Search for a repressor regulator of invasion in *Mycobacterium avium*

**INTRODUCTION AND LITERATURE REVIEW**

**Organisms of the genus Mycobacterium.** Mycobacteria are nonmotile, high G+C gram-positive bacteria encased in a thick cell wall with attached mycolic acids, characteristic of the ancient family actinomycetes (19, 61).

According to “J.P. Euzéby: List of Bacterial Names with Standing in Nomenclature,” the genus mycobacteria presently includes 113 known named species, and the list is expanding rapidly (http://www.bacterio.cict.fr/m/mycobacterium.html).

Mycobacterial species fill a wide variety of evolutionary niches. The most vigorously studied member of the genus is *Mycobacterium tuberculosis*, the causative agent of human tuberculosis (13). *M. tuberculosis* was responsible for an estimated 1.6 million deaths worldwide in 2002, making it second to only HIV in mortality attributed to an infectious disease (2). *Mycobacterium bovis* and *Mycobacterium microti*, causative agents of bovine and murine tuberculosis, respectively, along with *M. tuberculosis* and a few others make up an evolutionary clade of obligate pathogens of animals. Closely related are *Mycobacterium marinum*, a common pathogen of fish and frogs, and *Mycobacterium ulcerans*, responsible for Buruli’s ulcer (18).

Other mycobacterial species are thought to be purely environmental organisms, such as *Mycobacterium smegmatis*, a saprophyte with minimal known virulence in
mammalian hosts. Still other species are environmental, but maintain some capacity for virulence in eukaryotes. These include a clade that includes *Mycobacterium avium* subspecies *avium*, *Mycobacterium scrofulaceum*, *Mycobacterium intracellulare*, and *Mycobacterium kansasii*, the latter being from the next nearest evolutionary branch (18). *Mycobacterium leprae*, the agent responsible for leprosy, is found in a deep branch off of this group (58).

**Overview of mycobacterioses.** Infectious mycobacteria commonly enter the body of the host through mucosal surfaces by invading epithelial cells. *M. avium* subspecies *paratuberculosis* (herein referred to as *M. paratuberculosis*) is the causative agent of Johne’s disease, an inflammatory bowel disease of cows. The bacterium gains entry by the oral route and infects M cells, the specialized antigen-sampling epithelial cells of the Peyer’s patches, at the distal ileum (39). *M. leprae* is believed to infect the nasal mucosa or the skin itself, where it can cause a chronic granulomatous infection. *M. tuberculosis* enters the lung through droplets from the cough of an infected patient. While is believed that this organism initially targets primarily alveolar macrophages, *M. tuberculosis* is also thought to invade lung epithelial cells, from the observations that it can invade both type II alveolar cells *in vitro* and recently removed intact respiratory mucosal tissue, even in the absence of phagocytic cells (6, 38).

In all three of the above infections, mycobacteria replicate at the site of infection, resulting in pathologies with still further commonalities. All utilize and enter macrophages associated with the site of infection, where it is believed the
bacteria can survive by preventing acidification of the vacuole and phagosome-lysosome fusion (55). The survival of bacteria associated with host recognition and response commonly leads to granuloma formation, an aggregation of macrophages and T cells. These structures usually have a caseous center and calcified exterior, and when in the lung are often visible on X-ray images of the affected area. Also, bacteria are frequently found in the lymph nodes draining the site of infection. Mycobacterial infections are often long-lived, resulting in years- or even decades-long chronic disease or latent periods.

In severe cases, local mycobacterial infection progresses to systemic disease, resulting in colonization of varied disparate body tissues with high associated morbidity and mortality. Indicators of this condition include bacteremia and the ability to isolate bacteria from other tissues, such as the spleen and liver.

**M. avium and the Mycobacterium avium complex**

*M. avium* subspecies *avium* is a slow-growing opportunistic pathogen, ubiquitous in soil and water. Clinically, the organism is often referred to in the context of the *Mycobacterium avium* complex (MAC). The MAC consists of *Mycobacterium intracellulare* and *M. avium* subspecies *avium*. The latter is the primary subject of this thesis, and will be hereafter in this study simply referred to as *M. avium*. 
In humans, MAC is known to have two basically distinct sites of infection. Before the advent of the AIDS pandemic, most MAC infections were associated with the lungs and were often referred to simply as a subgroup of the nontuberculous mycobacterial (NTM) or atypical mycobacterial infections. These infections occur in specifically immunocompromised hosts, such as persons with lung pathologies such as silicosis, emphysema, bronchiectasis or previous tuberculosis or other lung infections (20, 61). The MAC also infects the lungs of immunocompetent hosts, with a higher incidence in women, the elderly and patients with low body weights, but with no other known preexisting risk factors (33, 48).

With the emergence of HIV, and specifically the increased prevalence of T cell lymphopenic AIDS patients, there was a sharp increase in the incidence of disseminated MAC (DMAC) infections (5). After the advent of effective antiretroviral therapies in the 1990s, restoring the immune systems of HIV positive persons where available, the incidence of DMAC in declined dramatically (10, 22, 30).

AIDS related DMAC infections are primarily *M. avium*, while non-AIDS related cases are often, if not predominantly, *M. intracellulare* (20, 25). With a similar but not identical pattern of etiology, other observations indicate that intestinal infections are predominantly *M. avium*, while pulmonary infections are often, if not mostly attributable to *M. intracellulare* (26, 48). While the patterns of disease of these two closely related organisms do overlap, there are important distinguishing differences in the predominant behavior of each.
**M. avium infection and invasion.** AIDS related DMAC infections are believed to be primarily the result of an initial infection at the intestinal epithelium, although some evidence suggests the organism can disseminate from a pulmonary infection as well (15, 20, 59).

Specifically, *M. avium* is thought to gain initial entry into body tissues by invading intestinal epithelial cells, enterocytes, of the terminus of the ileum (52). This is in contrast with the site of entry of the closely related species *M. paratuberculosis*, which likely enters the more specialized M cells (39).

It has been shown *in vitro* tissue culture models that *M. avium* attaches to and invades intestinal epithelial cell lines such as Henle 407 and HT-29, while saprophytic mycobacteria such as *M. smegmatis* and *Mycobacterium phleii* do so with a much lower efficiency (8, 36). *M. avium* killed by autoclaving, formalin or UV-light exposure are also unable to attach or invade, suggesting these are active processes for the bacteria (36). Additionally, pre-exposure of tissue cultures to cytochalasin B, an actin cytoskeleton rearrangement inhibitor, and eukaryotic signal transduction inhibitors staurosporin, H7 and genistein all strongly inhibit *M. avium* invasion (8, 36). These data suggest uptake of the bacteria is an active process for the host as well. Additional work investigating involvement of host cell signaling pathways utilizing phosphatidylinositol-3 kinase (PI-3K) and Rho-family small GTPases using pharmacological inhibitors found that PI-3K and Rho, but not Rac or Cdc42, are involved in *M. avium* invasion and no membrane ruffling was detected, while by
contrast *Salmonella typhimurium* required Cdc42 activity only and does induce membrane ruffling (51). However, later work has suggested Cdc42 and membrane ruffling may be involved in *M. avium* invasion as well (14).

In one ultrastructural study, transmission electron microscopy showed *M. avium* enters the cell and is surrounded by an endocytic vesicle (53). Following initial uptake multiple bacteria are found in some vacuoles, however they tend to segment to leave individual bacteria in each vacuole, which are large and near to the nucleus. Importantly, *M. avium* was not found outside the vacuoles after 5 days, suggesting vacuole lysis does not occur early in the infection, if at all.

Days after infection in a murine model *in vivo, M. avium* can be found in mesenteric lymph nodes and the spleen. The present model of *M. avium* infection is thus posits the bacteria is ingested, and invades and colonizes the distal ileum on the way through the gastrointestinal tract. From there, the organism escapes the intestinal mucosa without lysing the vacuole, and is eventually found in mesenteric lymph nodes, the blood and the spleen, indicating disseminated infection and resulting in high morbidity and mortality. Present studies seek to advance our knowledge of this model by elucidating the molecular mechanisms of the infection process.

**Polarized epithelial cells.** One of the important differences between epithelia *in vivo* and our standard *in vitro* models of epithelial cells in tissue culture is that, unlike cells attached to a flask or the bottoms of multi-well plates, the cells at mucosal surfaces in living organisms are polarized, which can have important consequences for our
understanding of the initial host-pathogen interaction (31). For example, *Listeria monocytogenes* was found to invade basolateral but not apical surfaces of Caco-2 cells, suggesting the organism initially must enter at some other location than differentiated enterocytes of the vili in which they are found after necropsy (23).

In polarized cells, the apical environment-facing and basolateral inward-facing surfaces are differentiated by targeted membrane trafficking, leading to unique lipid, protein and macromolecule compositions at each surface (40). A convenient *in vitro* model of a polarized membrane is achieved by growing epithelial cells to confluence on a filter support, such that the basolateral side is exposed to nutrients. Notably, in such a model, *M. avium*, like invasive Salmonella, efficiently enters cells via the apical but not the basal side (21, 51). In these ways, experiments utilizing polarized monolayers grown on filter supports might represent a better model of host-pathogen interaction at epithelial barriers.

**Other common gastrointestinal pathogens.** As a busy point of interaction between our bodies and foreign objects, the gastrointestinal tract provides a location many pathogens use to cause infections. Most gastrointestinal pathogens are transmitted by the fecal-oral route, which one would expect given an organism adapted to live or replicate in the mammalian intestinal tract, and commonly induce gastroenteritis and diarrheal disease in the host. There are two major strategies, not exclusive of each other, that gastrointestinal pathogens use; adherence followed by replication and fecal release of large number of bacteria, or invasion and colonization of host tissues.
*Vibrio cholerae*, enteropathogenic *Escherichia coli* strains and *Bacillus cereus* attach to the intestinal wall without invading, and produce toxins that cause diarrhea. While sometimes severe, this condition is generally self-limiting and survivable with supportive treatment such as oral rehydration solution (1). In some cases however, such as enteropathogenic *E. coli* strains and some *Brucella* and *Clostridium* species, release cytotoxins that cause such severe damage that the intestine that barrier function is lost, and dysentery and septicemia can result.

In contrast, other pathogens including enteroinvasive *E. coli*, *Salmonella*, *Shigella*, *Yersinia* and *Listeria* directly invade the cells of the intestinal wall, often via the M cells of Peyer’s patches (43). From there, depending on the infectious organism, infection may progress laterally through the intestinal wall or disseminate to the mesenteric lymph nodes, the bloodstream, and to other tissues.

**Virulence gene regulation by signal transduction systems.** Often, genes involved in virulence in bacterial pathogens are coregulated via a global transcriptional activator or series of transcriptional regulators. These in turn are induced by an array of sensor molecules. The result is a broad phenotype switch that occurs in the presence of one or more environmental cues.

OmpR is a transcription factor in *E. coli* responsive to an osmolarity-regulated sensor kinase, EnvZ, that mediates production of various porins (57). EnvZ is a membrane-spanning protein with a histidine kinase ATPase domain. Environmental cues induce autophosphorylation of this sensor at the histidine residue, utilizing the γ-
phosphate from an ATP molecule. The sensor kinase in turn phosphorylates the response regulator at a conserved arginine residue, which induces a conformation shift that promotes DNA binding of the response regulator at consensus promoters of the regulon, thus leading to altered gene expression at any number of loci (28).

After the discovery of the OmpR/EnvZ two-component regulatory system, it was soon revealed that many gram-negative bacteria harbored directly homologous and paralogous systems (50). BLAST searches using the *Salmonella enterica* serovar *typhi* OmpR and EnvZ homologue amino acid sequences as queries reveal that proteins with detectible similarity are widely present in the tree of life, including in gram-positive Eubacteria, Archaea and Eukaryota (unpublished results).

Many two component regulatory systems are thought to be involved in virulence. For example, *M. tuberculosis* is known to have extremely long potential periods of latency and remission. It is believed the organism can enter a “nonreplicating persistant” state inside the hypoxic environment of blocked pulmonary lesions (60). Presumably, the bacterium must have a way to recognize the macrophage environment in order to induce that state, as a nonreplicating phenotype is one that must be tightly regulated.

A LuxR-family transcriptional activator, Rv3133c/DosR, induces an approximately 50 gene regulon involved in latency and long term survival inside a phagosome by binding to a specific promoter sequence and is essential to prolonged survival in the absence of oxygen (9, 42, 46). DosR, in turn, has been found to be activated by at least two sensor kinases, Rv3132c/DosS and Rv2027c/DosT, in the
presence of hypoxic conditions or nitric oxide, which are suspected to be the conditions inside the phagosome (49). Interestingly, DosR knockouts have been implicated in increased mortality in a murine model, but decreased morbidity in a hamster model in different studies (35, 45).

Somewhat similarly, a gastrointestinal pathogen must have regulatory elements to recognize the intestinal lumen environment. Recent studies have shown that dissecting the regulatory pathways leading to this phenotypic shift in these organisms can lead to further discoveries regarding the components and the mechanisms of the disease process. Again we see that different environmental signals, which lead to regulation of often many virulence factors, are connected through just one or very few transcription factors. With such broad effects, one can call these transcription factors “global regulators” of virulence.

For a well-adapted gastrointestinal pathogen, the distinct conditions of the intestinal lumen, such as hyperosmolarity and hypoxia, would seem to provide powerful and specific environmental signals from which to activate the invasive phenotype.

The spore-forming bacterium *Bacillus cereus* must sense the gastrointestinal environment to signal the return to the virulent vegetative and toxin-producing state. Alternately, the evolutionarily related pathogen *Clostridium perfringens* senses the same environment to induce toxin production and sporulation. The Spo-family transcriptional regulators required for spore formation in both *Clostridium* and *Bacillus* have phosphate binding sites and are activated upon phosphorylation,
indicating the presence of conjugate sensor kinase mechanisms (44, 47). Like mycobacteria, these organisms also have eukaryotic-like serine and threonine kinases, as well as the classical bacterial histidine kinases. So while this information does not allow us to presume the involvement of classic two-component regulatory systems in spore formation, it does suggest the presence of a phosphorylation-based signal transduction system.

**ToxT and HilA: global regulators of virulence.** *Vibrio cholerae* is an important human pathogen causing often severe diarrheal disease. It is transmitted from person to person by the fecal oral route, usually via drinking water sources lacking sufficient sanitation.

While *V. cholerae* is not an intestinal invader, it does live a dual niche lifestyle that suggests a requirement for regulation of virulence. *V. cholerae* expresses a number of factors, including toxin production, toxin coregulated pilus production, motility and chemotaxis, that are regulated by the ToxT transcriptional activator. In turn, ToxT is known to be regulated by another transcriptional activator, ToxR, which responds various environmental cues such as salinity, osmolarity and temperature, via two-component regulatory systems (54).

While there is significant evolutionary distance between the gram-negative, enteric γ-Proteobacteria genus Salmonella and the genus Mycobacterium, some salmonelloses are at least superficially analogous to GI acquired DMAC infection. Both invade and colonize intestinal cells that are non-professional phagocytes, both
have species capable of translocating that epithelial barrier and disseminating to mesenteric lymph nodes and splenic macrophages. In macrophages, both have been found to partially inhibit phagosome-lysosome fusion, and hosts with congenital defect in IFN-γ production have been found to be susceptible to infections from both genera.

In S. typhimurium aerobic conditions repress invasiveness in intestinal epithelial cells. It was hypothesized that some gene or genes directly contributed to this repression. Screening of a transposon knockout library revealed three loci that contribute to diminished invasion in aerobic conditions. One of those is the hil locus in a region dubbed Salmonella Pathogenicity Island 1 (SPI-1) (34). The crux of the system is hilA, coding for an OmpR/ToxR-family transcription factor that acts as a global regulator of invasion genes, including an invasin locus and a type III secretion system coded on SPI1. Expression of hilA has been found to increase in neutral to alkaline pH, high osmolarity and low oxygen tension and the presence of acetate: factors similar to the lumen of the small intestine. High levels of bile, propionate, butyrate, or cationic peptides, as one would find in the proximal small intestine, the cecum, the colon and inside macrophages, respectively, all inhibit hilA expression. Knockout studies have demonstrated invasiveness in vitro, and virulence in vivo, are both sharply dependent on hilA expression and upstream regulators of hilA expression, which include two component regulatory systems, transcriptional regulators and other proteins (3).
Interestingly, some genes act to repress invasion, indicated by the discovery of knockout mutants with increased invasion and virulence. Most of these, including Hha, the PhoP/PhoQ Mg$^+$-sensing two-component regulatory system, the transcriptional regulator HilE, and the ubiquitous Lon protease, act by repressing HilA activity or expression (3, 29).

**M. avium virulence gene regulation and this project.** Like Salmonella, *M. avium* invasion of intestinal epithelial cells *in vitro* also increases in bacterial culture conditions similar to the intestinal lumen, such as low oxygen tension and high osmolarity (7). This suggests the possibility of a mechanism of invasion gene expression responding through an environmentally sensitive global regulatory pathway.

The specific components of this pathway are largely unknown. Elucidation of some parts of this hypothetical sensor-regulator-effector cascade can provide a starting point to help us identify other important virulence factors and bolster our understanding of the mechanism of the initial interaction of *M. avium* and the host.

In this project, we hypothesized that a repressor regulators of invasion exist in *M. avium*, and that finding one would lead to further discoveries regarding the regulatory pathways and effector molecules. To this end, we screened a transposon library for hyperinvasive clones using positive selection. A clone was isolated with an apparently constitutive high rate of invasion and was characterized by various methods. However, it eventually became apparent that the mutant is broadly
aminoglycoside resistant but not highly invasive, and was initially mischaracterized as
highly-invasive due to experimental design error in the initial screens. Still, our
results suggest that this mutant, dubbed HI5, is deficient in dissemination after oral
infection in a murine model, and may prove to be a useful tool in studying the as yet
unmapped dissemination process.
MATERIALS AND METHODS

**Bacterial culture.** *M. avium* strain A5, a non-plasmid containing, smooth and opaque colony-forming blood isolate from an AIDS patient (27), and the mutant strain HI5 were used in this study. Both were grown on Middlebrook 7H10 agar (Difco) at 37ºC for 4-6 days or in 7H9 broth (Difco) for 3 days prior to experimentation. For HI5 culture, the media were supplemented with 400µg/ml kanamycin to maintain transposon stability. For all quantifiable assays, bacteria were agitated and suspended at high concentration in approximately 2ml HBSS and allowed to settle for 15 minutes, to remove clumps by sedimentation. After that time dispersed bacteria were pulled from the top 1ml of the settled suspension and mixed to the desired inoculum concentration. Concentrations were confirmed by serial dilution, plating and CFU determination.

**pTNGJC construction.** Temperature-sensitive plasmid pTNGJC was constructed based on the pUC18 vector. The transposon Tn5367 from pYUB285 was cloned into the EcoRI and HindIII restriction sites making the plasmid pUC18-Tn5367 (37). This transposon contains the *aph* kanamycin resistance gene. The temperature sensitive mycobacterial origin of replication was removed from pCG79 (24) (provided by B. Gicquel, Insititute Pasteur, Paris) and inserted in pUC19-Tn5367 to create pTNGJC.
Transposon mutagenesis. Transformation of *M. avium* A5 with plasmid pTNGJC was carried out by electroporation, then plated on 7H11 agar with 400 μg/ml of kanamycin. A clone containing the plasmid was grown at 30°C for 3 weeks in 7H9 broth with kanamycin. After the number of bacteria reached approximately 10^9 CFU, the culture was placed in a 41°C incubator for 3 days. The suspension was then diluted and plated onto 7H11 agar with kanamycin at 37°C. Colonies were harvested and screened by PCR for the presence of the pTNGJC-KAN plasmid. Primers for the Km gene were: 5’-TGTTCAACAGGCCAGCCA-3’ and 5’-TAATGTCGGGCAATCAGGTG-3’. Twenty colonies were selected and tested for the presence of the transposon-Km gene. All 20 contained the transposon.

Identification of unknown transposon-flanking regions. To identify the gene or genes putatively interrupted by transposon insertion, a non-specific, nested suppression PCR was used (56). The primer used was 5’-CCATCATCGGAAGACCTC-3’. PCR cycling was as follows: 35 cycles of 94°C for 30s, 50C for 1 min, and 72°C for 4 min. Prior to the first cycle, a temperature of 94°C was held for 5 min, and at the end of the last cycle, a temperature of 72°C was maintained for 7 min. The primer used for the second PCR was 6 nucleotides (GACCCC) longer at the 3’ end. The PCR cycling was the same as the first PCR, except the annealing cycle was for 30s at various temperatures using *Pfu* DNA polymerase (Stratagene). The PCR products were electrophoresed through a 1% agarose gel, and each PCR band that appeared on the gel was excised and extracted
using the gel-extraction kit (Qiagen). The PCR amplifications were cloned into pCR2.0 TOPO vector (Invitrogen, Carlsbad CA), and submitted for sequencing.

**Tissue Culture.** Stock culture of HT-29 cells (ATCC TIB-71) were grown in McCoy’s 5a media (Gibco) supplemented with 10% heat-inactivated FBS at 37°C in the presence of 5% CO₂. For cell subcultivation and propagation, exponentially growing cells were trypsinized, centrifuged at low speed (approximately 150g for 6 minutes), resuspended in warm media, and then dispersed according to the needs of each experiment.

RAW 264.7 macrophages (ATCC) were grown in Dulbecco’s modified eagle medium (DMEM, Gibco) supplemented with 10% heat-inactivated FBS at 37°C in the presence of 5% CO₂. A cell scraper was used to manually remove the cells from the culture flask. For bacterial intracellular survival assays, approximately 10⁵ cells were transferred to experimental wells in a 24-well tissue culture plate, and grown to approximately 70-90% confluence.

**HT-29 invasion assay.** HT-29 cells were grown to near confluence (70-90%) in the wells of a 24-well plate. The monolayers were washed once with HBSS using a transfer pipette, to remove any loose or dead cells. Bacterial inocula were mixed as described above to an approximate concentration of 2 x 10⁷ CFU/ml, and 0.5 ml was placed in each experimental well and incubated for 1 hour at 37°C. After the invasion,
cells were washed again with HBSS to remove unattached bacteria. New media with
200 mcg/ml amikacin was added, to inhibit bacterial protein synthesis and remove
attached, but not endocytosed, bacteria. Cells were washed a final time and lysed in
0.5 ml 0.5% Triton X-100 for 10 minutes. The lysate was then diluted and plated and
compared to the inoculum concentration to calculate percent invasion.

**HT-29 invasion assay (alternate).** Cells were grown to near confluence (70-90%) in
the wells of a 24-well plate. Wells were washed once with HBSS using a transfer
pipette, to remove loose or dead cells. Bacterial inocula were mixed as described
above to an approximate concentration of $6 \times 10^7$ CFU/ml, and 0.5 ml was placed in
each experimental well and incubated for 1 hour at 37°C. After the invasion, cells
were washed again three times with HBSS to remove unattached bacteria. To loosen
the tissue culture cells, 0.4 ml of a prewarmed 0.25% trypsin-EDTA suspension was
added to each well and allowed to incubate for 10 minutes or until the cells were
visibly loosened. The solution was transferred to a microcentrifuge tube and
centrifuged at 10,000g for 90 seconds to pellet the cells. The fluid was removed and
0.5 ml of a 0.5% Triton X-100 was added and allowed to incubate at room temperature
for 10 minutes to lyse the cells. The lysate was then quickly diluted, plated and CFU
determined to calculate percent invasion.

**Murine oral infection model.** To compare the dissemination of the A5 and H15
strains *in vivo*, C57/Black mice (Jackson Laboratories) were infected by gavage with
approximately $10^7$ CFUs suspended in 0.2 ml saline. Depending on the experiment, the mice were sacrificed after 4 hours or 7 days. A 2 cm section of the terminus of the ileum was removed and washed in HBSS, then homogenized and plated to enumerate CFUs. To inhibit contaminant growth, 7H10 agar plates supplemented with PACT (5.5 mcg/ml polymyxin B, 11mcg/ml amphotericin B, 25mcg/ml carbenicillin and 2.5mcg/ml trimethorphin) were used. In the 7 days infection model, the spleens were also harvested, homogenized and plated to measure dissemination. The organs were weighed before homogenization, and CFU/g of organ was calculated.

**Macrophage survival.** RAW 264.7 macrophages were infected with plate-grown A5 or the mutant HI5 for 1 hour, and then washed 3 times with HBSS to remove any bacteria that had not entered the macrophages. Macrophages were lysed at selected time points, by immersion in 0.5 ml sterile water for 10 minutes followed by scraping with a micropipette tip, to measure the survival and growth of intracellular bacteria over time. At each time point, the lysate was plated on 7H10 agar plates at the appropriate dilution, to enumerate CFUs/ml of lysate.

**Transwell monolayer translocation and survival.** Approximately $3 \times 10^5$ HT-29 cells suspended in 100 µl media were transferred to the top of a 6.6 mm diameter polycarbonate transwell membrane (Corning Costar 3415) suspended in a 24-well plate well with 0.6 ml pre-warmed media and allowed to settle. Each day the upper chamber and lower chamber media were changed, and after 2 days, confluence of the
monolayer was tested by adding extra media to the top and measuring the ability of the monolayer to prevent the fluid from returning to equilibrium. Confluence was also confirmed with a volt-ohm meter (Millipore Millicell ERS), with an expected confluence threshold of approximately 500 mΩ/cm². After confluence was reached, to prevent bacteria from crossing the membrane extracellularly, bacteria were allowed infect for one hour, then washed off with HBSS as in the macrophage assay. At selected time points, the lower chamber media was collected and plated to measure the propensity of the bacteria to translocate the monolayer membrane. After 4 days, the monolayer on the membrane was lysed, by cutting out the filter membrane and immersing it in 1 ml of 0.5% Triton X-100 for 15 minutes, then vortexing brusquely for 20 seconds. The lysate was plated to enumerate the bacteria remaining inside the cells of the monolayer.

**RNA isolation.** Broth-grown mid-log phase bacteria were split into 50ml aliquots, then spun down at 4°C. The pellet was resuspended in Trizol reagent (Invitrogen), lysed with glass beads and a bead-beater, and spun again in a microcentrifuge at high speed. The fluid was taken off and successive acid phenol-chloroform purifications were performed until protein residue was no longer visible in the interface layer, usually after 2 more extractions. The solutes were then precipitated by adding an equal volume cold isopropanol, followed by a careful washing of the pellet with 80% ethanol. After the pellet was allowed to dry, the samples were resuspended in DEPC water and DNase treated, then phenol-chloroform/isopropanol-ethanol purified a final
After a second resuspension, optical density readings were taken to measure quantity and purity.

**DNA macroarray analysis.** From mid-log phase broth-grown bacteria, approximately 1µg of purified RNA was used with P³² labeled dCTPs and a Panorama *M. tuberculosis* cDNA labeling kit (Sigma-Genosys) to synthesize cDNA, as per the manufacturers instructions. Briefly, a mixture of unique primers for 3,875 known non-repetitive *M. tuberculosis* ORFs is used to prime cDNA synthesis by reverse transcriptase. The cDNA was then hybridized to Panorama *M. tuberculosis* gene arrays (Sigma-Genosys) for 15 hours. The arrays were washed and set against a phosphor screen (Molecular Dynamics) for 18 hours. The screens were then read with a phosphorimager (Molecular Dynamics). The images were analyzed to estimate expression fold-change between paired samples using software (Sigma-Genosys).

**Real-time PCR.** Quantitative PCR was performed to confirm expression changes indicated by the arrays. For each sample, 5 µg of RNA from mid-log phase broth-grown bacteria was used as template for single strand synthesis with a Superscript III First-Strand Synthesis System for RT-PCR kit (Invitrogen). Primers were designed with 100-400bp lengths, using Oligo 6.8 or Primer3 software. A Biorad iCycler was used to perform the qPCR. A total reaction volume of 25 µl was used, with 12.5 µl SYBR green enzyme supermix (Biorad or Invitrogen), 1 µl template suspension from the cDNA synthesis reaction, about 6.25 pmol of each primer and 0.5 µl DMSO (for
GC-rich sequences) with PCR-grade water to fill the remaining volume. The following qPCR protocol was used: one cycle at 95°C for 5 minutes, followed by 35 to 50 cycles with the steps; 95°C for 30 seconds, 62°C for 30 seconds, 72°C for 30 seconds. Efficient and accurate amplification of select fragments was confirmed by melting curve analysis and visualization by gel electrophoresis.

**Selective aminoglycoside resistance screen.** Two separate screens were used in an attempt to isolate kanamycin resistant, but amikacin and apramycin sensitive subclones of the HI5 strain culture. In one assay, isolated colonies were picked and used to seed 200µl media in wells of 5 96-well plates. Two wells were left unseeded, as sample blanks, and one was seeded with wild-type A5 as a negative control. Each well was mixed lightly by pipette to break large clumps, and allowed to grow 2-3 days at 37°C. Subsequently, 35µl from each well was seeded in 3 new 96-well plates with 150µl media, one each containing 200µg/ml amikacin, apramycin or kanamycin. Optical density was measured with a plate reading spectrophotometer at different time points to screen for subclones able to grow in kanamycin, but not the other antibiotics. Similarly, in another assay, isolated colonies were picked and seeded by toothpick on gridded agar plates. Approximately 50 colonies were placed on each plate. After 6 days, replica plates were produced using a disposable-velvet wrapped cylinder. Colonies were replica plated to media containing 200µg/ml kanamycin, apramycin, or amikacin, in that order. Plates lacking antibiotic were stamped last, as a positive control, and colonies were screened for ability to grow on plates with kanamycin or
lacking antibiotic, but lacking ability to grow in the presence of amikacin and/or apramycin.
RESULTS

Identification and characterization of hyperinvasive *M. avium* mutants. A heterogeneous mixture of mutants in *M. avium* A5 was created using Tn5367-mutagenesis. The mutants were screened for increased invasive ability when cultured using standard growth media and in the presence of atmospheric oxygen. Positive selection of the mutants from large pools was measured by their ability to resist washing and amikacin-treatment after 1-hour invasion of HT-29 cells in culture, in serially conducted invasions. This method is similar to that used in the identification of the hilA invasion regulator in Salmonella (34).

Four clones were isolated with approximately five-fold increased invasion rates compared to the wild-type in initial experiments (data not shown), and the transposon was located using a nested suppression PCR technique (56). In each clone, the transposon was found in the same ORF, and one of these clones, dubbed HI5, was further characterized in this study.

It has previously been observed that culture in an anaerobic chamber induces an invasive phenotype in *M. avium* (7). When cultured in the absence of oxygen, both wild-type A5 and mutant HI5 show approximately the same rate of invasion as the mutant displays in the presence of oxygen (data not shown). The mutant, unlike wild-type *M. avium*, is insensitive to oxygen suppression of the invasive phenotype, suggesting a defect in an as yet uncharacterized invasion repressor-regulatory pathway.
Upon sequencing and comparative genomic analysis, the putative transposon-interrupted ORF is similar to MAP1701c from *M. paratuberculosis*, and Rv1722 in *M. tuberculosis*. The conserved hypothetical gene product of this ORF contains an AccC biotin carboxylase conserved domain, and is a member of a ubiquitous ATPase grasp-fold superfamily of proteins.

In *M. avium*, *M. tuberculosis* and *M. paratuberculosis*, these similar ORFs overlap the ORF immediately downstream by 4 nucleotides, suggesting polycistrionicy and coregulation of expression. This downstream ORF is similar to conserved hypothetical genes Rv1722 from *M. tuberculosis* and MAP1700c from *M. paratuberculosis*. The translated protein sequences of these genes contain conserved domains of class C Beta-lactamases, the xenobiotic degradation enzyme 6-aminohexanoate dimer hydrolase, and other hydrolases found in other bacteria. In *M. avium* and *M. paratuberculosis*, the hypothetical genes immediately surrounding this region are homologous and in the same order, while they are completely different in *M. tuberculosis*.

This apparent two-gene operon is not found to share identity to genes in any other Actinomycetes or even other Mycobacteria in the sequence database, besides those listed above and *Brevibacterium Linens* BL2, a nonpathogenic gram-positive coryneform bacterium found to be involved in ripening of some cheeses. From all these organisms, the upstream possible carboxylase, but not the downstream hydrolase is also highly similar to a gene in *Acinetobacter baumannii*, a gram-negative γ-proteobacterium commonly associated with opportunistic nosocomial infections. It is
not clear how all those different organisms, some separated by appreciable evolutionary distance, came to share these presumably homologous genes.

**Oral infection of mice.** To examine the possibility of altered virulence in the HI5 mutant, *M. avium* A5 and HI5 were fed to C57/Black mice by gavage injection. The mice were sacrificed after 4 hours or 7 days, in separate experiments, and organs were harvested, homogenized and plated to quantify prevalence of bacterial CFUs.

In both experiments, after the requisite amount of time after infection, the termini of the ileum were collected. This is believed to be the initial location of *M. avium* entry into the intestinal wall. The organs were washed to remove extracellular bacteria, then homogenized and plated. At the 4 hour time point, both mutant and wild-type strains were found in equivalent amounts, suggesting the strains invade at similar rates *in vivo* (Fig. 1). In the 7 day set spleens were also harvested, to measure invasion as well as dissemination. At this time point, HI5 was more numerous in the ileum, but found in much lower numbers in the spleens compared to the wild-type (Fig. 2). Taken together, these data suggest that both strains initially enter the intestine at equivalent rates, but the mutant is attenuated in the ability to disseminate to the spleen.

Additionally, it was observed that colony morphology of the mutant HI5 differs from A5 when grown for at least 3 weeks on agar plates (Fig. 3). Wild-type A5 is a smooth-edged opaque strain, with colonies that grow into regular-shaped mounds. The HI5 strain is also smooth-edged and opaque, but the colonies grew to
have dimpled tops. The nature of the dimpling is variant, seemingly affected by such factors as plate drying, colony size and proximity to other colonies. Yet, the strain differences are readily apparent, and unaffected by the presence or absence of antibiotic selection.

**Invasion and survival in macrophages.** As the mutant appears dissemination deficient, *in vitro* tissue culture models were used to dissect the possible dissemination pathway. One explanatory hypothesis for the reduced number of mutant bacteria in the spleen is that the mutant is unable to enter or survive in splenic macrophages. To test this hypothesis, A5 and mutant H15 were used to infect RAW264.7 murine macrophages in tissue for one hour, and CFU were measured at different time points, from 1 hour to 4 days. Bacteria cultures in different growth states, as determined by their age on agar plates, were used in separate experiments ([Fig. 4](#) and [Fig. 5](#)).

Interestingly, the growth and survival curves for 6-day old cultures and 18-day old cultures were very different, suggesting the possibility of markedly different outcomes if observed for longer time periods. However, irrespective of bacterial culture age, both strains appeared to enter and survive in macrophages at equivalent rates, indicating diminished macrophage survival is not the proximate cause of attenuated dissemination of the H15 mutant in our *in vivo* assay.
Translocation of HT-29 polarized epithelial cells. Another probable step in the dissemination process of *M. avium* is the exit of bacteria from the their initial entry site of intestinal epithelial cells. A5 and mutant HI5 were used to infect the apical side an HT-29 polarized monolayer grown on a filter membrane, and bacterial CFU from the lower chamber were enumerated at different time points to measure translocation. The mutant was found to be significantly attenuated in translocation by this method at 3 and 4 days (Fig. 6). This suggests perhaps a diminished ability of the mutant to induce host vesicle trafficking or transcytosis.

Macroarray expression analysis. The global regulators of virulence, such as HilA and ToxT, have downstream effects on transcription of numerous genes, with some upregulated and others downregulated. To attempt to characterize this regulon in a Panorama™ *M. tuberculosis* gene array was used to compare expression in *M. avium* A5 and mutant HI5. This commercially available array was selected for simplicity and the high degree of sequence similarity of *M. tuberculosis* and *M. avium*.

Three replications were conducted, using independently synthesized cDNA from separate RNA samples. In each, different sets of genes were indicated to have altered transcription, and there was no apparent consensus regulon among them (data not shown).
**Second intestinal cell invasion.** In preparation for complement screening, invasion experiments were conducted with wild-type *M. avium* A5 and mutant HI5 by different personnel than the previous experiment. HT-29 cells in culture were exposed to bacteria for one hour, and then washed vigorously with HBSS. In some experiments, amikacin was used to kill extracellular bacteria (data not shown). In other experiments, Hep-2 cells were used, but in all of them, the HI5 mutant was not found to increase at a significantly higher rate than the wild-type. In fact, generally the mutant was found to invade at a slightly lower rate (Fig. 7).

**Real-time PCR.** To clarify the array results, real-time PCR was performed. RNA was extracted from log-phase, atmospheric air-exposed broth-grown wild-type and mutant HI5 bacteria and used as a template for cDNA synthesis using random primers. Amplification of selected genes was measured and compared to that of 16S baseline-control. Genes were selected with indicated altered transcription on one or more of the arrays, along with the 16S control and those of the two-gene operon examined in this study. It was found that none had significantly altered transcription.

**Selective aminoglycoside resistance screen.** One possibility to explain the disagreeing invasion experiment and the transposon-locating and real-time results was that the HI5 strain mostly lost the transposon in culture, due to instability caused by a random aminoglycoside-resistance mutation. Spontaneous resistance to aminoglycosides has been frequently observed in Mycobacteria, and such a mutation
in the HI5 cell culture would greatly reduce the selective pressure preserving the transposon, with its kanamycin-resistance marker. If the hypothetical resistance mutation had not resulted in complete fixation of the transposon, then the HI5 culture should be heterogeneous.

To test this hypothesis, two separate screens were performed to isolate kanamycin resistant, but amikacin and apramycin sensitive colonies of HI5. Both utilized colony isolation and replica plating, one using broth culture in 96 well plates, with mechanical screening, and the other using selective agar plates and screening visually. In all, approximately 900 kanamycin resistant colonies were screened, and none were found to have impaired growth in the presence of apramycin or amikacin.

Furthermore, 10 of these HI5 colonies were screened for the presence of the kanamycin gene in genomic extract, and the marker was detectible in 9 of them. Taken together, these data suggest the transposon was not lost, but rather is present in a location besides the \textit{Rv1722}-homologous ORF.
Figures

**Fig. 1** Four hour oral infection of mice. In one experiment, C57/Black mice were sacrificed 4 hours after gavage infection with approximately $2 \times 10^6$ wild-type A5 and mutant H15 *M. avium*. From this sample (n=20), the termini of the ileum were collected, weighed, and homogenized to quantify CFU/g as a measure of entry of the bacteria into the intestinal wall.
Colonization and survival of M. avium A5 and HI5 in specific tissues 7 days post-infection

![Graph showing colonization and survival in ileum and spleen]

**Fig. 2** Seven day oral infection of mice. C57/Black mice were infected with approximately $2 \times 10^6$ wild-type A5 and mutant HI5 by gavage injection, and sacrificed after 7 days. Specific tissues were harvested, weighed, homogenized and plated on agar to enumerate bacterial CFU/g. The ileum was harvested to measure persistence in the intestine. Spleens were harvested to measure dissemination. Both organs collected from each mouse, and n = 5 for each strain.
Fig. 3  Colony morphology of mutant and wild-type strains. Standard digital photography was used to view *M. avium* A5 and mutant H15 colonies on agar plates. Colonies were grown 41 days, and colonies of the wild-type (upper and lower left panels, large colonies) are smooth, while mutant colonies (large colonies in right panel) have a polymorphic dimpled top.
**Fig. 4** Stationary phase bacteria in macrophages. Confluent monolayers of RAW 264.7 macrophages in 24-well plates were infected with approximately $3 \times 10^6$ CFU of 18-day-old plate-grown bacteria for 1 hour, washed to remove extracellular bacteria, lysed at different time points and the lysate plated to quantify bacterial entry and survival over time. No significant difference between wild-type A5 and mutant HI5 CFUs was observed at three of the time points ($p>0.05$). The four hour time point is likely an anomaly. Each point represents triplicate wells and these results are representative of at least 3 separate experiments.
Fig. 5 Growth phase bacteria in macrophages. Bacteria were grown for 6 days on agar plates and approximately $6 \times 10^6$ CFU were used to infect RAW 264.7 macrophages. Entry rates and survival over time were measured by lysing the cells and plating the lysate to enumerate CFU up to 4-days after infection. No significant difference in CFU values was observed between strain at any time point (p>0.05). Each data point represents triplicate wells, and these results are representative of at least 3 separate experiments.
Fig. 6  Polarized monolayer translocation. Comparison of wild-type A5 and mutant HI5 translocation of an intact polarized HT-29 monolayer over time. Confluent monolayer membranes were exposed to bacteria in the upper chamber for 1 hour, and media was collected from the lower chamber to measure bacterial translocation. Error bars indicate standard deviation, * indicates statistically significant difference between the strains (p<0.05 by student's t-test). Data is the combined result of 3 experiments, for each strain n = 5.
Fig. 7  Repeat of intestinal cell invasion. Approximately $10^7$ wild-type A5 or mutant HI5 *M. avium* were used to infect HEp-2 and HT-29 cells for 1 hour, then cells were lysed and lysate plated and compared to inoculum concentration to calculate percent invasion. No significant difference in invasion rates between strains was observed in either cell type ($p>0.05$). Triplicate wells were used for each data point, and $n=3$ for each strain in each experiments. These results are representative of at least 3 experiments.
DISCUSSION AND CONCLUSIONS

**Invasion and virulence gene regulation.** There is evidence that pathogenic mycobacteria such as *M. avium* and *M. tuberculosis* assume a high invasive phenotype following exposure to such diverse environments as macrophage and epithelial cell phagosomes, from exposure to certain conditions present in the intestinal environment such as hypoxia and high osmolarity, and from coculture with amoeba. We are only beginning to understand the characteristics of these invasive phenotypes, in the form of alterations to expression of different genes and other activity. It is tempting to hypothesize these invasive phenotypes, in response to different environments, are in fact the same phenotype, with different stimuli activating convergent pathways and leading to a similar array of invasion effectors being activated.

Numerous examples of virulence regulatory circuits have been described, including those centered on transcriptional regulators ToxR in *Vibrio* and HilA in *Salmonella* (3, 29, 54). From these systems we can build a simple hypothetical model of virulence gene regulation in an intestinal pathogen. Sensor histidine kinases process environmental signals by hydrolyzing ATP and undergoing autophosphorylation. These in turn pass the phosphate to a direct DNA binding transcriptional regulator, a response regulator, which modulates the virulence phenotype by affecting expression of virulence effectors.

There is a parallel system in *M. tuberculosis* with the DosR response regulator. This protein controls dormancy in response to both hypoxia and nitric oxide such as is
found inside macrophages and is necessary for prolonged latency of infection (49).

The dosR gene exists in M. avium as well, although given their different evolutionary niches, the regulon activated may be somewhat different in M. avium compared to M. tuberculosis. It has been demonstrated that DosR can be phosphorylated by two paralogous sensor kinases, DosS and DosT in M. tuberculosis, while only one direct homologue to these exists in M. avium, with roughly equal similarity to both.

It is possible that there is an analogous system regulating the invasion in M. avium. Regarding the specific mechanisms, there are now at least two alternate hypotheses. The first is that there is a strong consensus regulon activated by different environmental cues, which increases entry into both macrophages and epithelial cells. In this hypothesis, we expect the response regulators and effectors would be mostly present and conserved in M. tuberculosis, M. paratuberculosis and M. avium, as they all are invasive. In contrast, we would allow that the sensory molecules may differ significantly to account for specific environmental adaptations. This hypothesis is supported by the observation that general exposure to different intracellular environments in tissue culture has been found to activate invasiveness in different mycobacteria, in many different experiments.

The second hypothesis is that the different invasive phenotypes do not significantly overlap. In this case numerous possible circuits would exist in different mycobacteria, and while the different organisms may share conserved sensors and response regulators, the lack of stabilizing selection would allow divergence in both
amino acid sequences of the effectors, and the array of effectors affected by each transcription regulator in each organism.

There are at least 11 sensor kinase/response regulator pairs, 5 orphan response regulators and 1 orphan sensor kinase conserved in *M. avium*, *M. tuberculosis* and *M. paratuberculosis*. In *M. tuberculosis* and *M. bovis* BCG, some of these have been implicated in virulence, but none so far have been associated with invasion into epithelial cells.

Present in *M. avium* and *M. paratuberculosis* but missing in *M. tuberculosis*, there are three response regulator/sensor kinase pairs with observable significant similarity to others (MAP1221/MAP1222, MAP3274/MAP3275, MAP4317/MAP4318), one putative sensor kinase with a response regulator both upstream and downstream (MAP3390c with MAP3391c and MAP3389c), one orphan sensor kinase (MAP0153), and finally one pair of very large genes with motifs similar to the DosS/DevS sensor kinase (MAP2078) and other bacterial transcriptional regulators (MAP2079) (unpublished BLAST search). While none have yet been associated with any phenotype, it is possible these genes are associated with responses to environments encountered by *M. avium* and *M. paratuberculosis*, but not *M. tuberculosis*, such as the intestinal lumen. While many other genes besides can affect regulatory pathways, similarities and differences in sensor kinases found in related organisms provide a pragmatic target for investigation.
Different methods to isolate genes involved in a phenotype. When we set out to
learn how M. avium launches an invasion of the cells of the host’s intestinal wall, we
knew there were multiple ways to discover which proteins are involved in a particular
bacterial phenotype.

Transposon mutagenesis, followed by screens for loss of function, remains the
workhorse of modern molecular microbiology. This method is effective in identifying
genes that contribute to a phenotype, but the screening process, which often involves
screening thousands of clones, generally requires considerable time and challenging
procedures.

Comparative genomics offers the possibility of more targeted search.
Presently, because of their medical importance, most of the more broadly-studied
bacteria are pathogens from the groups Proteobacter and the low G+C gram-positives.
While even super-tree approaches give conflicting accounts of the exact phylogeny of
the high G+C gram-positives as a group, which includes Mycobacterium, they
certainly diverged from those other more well-studied groups long ago (11, 12, 17).
Yet, even though mycobacterial pathogens are evolutionarily distant from the
mainstream, the presence of organisms in different niches, such as M. avium, M.
paratuberculosis and M. tuberculosis, offer a useful sampling. As discussed in the
previous section, comparing the genomes of organisms that are close, but that differ in
the areas of interest, can provide a list of genes potentially involved with a process.

However, while this clean approach can provide some insight, pure in silico
studies are rarely convincing. To confirm the function of genes with predicted
involvement in a phenotype requires further testing. Targeted mutagenesis methods have been used effectively to study genes of interest in many organisms. However, this remains difficult in mycobacteria. Since at least the 1880’s, when Robert Koch complained of having to create a new staining method to locate *Mycobacterium tuberculosis* in diseased tissues, the study of Mycobacterium has been a process of making difficult adaptations to widely used microbiological techniques (32). A method exists to knockout specific mycobacterial genes, using simple allelic exchange, but this remains inefficient.

Presently, nucleotide array approaches are showing great promise in the study of proteomics and gene regulation. With the generally accurate presumption that protein production is modulated by altered expression and phenotype is related to protein abundance, powerful expression studies using nucleotide arrays can potentially illuminate entire regulons at once.

However, acquiring array data often leaves many more questions. Altered expression does not itself indicate the involvement of a specific gene in a process, and construction of a gene-knockout and complement strain is required to make an inference of causality and satisfy Koch’s molecular postulates. Additionally, arrays are still a relatively new technology, and are often expensive, unavailable for certain organisms, or simply unreliable. While powerful and certain to increase in reliability and popularity in the foreseeable future, they are at present difficult to use and often don’t tell us anything with publishable certainty.
In this study, we utilized a different, but previously-tested basic approach. Acting on the hypothesis that a repressor regulator of invasion genes exists in *M. avium*, such that when knocked out it would result in constitutively increased invasion, we screened a heterogeneous culture of transposon mutants using positive selection. This has the obvious advantage over screening isolated clones for loss of function, in that natural selection can be utilized to isolate mutants with the desired phenotype. This approach is similar to those used previously to discover repressors of *hilA*, a global regulator of intestinal cell invasion in pathogenic Salmonella (34). In that organism, the discovery of the central components of the regulatory pathway facilitated the further discovery of downstream effectors and upstream sensors involved in the process. We had hoped to achieve a similar breakthrough finding in the study of *M. avium* invasion.

**Technical challenges during the course of this study.** While the theoretical approach taken remains promising, and if repeated, may yet prove to be fruitful, this study was not successful in its goal, and was beset by some terminal technical problems. In the initial screen, utilizing positive selection to find hyperinvasive mutants, amikacin was used to kill extracellular bacteria. In subsequent invasion assays, the antibiotic was also used. While this was done in an attempt to isolate the processes of invasion and attachment in assays, it also had the unintended effect of creating a confounding selective pressure for amikacin resistance, rather than just for invasiveness.
The marker carried in the transposon used in this study is a 3’-aminoglycoside phosphotransferase II kanamycin resistance (kan') gene from a transposon in *E. coli* (4). This marker is very widely used, and should provide specific resistance to only certain aminoglycosides, not including amikacin and apramycin. However, it was observed that the HI5 mutant is in fact resistant to amikacin and apramycin. Additional invasion assays conducted years later, during the course of this project, using a vigorous washing technique and in experiments both in the presence and absence of amikacin, failed to detect a hyperinvasive phenotype in the mutant compared to the wild-type. It thus appeared likely that an amikacin resistant mutant was originally isolated and mistaken for a hyperinvasive one.

Still, it was later alternately hypothesized that ongoing cultivation of the hyperinvasive HI5 mutant, lacking in stabilizing selective pressure on the transposon due to a spontaneous nonspecific aminoglycoside resistance mutation, resulted in transposon instability and a culture that was heterogeneous. The invasive phenotype may not be detected if too small a proportion of the culture retained the transposon. To test this hypothesis, two methods were used. First, approximately 900 colonies of the mutant HI5 were isolated, and cultured on replica plates, using broth and agar in different experiments, to isolate a colony with sensitivity to amikacin and apramycin, but resistant to kanamycin. Such a colony would have both the transposon and the hyperinvasive phenotype intact. However no such specifically resistant colony could be found among the number screened.
Additionally, a repeat of the original positive selection assay, without using amikacin to kill extracellular bacteria, was used to screen the HI5 culture for highly invasive subclones. Both A5 and HI5 were used to seed four pools, which were used for four successive invasions of HT-29 cells in tissue culture. If a high-invasive mutant existed in the HI5 pools, we would expect to observe higher increase in invasion among the final output from those pools than the wild-type negative control, due to selection on a heterogeneous compared to a homogeneous population. In all pools an approximately 2-fold higher invasion rate was observed in the final invasion compared to initial. However, there was no significant difference between the mutant and the wild-type, indicating no unique subpopulation was present in the HI5 culture with a significantly higher invasion rate than the wild-type (data not shown).

In preparing for the aforementioned assay to screen mutant HI5 colonies for subclones resistant to kanamycin but sensitive to other aminoglycosides, some wild-type A5 was streaked on each agar plate with different antibiotics as confirmation of antibiotic efficacy. On each of the three different antibiotic plates, a small number of spontaneous mutants arose. However, on only the amikacin plates, these mutants grew to approximately normal size and had dimpled colony morphology, similar to that observed in the HI5 mutant. Taken together, these observations suggest the HI5 strain is simply amikacin-resistant rather than highly invasive, although it is unclear whether spontaneous mutation or transposon mutagenesis accounts for the resistant phenotype.
An additional problem arose locating the transposon in the mutant. Initially, the transposon was determined to be in an ORF homologous to \textit{Rv1722} from \textit{M. tuberculosis}, with the expected effect of knocking out that gene and the one immediately downstream. However, subsequent PCR-based assays cast doubt on that finding. Using genomic DNA from the HI5 strain as a template, the entire \textit{Rv1722}-like ORF could be amplified, and quantitative real-time PCR indicated that transcription levels of both the \textit{Rv1722} and \textit{Rv1723}-like genes in the HI5 mutant were comparable to those in wild-type A5 (data not shown). Also, three complement plasmids, one with each of the hypothetical ORFs and one with a fragment containing both, were constructed, transformed into HI5, and transformation was confirmed by PCR. As yet, a different phenotype among these three strains compared to the HI5 mutant has not been detected. They all have dimpled colony morphology and unchanged invasion rates.

The nested suppression PCR technique can be difficult and complicated, and it appears there was likely an error in this process. The HI5 mutant does carry a copy of the transposon, as PCR screens using primers specific for the transposon \textit{aph} gene indicate, but it remains to be seen exactly where it is.

Another minor but important unexpected hurdle occurred in the process of making serial dilutions to quantify both the input inocula and the output of various experiments. Unlike the more popular 101, 104 and 109 strains of \textit{M. avium}, it was eventually discovered that A5 requires the addition of a surfactant to accurately perform serial dilutions for quantification by plating. Previously, only water was used
as a dilution medium, and bacteria seemed to stick to the side of the tubes, resulting in erroneously and unpredictably low colony numbers were attained when quantifying the CFU density of mixtures with high concentrations of bacteria. The addition of 0.1% Tween solves this issue (unpublished findings, our lab).

Also in this study, a Sigma-Genosys *M. tuberculosis* DNA macroarray was utilized, comparing the wild type A5 strain with the mutant HI5. Hypothetically, if the HI5 strain did have a disruption in a repressor regulator gene, then a host of downstream genes involved in invasion would have altered expression. While the array is not specific for *M. avium*, it was expected that the high degree of sequence similarity between the two organisms should allow for differential expression to be observed using a hybridization-based measure.

However, in this case the array did not prove to be informative. In three repetitions there was no observable similarity in the array analysis results. The source of the difference and error in each is not known. If a regulatory gene knockout was not selected in the initial screen, we would expect to see roughly no change in expression between A5 and mutant HI5 for the vast majority of genes, and a different level of expression in the one or few genes knocked out by the transposon and/or involved in aminoglycoside resistance only if they are among the genes with a high level of homology between *M. avium* and *M. tuberculosis*. What was seen was roughly consistent with this hypothesis, although the presence of indicated up or downregulation (>3-fold) on two arrays, but not the third, suggests a significant amount of error was present. Those two arrays utilized cDNA synthesized from the
same RNA sample, but the genes with apparent altered transcription still did not agree among them.

One noticeable trend among the genes with the most widely variant results among the three arrays is that they were predominantly from short ORFs, less than 420bp in length. Perhaps the short gene length required the use of suboptimal oligomers by the manufacturer, resulting in a smaller margin for error.

It has been observed that kanamycin, when added to standard 7H9 with OADC broth, such as is done to culture the HI5 mutant, caused a yellowish precipitate to appear. This isn’t immediately noticeable in the presence of bacteria, but is clear when observing broth that has set for some hours after the addition of the antibiotic.

In the course of this study, during the RNA extraction process preparing for macroarray and real-time analysis, it was noticed that the yellowish color from the precipitate persisted during phenol-chloroform extraction and often was visible after alcohol precipitation. When synthesizing radio-labeled cDNA for the array, using the same amount of RNA from each strain as templates, the intensity of radioactivity of the labeled HI5 RNA sample was considerably lower, about one-half, even after multiple attempts.

Real-time PCR was used to check the results of the arrays. Three genes, one each with indicated altered transcription from each array and each being of greater than 1kb nucleotides in length, were selected. During real-time qPCR analysis, the threshold fluorescence value for the 16S baseline-control amplicon was usually about one cycle higher in the HI5 mutant compared to the wild-type, despite using equal
amounts of RNA as a template for cDNA synthesis, indicating that this cDNA synthesis also appeared to be only half as efficient using RNA derived from the mutant culture. Additionally, there was some variability in the real-time expression fold-change values for different genes from experiment to experiment.

When HI5 was cultured in broth without kanamycin, for only a few days so as to avoid significant transposon instability from lack of selective pressure, the problem was resolved. The 16S baselines were nearly identical, the fold-change results were more consistent. Going forward, this may prove to be an important observation to aid future attempts achieving expression array and real-time PCR assay reproducibility. Not incidentally, the real-time results indicated that expression for the three genes selected was roughly equal in the A5 and HI5 strains.

Another couple minor observations that may be useful going forward regard the process of complement construction. In the course of this study, three fragments, one with the \textit{Rv1722}-like gene from \textit{M. avium}, one with the \textit{Rv1723}-like gene, and one with both genes in one amplicon, were cloned into a complement plasmid containing a mycobacterial \textit{hsp60} promoter for moderate constitutive expression. Of the three, only the one with the \textit{Rv1723} homologue could be cloned quickly and easily into the plasmid. Using similar protocols, repeated many times, the other fragments simply would not clone. A fragment containing the \textit{Rv1722} fragment oriented anti-parallel to the promoter was processed in parallel with the others. This fragment was cloned into the plasmid with relative ease. Negative and positive controls were used to confirm digestion, ligation and transformation efficiency, and all were working as expected in
those groups. Both chemical and electrical transformations were attempted. However, in repeated trials plasmids could be isolated from the transformed *E. coli*, but they never had the desired insert.

One hypothesis for this occurrence is that the *Rv1722* homologue gene product is toxic to the *E. coli* shuttle. The observation that it was easily cloned antiparallel but not parallel to the promoter supports this hypothesis. Although mycobacterial promoters generally don’t function well if at all to express genes in *E. coli*, the more conserved heat-shock promoters, such as the *hsp60* promoter used in the pmv261-apr. II complement plasmid, are a possible exception (16, 41). Our chemical transformation protocol explicitly requires heat shocking. Similarly, the electrical transformation protocol recommends incubating the cold *E. coli* in a warm shaker incubator to allow time for the resistance marker to express before plating on antibiotic agar plates. Acting on the hypothesis that these procedures temporarily induced expression via the heat shock promoter, the electrical transformation was repeated with the transformant mix plated directly onto room-temperature plates. After that alteration to protocol, the fragments containing the *Rv1722* homologue gene and the combined *Rv1722-Rv1723* homologues fragment were successfully cloned.

**Outlook.** The ability of *M. avium* to sense the intestinal lumen environment and activate an invasive phenotype remains a tempting avenue for investigation, despite remaining unknown through the course of this study. A regulatory mechanism must
exist, and when some pieces are discovered, they will likely prove to provide impetus for still more fruitful studies.

The theoretical basis used to conceive the initial screen for a hyperinvasive mutant remains sound. However, if repeated in the future, it may prove beneficial to forgo the use of amikacin or other antibiotic protection in the invasion assays. This method is used to differentiate invasion rates from attachment rates, but in this search that is not absolutely necessary. In fact, it is possible that *M. avium* upregulates attachment effectors in the intestinal lumen environment, which also remains to be investigated.

While the search for a regulatory pathway did not progress as anticipated, assays for translocation *in vitro* and dissemination *in vivo* suggest the HI5 strain described in this project does have a mutant phenotype relevant to pathogenesis. These assays had relatively low sample sizes, but are statistically significant.

The process of translocation of the intestinal wall is not well-studied, in any pathogen. Some organisms may lyse the endosome, and escape by endocytosis in a different vacuole, by inducing inflammation and immune-mediated host cell lysis, or by some other mechanism. Presently, the process of escape from intestinal cell endosomes by *M. avium* has not been mapped or directly observed.

Presumably, one mechanism potentially used by *M. avium* to cross the monolayer is the host cell’s apical-to-basal side transcytosis process. However, traffic in this direction is less common than basal-to-apical, and the host’s own native effectors for traffic in this direction are not well-understood.
Dissemination is also a poorly understood process, in both *M. avium* and other bacterial pathogens, as it is generally not conducive to modeling with simple tissue culture or selection assays. In this way, a mutant deficient in translocation and dissemination may prove valuable in mapping and understanding these important aspects of pathogenesis, and merits further investigation.
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