The \textit{fadD2} Gene Is Required for Efficient \textit{Mycobacterium avium} Invasion of Mucosal Epithelial Cells

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\textbf{Objective.} \textit{Mycobacterium avium} is capable of invading the intestinal epithelial cells, which requires cytoskeleton rearrangement and protein phosphorylation in the host cell. However, little is known about the mechanism.

\textbf{Methods.} A transposon bank was screened for invasion-impaired mutants.

\textbf{Results.} Among the genes identified, inactivation of the \textit{fadD2} gene resulted in \raisebox{-.25ex}{\(\sim\)}50\% reduction in invasion in vitro and 100-fold reduction in invasion in vivo, compared with the wild-type (wt) strain. Invasion by wt \textit{M. avium} led to the recruitment of neuronal Wiskott-Aldrich syndrome protein (N-WASp), which was not observed with mutant lacking a functional \textit{fadD2} gene. \textit{M. avium} entry resulted in the phosphorylation of N-WASp and activation of the Arp2/3 complex. Supernatant obtained from wt \textit{M. avium} incubated with HEp2 epithelial cells rescued the mutant 1B2 ability to enter the cells, which suggests that activation of Cdc42 probably follows the secretion of \textit{M. avium} proteins.

\textbf{Conclusion.} \textit{fadD2} is a regulator of \textit{M. avium} invasion, and its effect is through Cdc42.

Organisms of the \textit{Mycobacterium avium} complex (MAC) are commonly encountered in the environment [1]. The bacterium is a common cause of opportunistic infection in patients with AIDS, whereas, in patients without AIDS, the infection is usually secondary to an underlying chronic lung disease. In persons with AIDS, the majority of \textit{M. avium} infections are acquired through the intestinal tract [2]. Earlier, it was shown that \textit{M. avium} is able to bind to and invade epithelial cells in vitro [3, 4] and that, in mice, \textit{M. avium} invades preferentially the terminal ileum mucosa [5]. After invasion of epithelial cells, \textit{M. avium} suppresses the production and secretion of such chemokines as interleukin-8 and RANTES [6]. This characteristic of the organism is associated with the absence of an inflammatory response early during infection [7].

A few mechanisms for \textit{M. avium} invasion have been suggested. Studies have proposed, for example, that \textit{M. avium} interacts with the intestinal mucosal cells by using tissue fibronectin to bind to an integrin receptor [8]. A fibronectin attachment protein (FAP) has been cloned, and antibodies against FAP prevented the association of \textit{M. avium} with bladder epithelial cells [8]. Another protein identified to bind the epithelial-cell surface is the 27-kDa \textit{M. avium} protein, which shares identity with a 27-kDa protein of \textit{M. tuberculosis} and Mn superoxide dismutase (SOD) [9, 10].

After \textit{M. avium} binds to enterocytes, cytoskeleton rearrangement is required for the invasion of epithelial cells [3]. However, the signal-transduction pathways that lead to actin polymerization and \textit{M. avium} invasion are not well defined. The role of the Rho family of small GTPases, acting as modulators between membrane receptors and the downstream effectors involved in regulating actin dynamics, during the invasion of a pathogen into the host cell has been established for various organisms [11–14]. The involvement of Rho GTPase Cdc42 in actin organization occurs through neuronal Wiskott-Aldrich syndrome protein (N-WASp) and the Arp2/3 complex. The N-WASp family provides focal points at which multiple signals converge and regulates...
the reorganization of actin through a common effector Arp2/3 complex [15]. A number of pathogens have recently been reported to activate these pathways during entry into the host cell [14, 16, 17].

To identify the bacterial genes involved in the process of epithelial-cell invasion, we screened an M. avium transposon mutant library for clones with impaired invasion. Inactivation of the fadD2 gene excluded the involvement of the Cdc42 signaling pathway during M. avium invasion.

MATERIALS AND METHODS

Reagents. Unless otherwise stated, all reagents were obtained from Sigma Chemical. Mycobacteria were cultured on 7H9 Middlebrook broth or 7H10 Middlebrook agar (Difco Laboratories). Luria-Bertani medium (Difco Laboratories) was used for the culture of Escherichia coli. ECL chemiluminescence kits were purchased from Amersham Pharmacia Biotech UK.

Cell culture. HEP-2 cells (laryngeal cells) were obtained from American Type Culture Collection and cultured in RPMI 1640 medium (BioWhittaker) supplemented with 10% heat-activated fetal bovine serum (Gemini BioProduct) with 2 mmol/L glutamine. The cells were used for passages 2–10 [3]. Chambered slides were purchased from Nunc, and cells were cultured as described elsewhere [18].

Bacteria. M. avium strain 109 (serovar 4) is a clinical isolate obtained from the blood of a patient with AIDS. Mutants were generated as described below. For the experiments, bacteria were grown in 7H10 Middlebrook agar enriched with 10% oleic acid albumin dextrose catalase (Difco) for 10 days and either washed with Hanks’ balanced salt solution (HBSS) and used or inoculated in 7H9 Middlebrook broth for an additional 5 days to achieve the logarithmic phase of growth [3, 6, 9, 18].

Antibodies and restriction enzymes. The monoclonal antibodies (MAbs) anti-paxillin, anti-ezrin, and anti-cortactin were purchased from Zymed. The polyclonal antibodies anti-N-WASp and anti-Arp2 were obtained from Santa Cruz Biotechnology. Secondary antibodies conjugated with horseradish peroxidase (HRP)/fluorescein isothiocyanate and Texas Red were purchased from Amersham Pharmacia Biotech and Sigma Chemical. The restriction enzyme EcoRI was purchased from Life Technologies. Taq polymerase and Pfu polymerase were obtained from Invitrogen. Oregon Green (Phalloidin-514) was obtained from Molecular Probes.

Generation of transposon mutants and screening for impaired invasion. A transposon mutant library was created in M. avium strain 109 by using the temperature-sensitive phage phAE94, which contains the transposon Tn5367 harboring a kanamycin-R–resistant gene, as described elsewhere [19]. Approximately 3000 clones were screened individually for the ability to invade epithelial cells. Wild-type (wt) M. avium 109 was used as the control in invasion assays. Mutants with an efficiency of invasion of ≤1% (vs. 2.5% ± 0.4% of the wt bacterium) for 1 h were selected as poor invaders for further study [3, 18]. Briefly, HEP-2 cells grown to 80% confluence were infected with wt M. avium 109 and mutants for 15 m, 30 m, 1 h, and 2 h at 37°C in 10% CO₂. The epithelial-cell monolayers were then washed 2 times and incubated with amikacin (200 µg/mL) for 2 h at 37°C to kill the extracellular bacteria [3, 6]. Kanamycin resistance does not affect susceptibility to amikacin. The medium was then removed, and the monolayer was washed once with HBSS and lysed with 1.0% Triton X-100 for 30 min. The cell lysate was diluted and plated onto 7H11 Middlebrook agar. The efficiency of invasion of mutants and wt...
Table 1. Strains and plasmids used in the study.

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAC 109</td>
<td>Wild type</td>
<td>5</td>
</tr>
<tr>
<td>1B2</td>
<td>Derivate of MAC 109, Ma fadD2::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>6F9</td>
<td>Derivate of MAC 109, Ma 1280::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>2D10</td>
<td>Derivate of MAC 109, Ma 0020::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>8E10</td>
<td>Derivate of MAC 109, Ma 0271::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>2G3</td>
<td>Derivate of MAC 109, Ma 3529::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>7E2</td>
<td>Derivate of MAC 109, Ma sodC::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>8D1, 2D6</td>
<td>Derivate of MAC 109, Ma 0018::Kan</td>
<td>Present study</td>
</tr>
<tr>
<td>Plasmid pMV261-Apr II</td>
<td>Kanamycin and apramycin resistance</td>
<td>Present study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> XL1-blue</td>
<td>Host strains for general</td>
<td>Stratagene</td>
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**NOTE.** MAC, *Mycobacterium avium* complex.

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bacterium was calculated as the percentage of the inoculum that entered the cell in a determined period of time (table 1).

**Mice infection.** Female C57BL/6 mice, 8 weeks old, were infected orally with 200 μL of bacterial suspension (1 × 10⁷/mL), as described elsewhere [5]. Two mutants (1B2 and 6F9) were used for the experiments. The clones were evaluated for acid resistance, as reported elsewhere [20], and were found to behave like the wt bacterium (data not shown). The wt MAC 109 strain was used as a control. After 4 h, mice were killed, and the intestinal segment corresponding to the terminal ileum was harvested, cut open, washed in HBSS, homogenized, and plated onto a 7H11 Middlebrook agar plate for the determination of the number of bacteria associated with mucosa, as described elsewhere [5, 21]. Five mice were used in each group, and the experiment was repeated 5 times.

**Microscopy.** To detect host-cell proteins that colocalize with *M. avium*, we stained the proteins involved in signal-transduction pathways. Monolayers were infected at a bacteria-to-cell ratio of 10:1. Invasion was performed for 1 h at 37°C. Extracellular bacteria were removed by washing the monolayers 3 times with HBSS. Cells were fixed with 2% paraformaldehyde for 30 min and permeabilized with 0.1% Triton X-100 for 15 min. Cells were incubated with specific primary antibody (dilution, 1:40)—mouse anti-paxillin, anti-ezrin, anti-cortactin, anti–N-WASp, and anti-Arp2—for 1 h at room temperature. The monolayer was washed 3 times and incubated with the secondary antibodies (rabbit anti-mouse) for 1 h at room temperature. Non-specific antibody from the same isotype was used as a control. Actin filaments were stained by incubating the monolayer with Oregon Green at a 1:10 dilution (Molecular Probes). We used a Nikon microscope equipped with an optronics camera and a video microscopy device.

**Immunoprecipitation and Western blotting.** HEp-2 cells (80% confluence) were infected with *M. avium* (MAC 109) or mutants at 37°C. After 15 min, 30 min, 1 h, and 2 h, monolayers were washed with HBSS and lysed with sterile water in the presence of 0.05 mol/L phenylmethylsulfonyl fluoride in isopropanol. Uninfected HEp-2 cells were used as a control. The lysate was passed through a 21-gauge needle 10 times and placed on ice for 1 h. Subsequently, it was centrifuged at 6000 g for 5 min. The supernatant was collected for further work. Protein estimation in the supernatant was performed using the Bradford method. An equal amount of protein from each lysate was used for immunoprecipitation. Mouse MAbs to paxillin, phosphotyrosine, and N-WASp were added to the lysate. Cross-linked antibody was immobilized with protein A/G beads. Immune precipitates were washed several times with PBS, and the captured antigen was extracted in 1× SDS buffer, separated on a 12% SDS gel, and submitted to electrophoresis at 120 V for 2 h. The protein(s) were transferred to a nitrocellulose membrane.

Table 2. Genes identified as playing a role in *Mycobacterium avium* invasion of epithelial cells.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Gene homologous to <em>M. tuberculosis</em></th>
<th>Similarity/identity, %</th>
<th>Description/function (% invasion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B2</td>
<td>Rv0270</td>
<td>82/80</td>
<td>Acyl CoA Synthase/unknown (0.8)</td>
</tr>
<tr>
<td>6F9</td>
<td>Rv1280</td>
<td>83/82</td>
<td>Periplasmic solute binding protein/unknown (0.81)</td>
</tr>
<tr>
<td>8E10</td>
<td>Rv0271</td>
<td>93/77</td>
<td>Acyl CoA dehydrogenase/unknown (0.9)</td>
</tr>
<tr>
<td>2G3</td>
<td>Rv3529</td>
<td>82/78</td>
<td>Hypothetical protein/unknown (0.65)</td>
</tr>
<tr>
<td>2D10</td>
<td>Rv0020</td>
<td>89/64</td>
<td>Hypothetical protein/unknown (0.5)</td>
</tr>
<tr>
<td>1D6, 8D1</td>
<td>Rv0018</td>
<td>81/79</td>
<td>Highly similar to phosphoprotein phosphatase/unknown (0.72); transposon inserted in different sites of the gene in the 2 mutants</td>
</tr>
<tr>
<td>7E2</td>
<td>Rv0432</td>
<td>92/90</td>
<td>SodC (0.84)</td>
</tr>
</tbody>
</table>

**NOTE.** CoA, coenzyme A; Sod, superoxide dismutase.
was digested with EcoRI and HindIII and cloned into pMV261-Apr II. The pMV261-Apr II plasmid has the apramycin gene in place of the kanamycin gene of the pMV261 plasmid. The pMV261-Apr II plasmid with the inserted DNA was used to transform competent cells made from the 1B2 mutant. Transformed clones were grown on 7H10 Middlebrook agar plates that contained 200 μg/mL apramycin. The screening of the clones was performed by PCR using primers for the apramycin gene: upstream, 5′-TTAGAATTCGGTGTTTGACTTCGGAGC-3′, and downstream, 5′-TAGGTACGACTCGGTCTGTTGA-3′.

Statistical analysis. Each experiment was performed at least in triplicate and repeated at least 3 times, and means ± SDs were determined. The significance of the results between control and experimental groups was determined by Student’s t test. P < .05 was considered to be significant.

RESULTS

Identification of invasion-impaired mutants. We screened 3000 clones for their ability to invade HEp-2 cells. Twenty-four clones showing < 1.0% efficiency of invasion, compared with the 2.5% efficiency of the wt bacterium in vitro, were selected. Two clones (1B2 and 6F9) of the 24 identified were further evaluated in mice (oral infection) and showed to have an ~100-fold decrease in efficiency of invasion, compared with M. avium 109 (figure 1A and 1B). To rule out susceptibility to the acidic environment of the stomach, the mutants were evaluated for acid resistance, compared with that of the wt bacterium. All the mutants behaved like the wt bacterium (data not shown). None of the clones had impaired growth in vitro, compared with the wt bacterium (data not shown).

Invasion-associated genes. The sequence of 8 genes associated with M. avium invasion of epithelial cells (clones 1B2, 6F9, 8E10, 2G3, 2D10, 1D6, 8D1, and 7E2) was obtained by PCR amplification of the region containing the transposon Tn5367. All of the mutants had 1 copy of the transposon identified by Southern blot (data not shown). We compared the DNA sequence of the PCR product encoding the flanking region of the gene inactivated by the transposon with the pub
Phosphorylation of different signal-transduction intermediates has been described as a commonly used molecular switch during invasion by different enteropathogenic organisms [24–27]. In Salmonella species, the induction of tyrosine phosphorylation of host-cell mitogenic activating protein kinase has been shown during the invasion of epithelial cells [25]. EPEC induces the phosphorylation of host-cell proteins associated with actin polymerization during invasion. In vivo modific-
Figure 5. Restoration of wild-type phenotype in invasion mutants by using supernatant from *Mycobacterium avium* (MAC) 109 incubated with HEp-2 cells. Supernatant was obtained as described in Materials and Methods and added to the bacteria before the assay. The supernatant had no effect on invasion of the 7E2 mutant. Boiling the supernatant abolished its ability to increase invasion of mutant strains.

tions of the small GTPases Rac and Cdc42 by *Bordetella* species have been reported in recent years [28]. In the present study, invasion of *M. avium* was associated with N-WASp phosphorylation and actin polymerization; however, the role of phosphorylation to initiate actin polymerization is not known. WASp and N-WASp family members provide focal points downstream of Cdc42, where multiple signals generated by extracellular stimuli converge. Moreover, because Cdc42 has been shown to be involved in *M. avium* invasion, it may be possible that a bacterial product that behaves like guanine exchange factor initiates Cdc42 activation. For instance, *Salmonella typhimurium* activates N-WASp and transfers the signal to the actin cytoskeleton [17].

Recent studies have shown that interaction of Arp2/3 complex with the cytoskeleton needs N-WASp [15, 29]. In our experiments, we that observed binding of the N-WASp molecule to Arp2/3 during invasion by *wt M. avium* was not seen in cells infected with the mutant 1B2, which does not use the Cdc42 pathway [30, 31].

Bacteria use complex secretory systems involving the secretion of several proteins (including phosphatases) that facilitate the bacterial internalization into host cells [32, 33]. A number of these proteins are delivered to the host cell by "injectosomes," which are needle-like structures produced by gram-negative bacteria. Several Sip proteins from *Salmonella* species and Ipa from *Shigella* species are examples of proteins that are injected into the bacteria. Phosphatases from *Salmonella* species, to cite a few, have been reported as secreted products with important roles during the invasion process [34–36]. Phosphatase secreted by *Yersinia* species, however, inhibits entry into eukaryotic cells [35]. In the present study, restoration of the *wt* phenotype in several mutant strains with the supernatant collected from the HEp-2 cells exposed to *wt M. avium* 109 suggests the presence of secreted proteins or lipids with roles in *M. avium* uptake. In recent years, several investigators have demonstrated the secretion of lipids by mycobacteria, when both inside and outside cells [37, 38]. However, boiling of the supernatant abolished the effect on invasion, which supports the participation of proteins. No type III secretion system has been identified in mycobacteria. It is possible that these proteins are internalized by different mechanisms or that the proteins trigger a response after binding to a membrane receptor. Using the genomic approach to predict the secreted protein by comparing it with other secreted proteins in other bacteria may not yield significant information, because not all secreted proteins in mycobacteria contain classical signal peptides. However, other approaches, such as DNA array using RNA from *wt bacterium* and mutants, might provide significant information.

It has been shown that SODs are involved in protecting cells from free radicals. SODs are secreted in *M. tuberculosis* and *M. avium* [39, 40]. The role of the SOD in the mechanism of *M. avium* uptake is still unknown and deserves future study. Re-
cent work has proposed that SodC binds to a receptor on the host cell, before entry [10]. However, the proof for the role of SodC as an adhesin will need to wait for further studies.

We observed that the fadD2 gene has a significant role during M. avium invasion of HEp-2 epithelial cells. fadD2 encodes a fatty acyl synthase and is involved in the degradation of fatty acids. The fadD2 gene is located between 2 genes in opposite orientation. This suggests that fadD2 is not directly involved in invasion but, rather, that it regulates other genes. In Salmonella species, the role of fadD has been established as a hilA regulator during invasion [41, 42]. The expression of FadD protein leads to the degradation of fatty acids and the release of products that activate hilA. It is possible that fadD2 is a regulator in M. avium and, as in Salmonella species, that is controls the expression of secreted proteins. Future studies will be aimed at identifying the proteins regulated by this mechanism.

The other mutants identified in the present study had inactivation of different genes. The products of these genes are homologous to the products of several M. tuberculosis genes—for example, Acyl CoA synthase (fadE6), periplasmic solute-binding protein (oppA), ppp similar to phosphoprotein phosphatases, and Rv0020 (a gene of unknown function). The mutants of the genes homologous to M. tuberculosis Rv0018 (2D10 and 1D6 mutants) and Rv0020 (8D1 mutant) belong to an operon in both M. avium and M. tuberculosis, which contains a phosphatase (Rv0019). The role of different genes present in this particular operon in M. avium needs to be investigated further. We are presently attempting to generate allelic exchange mutants in that specific operon.

The study of the regulation of fadD2 and its role in M. avium invasion will certainly generate new knowledge about interactions between host and pathogen. At least some of the genes regulated by fadD2 are likely to be involved in the invasion of epithelial cells.

Acknowledgments

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References


