infection: microfold cells (M cells) and enterocytes. One study suggests that *M. avium* subsp. *paratuberculosis* uptake by M cells is greater than that by enterocytes in ileal and jejunal sections of the sheep intestine (10). *M. avium* subsp. *paratuberculosis* is similar to other enteroinvasive pathogens in that it takes advantage of the specialized antigen-sampling cells present in the Peyer's patches of the small intestine (11, 12). M cells also serve as portals of entry for a number of other enteropathogenic bacteria, including *Escherichia coli* and *Vibrio cholerae* as well as *Salmonella* and *Shigella* species (13). M cells are located in the follicle-associated epithelium of Peyer's patches. They display β1 integrins at high density, and it has been postulated that these integrins may serve to target *M. avium* subsp. *paratuberculosis* to M cells (12, 14). A detailed pathology study clearly demonstrated *M. avium* subsp. *paratuberculosis* entry via M cells of the bovine intestine using electron microscopy (11). Another study suggests a defined interaction of *M. avium* subsp. *paratuberculosis* proteins with M cell integrins through a fibronectin bridge (12).

However, it is clear from other studies that M cells are not the only means by which *M. avium* subsp. *paratuberculosis* invades the host (15–17). It is possible that Peyer's patches and M cells are not well developed in the neonatal calf, because of a lack of maturation and antigen exposure (18). The Peyer's patches of germfree mice are poorly developed for this reason (19). Therefore, this route may not be the primary method of *M. avium* subsp. *paratuberculosis* entry at a stage when the animal is thought to be most susceptible, i.e., newborn (20). Furthermore, when M cells are absent, such as in B-cell knockout mice, enterocytes are actively invaded by *M. avium* subsp. *paratuberculosis* (21). In addition, mucus-secreting goblet cells of the small intestine and goat enterocytes

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Address correspondence to John P. Bannantine, john.bannantine@ars.usda.gov.

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have been involved in *M. avium* subsp. *paratuberculosis* uptake (7, 17). This choice of cell type for entry likely contributes to the ability of *M. avium* subsp. *paratuberculosis* to propagate in diverse hosts. Because *M. avium* subsp. *paratuberculosis* can only persist, not grow, once outside the host, this diversity is critical to its survival.

M. AVIUM SUBSP. *PARATUBERCULOSIS* AFFECTS THE INTEGRITY OF THE APICAL EPITHELIUM

How does the presence of *M. avium* subsp. *paratuberculosis* affect enterocytes lining the mucosa? *M. avium* subsp. *paratuberculosis* shows a strong affinity for the apical surface of enterocytes and is efficiently internalized (16). Furthermore, the uptake of *M. avium* subsp. *paratuberculosis* activates the intestinal mucosa layer by secretion of the proinflammatory chemokine MIP-2 along with an increase in the expression of intracellular adhesion molecule 1 (ICAM-1). This *M. avium* subsp. *paratuberculosis* internalization-dependent tissue activation is also accompanied by inflammation from infiltrating neutrophils and macrophages at the site of invasion (7, 10, 22). Of note is that when calves are infected, no inflammatory manifestation of clinical relevance takes place. Uptake is dependent on the small GTPase RhoA in combination with Cdc42 (16, 23).

M. avium subsp. *paratuberculosis* may have a more profound effect on the epithelial cell layer than simply activation by proinflammatory cytokines, regulating Cdc42, or using it to process interleukin 1β (IL-1β) for efficient invasion. Khare and coworkers have recently examined how the early events of *M. avium* subsp. *paratuberculosis* infection affect the global gene expression of the host (24). By examining RNA extracted from biopsied bovine Peyer's patch tissues on microarrays containing bovine sequences, they found that several host pathways were suppressed within the first hour, and there were multiple pathway changes detected up until the final (12-h) time point. An interesting finding from this study was that infection increased the intestinal permeability. This was shown experimentally by measuring transepithelial resistance, which showed an initial increase but then decreased after 30 min of exposure to *M. avium* subsp. *paratuberculosis*. This occurs by marked suppression in the cell communication pathways, which includes genes in the tight junction, gap junction, and adherens junction pathways. These pathways form the intercellular links that seal the apical epithelium and create a semipermeable diffusion barrier. Thus, the observed suppression of genes in these pathways suggests a weakening of the muscosal barrier following *M. avium* subsp. *paratuberculosis* infection. These findings underscore the complexity of the host response to *M. avium* subsp. *paratuberculosis* infection.

Using a permeable support system, Lamont and coworkers were able to artificially construct an epithelial cell layer from a bovine mammary gland epithelial cell line, MAC-T cells, along with bovine monocyte-derived macrophages (MDMs) immediately underneath to represent subepithelial macrophages (25). They used this system to answer questions about transepithelial migration of *M. avium* subsp. *paratuberculosis*. In contrast with macrophages that have been shown to arrest phagosome acidification and subsequent maturation (26), *M. avium* subsp. *paratuberculosis* infection of MAC-T epithelial cells quickly leads to endosomal acidification (25). This acidification is necessary for IL-1β expression, which leads to macrophage transepithelial migration. It is still not clear how the pathogen exits the enterocyte to enter the subepithelial macrophages. Unlike the host gene expression study by Khare et al., which examined intestinal permeability (24), transepithelial resistance was not measured, but the authors acknowledge that MAC-T cells have more diffuse tight junctions which may lessen the barrier to transepithelial migration (25). In addition, the induction of apoptosis or pyroptosis by secreted IL-1β was not examined as a possibility to explain the partial loss of membrane resistance.

M. avium subsp. *paratuberculosis* apparently traverses the epithelial layer quickly. Studies have shown that the apical and basolateral membranes are crossed with equal efficiencies in MAC-T cells (27). By 30 min, *M. avium* subsp. *paratuberculosis* is seen inside bovine and murine epithelial cells (16, 22). But how fast does it bypass the epithelial barrier and enter the lamina propria? To examine this, Wu and coworkers surgically deposited approximately 10^7 *M. avium* subsp. *paratuberculosis* cells by injection into the lumen of the intestine of a cow (28). Passage through the epithelial layer occurred within 1 h, as shown by the ability to culture *M. avium* subsp. *paratuberculosis* from a regional lymph node of the ileocecal mesentery. This is much faster than observed in other studies, which only examined later time points. For example, Momotani et al. demonstrated the presence of *M. avium* subsp. *paratuberculosis* in subepithelial macrophages by 5 h postinfection (11). Furthermore, *M. avium* subsp. *paratuberculosis* disseminated to the liver and mesenteric lymph node tissues shortly after breaching the intestinal lining and then at later time points settled into the ileum and lymph nodes to maintain the infection (28). Another study has shown translocation of polarized Madin-Darby bovine kidney (MDBK) cell monolayers by 2 h postinfection and that dissemination to other tissues in the mouse occurs at a low level (21). Since the study by Wu and colleagues (28) was performed using intestinal loops, in contrast to the others, it is possible that vasodilation and blood flow could explain the different observation.

M. avium subsp. *paratuberculosis* encounters dendritic cells (21, 29, 30) and subepithelial macrophages (11) in the lamina propria after translocation across the intestinal epithelial layer. Dendritic cells may also transport *M. avium* subsp. *paratuberculosis* inside the lamina propria by sampling through tight junctions (9). Dendritic cells exist in these tissues in an immature state in which they capture antigens using highly flexible dendrites and pattern recognition receptors (31). With the antigen, dendritic cells are trafficked to the regional lymph nodes. This series of events is similar to that with the subepithelial macrophages, which engulf *M. avium* subsp. *paratuberculosis* and migrate to the lymph nodes. Both cell types either process and present antigens locally or migrate to adjacent mesenteric lymph nodes to interact with T cells. Several studies have shown that the bacteria are readily detected in mesenteric lymph nodes (32–34) before disseminating to other organs, such as the liver (28, 35). The early events of *M. avium* subsp. *paratuberculosis* infection of enterocytes and M cells are summarized in Fig. 1.

HOST INVOLVEMENT ENHANCES INVASION

Initial interactions between pathogen and host occur through receptor molecules on the host cells. The extracellular matrix produced by host cells includes vitronectin and fibronectin, among other components. *M. avium* subsp. *paratuberculosis* uses fibronectin as a bridge between the pathogen and host cell cytoskeleton (36, 37). This 220-kDa host protein consists of 5 well-defined

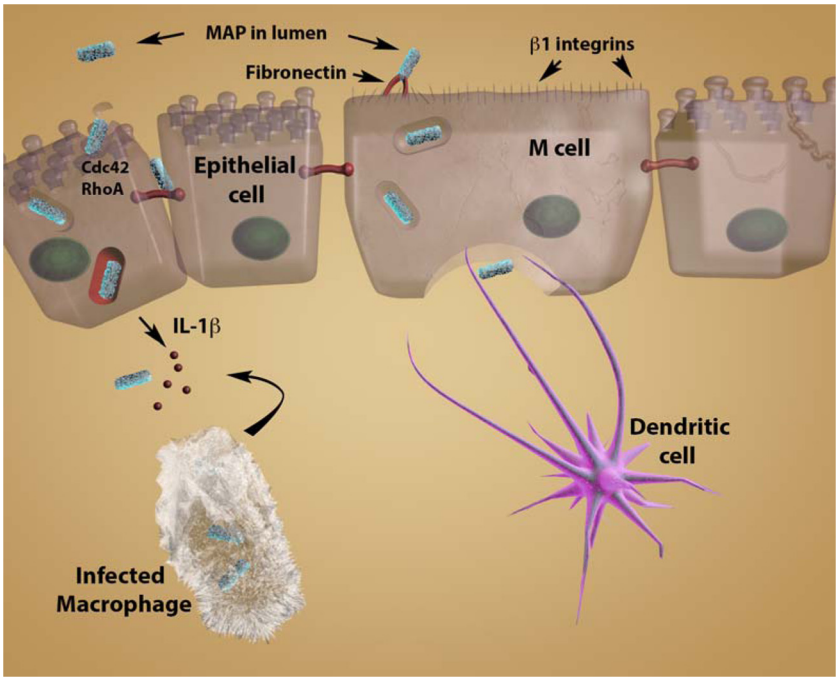

FIG 1 Overview of the initial events of *M. avium* subsp. *paratuberculosis* (MAP) infection. Shown are intestinal enterocytes and an M cell with the basolateral invagination. *M. avium* subsp. *paratuberculosis* interacts with the M cell through a fibronectin bridge. The bacteria (blue) are easily trafficked through the M cells, where they can be picked up by dendritic cells (purple) or macrophages (white) and carried to the mesenteric lymph nodes or other regional lymph nodes. In the enterocyte, the bacteria are taken up through the Cdc42-RhoA mechanism into an endosomal vacuole that becomes acidified (red-shaded vacuole). This signals IL-1β production and draws subepithelial macrophages. *M. avium* subsp. *paratuberculosis* disrupts cell communication pathways that loosen tight junctions, and thus the pathogen may also slip between enterocytes to gain access to the lamina propria (bottom half of figure). Epithelial cell nuclei are shown in green.

domains of which the 40-kDa domain interacts directly with *M. avium* subsp. *paratuberculosis* (36). While *M. avium* subsp. *paratuberculosis* uses fibronectin for adherence, studies have shown that only a small percentage of *M. avium* subsp. *paratuberculosis* organisms enter the epithelial mucosa by fibronectin-dependent mechanisms (21). Furthermore, the binding to vitronectin or other extracellular matrix proteins by *M. avium* subsp. *paratuberculosis* has not been studied and may provide additional means of access. As discussed below, a number of *M. avium* subsp. *paratuberculosis*-derived proteins, when disrupted or blocked, reduce the ability of *M. avium* subsp. *paratuberculosis* to invade cells, and not all of these are believed to interact with fibronectin.

The GTPase proteins Cdc42 and RhoA have been shown to be stimulated by *M. avium* subsp. *paratuberculosis* in a manner that promotes invasion. Involvement of these proteins was uncovered through studies with *M. avium* subsp. *paratuberculosis* strains containing defined transposon mutations, as discussed in the following section.

Host compounds appear to stimulate *M. avium* subsp. *paratuberculosis* invasion. This is demonstrated when *M. avium* subsp. *paratuberculosis* is exposed to cow milk; it is then more adept at invasion of epithelial cells than *M. avium* subsp. *paratuberculosis* cultured in laboratory media. This was initially shown with *M. avium* subsp. *paratuberculosis* immersed in whole milk and then exposed to MDBK cells (21). There was a significantly higher percentage of *M. avium* subsp. *paratuberculosis* organisms inside the MDBK cells when preexposed to the high-osmolarity conditions present in milk. The investigators followed up on this observation by examining gene expression changes of *M. avium* subsp. *paratuberculosis* in milk versus culture media. A LuxR regulator was identified that showed a 20- to 40-fold increase in transcription by reverse transcription-PCR (RT-PCR) analysis (38). Furthermore, the cell wall lipid profile of *M. avium* subsp. *paratuberculosis* changes when exposed to milk. A newly defined nonpolar lipid (lipid 550) was present in *M. avium* subsp. *paratuberculosis* exposed to milk, while a well-characterized lipid, para-LP-01 (39), disappeared following incubation under these conditions (38). Although not demonstrated, it is possible that these lipid changes enable *M. avium* subsp. *paratuberculosis* to more efficiently enter epithelial cells or have a direct effect on the inflammatory response. Clearly, increased expression of the regulator LuxR was linked to the expression of several proteins, some of them previously shown with other pathogens to be involved with invasion of epithelial cells. Unfortunately, host factors that induce these changes in *M. avium* subsp. *paratuberculosis* remain unknown and need to be uncovered through additional studies.

Of note is the fact that infection by *M. avium* subsp. *paratuberculosis* is associated with suppression of the majority of inflammatory proteins. This observation has been recently confirmed with cattle; upon *M. avium* subsp. *paratuberculosis* infection in the intestinal mucosa, expression of inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF), IL-6, and IL-12 is downregulated (24). The initial quietness of the immune system may help the bacterium to establish a niche. Additional studies are needed in the area to more fully document and understand why and how

this subversion of the immune response is a pathogenicity-related event.

MYCOBACTERIAL PROTEINS INVOLVED IN INVASION

In addition to the *luxR* gene mentioned above, a few other bacterium-derived mediators of internalization through surface proteins are now known for *M. avium* subsp. *paratuberculosis*. These proteins have been linked in some way to a role in the initial invasion of bovine intestine. Among these are fibronectin attachment proteins (FAPs) and the major membrane protein. It is known that opsonization of bacteria with bovine fibronectin enhances both adherence to and invasion of epithelial cells (40). ModD and antigen 85 are among the known fibronectin attachment proteins in *M. avium* subsp. *paratuberculosis*.

An *M. avium* subsp. *paratuberculosis*-expressed FAP was originally described by Secott and coworkers (37). They identified the sequence from a homolog in *Mycobacterium avium* subsp. *avium* and showed that whole *M. avium* subsp. *paratuberculosis* cells pretreated in pH 3 buffer bound fibronectin more efficiently. This acid pretreatment effect was never shown to be dependent on FAP. Although initially suggested to be embedded in the cell wall, but not surface exposed (37), FAP was later rediscovered in culture filtrate fractions and termed ModD (41) and then later Apa protein (42). The N-terminal signal sequence further suggests that the protein is secreted. MAP_1569 is the locus tag from the *M. avium* subsp. *paratuberculosis* strain K-10 genome for FAP and *modD* (43). This protein also has immunostimulatory effects and has been shown to induce the maturation of dendritic cells (44). A >70% reduction in M cell invasion was observed when FAP expression was attenuated, suggesting that this protein may be involved in targeting M cells (12). However, the same FAP mutant was able to invade epithelial cells with an efficiency equal to that of the wild-type strain. This experiment showed that M cells are not the only portal of entry and that other *M. avium* subsp. *paratuberculosis* proteins have an important role in the invasion process.

The antigen 85 complex consists of three additional fibronectin binding proteins that also catalyze the synthesis of a glycolipid in the mycobacterial cell wall (45). The genes encoding these three proteins are not linked on the *M. avium* subsp. *paratuberculosis* genome (MAP_0216 = Ag85A, MAP_1609c = Ag85B, and MAP_3531c = Ag85C); however, they do share sequence homology (46) and are thought to be secreted in *M. avium* subsp. *paratuberculosis* like in other mycobacteria (47). Recently, the interaction of Ag85 complex with fibronectin was characterized in molecular detail by measuring dissociation constants and through peptide inhibition assays (36). The investigators identified the 10-amino-acid region within a 40-kDa domain of fibronectin that specifically binds Ag85B. Finally, they showed a 44% reduction in binding of recombinant Ag85 to Caco-2 cells transfected with small interfering RNA, which lowered fibronectin expression. Whole *M. avium* subsp. *paratuberculosis* cell binding was reduced by 10% in the same cell line, suggesting that Ag85-fibronectin binding is not the only means for adherence to host cells. Collectively, these results suggest an important adhesive role for Ag85.

The major membrane protein (MMP) has an important role in invasion of bovine epithelial cells. Originally discovered as an immunodominant antigen in leprosy patients (48), this protein is present in high relative abundance in *M. avium* subsp. *paratuberculosis* membrane preparations (49) and elicits a cellular immune response in mice (50). Under conditions known to exist in the bovine intestine, such as low oxygen tension and high osmolarity, MMP transcription and translation were significantly increased in two independent studies (51, 52). When antibodies against this surface protein were used to block invasion of MDBK cells, the process was inhibited in a dose-dependent manner (51). In a subsequent study, the *Mycobacterium avium* subsp. *hominissuis* equivalent of MMP was enriched from a library of *M. smegmatis* clones that had increased ability to invade HEp-2 cells (52). Furthermore, when MMP was constitutively expressed in *M. avium* subsp. *hominissuis*, the recombinant had an increased ability to invade HT-29 cells. It is expected that a mutation in this gene would lead to decreased invasion, but so far a knockout has not been created in this gene. Therefore, the precise role this protein plays in invasion has yet to be defined.

M. avium subsp. *paratuberculosis* invasion of the intestinal mucosa is dependent on the expression of bacterial proteins upon contact with the epithelial cell surface. The chief mechanism is associated with the expression of genes present in an "invasion region" for *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *hominissuis*, and *Mycobacterium tuberculosis* (53). A putative oxidoreductase (MAP_3464) is required to deliver an effector protein, MAP_3985c, into the host cell and trigger a rapid entry. The inactivation of MAP_3464 makes *M. avium* subsp. *paratuberculosis* 6-fold less invasive than the wild-type bacterium. MAP_3464 has a β barrel domain that suggests a membrane location at some time before invasion takes place. Inactivation of MAP_3464 is associated with the failure to activate Cdc42, preventing cytoskeleton reorganization. MAP_3464 is a key component in the secretion of MAP_3985c, a small protein that binds to Cdc42, activating it. A delayed mechanism of entry also exists, and it is dependent on the activation of the small GTPase RhoA (23).

Attenuated mutants of *M. avium* subsp. *paratuberculosis* have also been identified that are unable to invade or colonize tissue. One of these mutants has a transposon insertion in the *gcpE* gene, which encodes a protein involved in isoprenoid biosynthesis (54). A second mutant had an insertion in *pstA*, a gene involved in biofilm formation (55). Both mutants were significantly less invasive than wild-type *M. avium* subsp. *paratuberculosis* in a competitive-invasion assay (28, 55). An oxidoreductase (MAP_3464) mutant along with five other mutants also showed an impaired ability to invade (23). All known *M. avium* subsp. *paratuberculosis*-derived effector proteins are summarized in Table 1.

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

More mechanistic details have recently been uncovered about the initial interactions of *M. avium* subsp. *paratuberculosis* with the host. *M. avium* subsp. *paratuberculosis* plays an active role in invasion of the intestinal epithelium through expression of effector proteins and by taking advantage of host components such as fibronectin and compounds present in milk. Specifically what compounds these are still needs to be defined. *M. avium* subsp. *paratuberculosis* appears to affect cell pathways that form tight junctions, leading to the increase of intestinal permeability. The bacterium is then concentrated in mesenteric and ileocecal lymph nodes before disseminating to other tissues. Just how these *M. avium* subsp. *paratuberculosis*-derived effector proteins, listed in Table 1, promote invasion is unclear, and future studies should be performed to address this question. The molecular detail uncovered for *Shigella* and *Salmonella* invasion of epithelial cells is much more advanced than what we know for *M. avium* subsp. *paratu-*

TABLE 1 *M. avium* subsp. *paratuberculosis* proteins implicated in invasion

Locus tag	Protein	Host cell type	Evidence	Reference
MAP_2121c	Major membrane protein I	MDBK	Antibody against protein blocks invasion	51
MAP_3464	NADH-dependent oxidoreductase	MDBK	Gene knockout lowers invasion, and complementation restores invasion	23
MAP_3212	NADH-ubiquinone oxidoreductase	MDBK	Gene knockout lowers invasion	23
MAP_3607	Mycobacterial cell entry (Mce1D)	MDBK	Gene knockout lowers invasion	23
MAP_0941	Arginine deaminase (ArcA)	MDBK	Gene knockout lowers invasion	23
MAP_2808	Potassium transporter (TrkA)	MDBK	Gene knockout lowers invasion	23
MAP_2938c	Isoprenoid biosynthesis	Calf intestine	Gene knockout lowers invasion	28
MAP_1242	Lipotripeptide biosynthesis (PstA)	Calf intestine	Gene knockout lowers invasion	55
MAP_0482	Transcriptional regulator (LuxR)	MDBK	Gene overexpression increases invasion	38
MAP_3985c	Hypothetical protein	MDBK	Secreted protein. Binds Cdc42 and activates it	23

berculosis. Does *M. avium* subsp. *paratuberculosis* invade by the zipper mechanism, where bacterial proteins bind receptors on the host cell membrane, similar to that proposed for *Listeria*? Or does it invade by the trigger mechanism, where type III or IV secreted bacterial proteins are injected into the host cell and interact directly with cellular proteins as observed with *Salmonella* and *Shigella* (56)? Regarding host factors that may promote the invasion process, there is involvement of host Cdc42 and RhoA GTPases, but it is not known if increased GTPase activity is required and actin-based rearrangements result from this involvement or if those rearrangements are even required for invasion. Also, what additional host factors are required? Studies with the LuxR regulator suggest that a lipid component in the mycobacterial cell wall is involved in the invasion process, but what those lipids are and how they promote invasion remain to be defined. Experiments that clarify these points should be considered the next steps to further define the pathogenesis of this bacterium. Finally, much of the knowledge on *M. avium* subsp. *paratuberculosis* invasion is based on studies using cell lines such as MDBK and MAC-T cells. To avoid potential artifacts, we urge extending these studies to the bovine intestinal loop model when practical.

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