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Phenotypic and Genomic Analyses of the *Mycobacterium avium* Complex Reveal Differences in Gastrointestinal Invasion and Genomic Composition

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***Mycobacterium avium* and *Mycobacterium intracellulare* are closely related organisms and comprise the *Mycobacterium avium* complex. These organisms share many common characteristics, including the ability to cause life-threatening respiratory infections in people with underlying lung pathology or immunological defects and occasionally in those with no known predisposing conditions. However, the ability to invade the mucosa of the gastrointestinal tract and cause disseminated disease in AIDS patients has not been epidemiologically linked to *M. intracellulare* and appears to be unique to *M. avium*. We compared the abilities of *M. avium* and *M. intracellulare* to tolerate the acidic conditions of the stomach, to resist the membrane-disrupting activity of cationic peptides, and to invade intestinal epithelial cells in vitro and in vivo. We observed that *M. avium* and *M. intracellulare* were both tolerant to the acidic conditions encountered in the stomach and resistant to cationic peptides. However, when strains of *M. avium* and *M. intracellulare* were examined for their ability to enter cultured human intestinal cells or mouse intestinal mucosa, we observed that *M. avium* could invade more efficiently than *M. intracellulare*. To elucidate the basis of this pathogenic difference and identify genes involved in the invasion of the intestinal mucosa, we performed chromosomal DNA subtractive hybridization using *M. avium* and *M. intracellulare* chromosomal DNAs. In all, 21 genes that were present in *M. avium* but absent in *M. intracellulare* were identified, including some that may be associated with the ability of *M. avium* to invade the intestinal mucosa.**

Mycobacterial diseases have been known for over 1,000 years and still pose serious health problems in both industrialized and underdeveloped parts of the world (34). Although *Mycobacterium tuberculosis* is the most aggressive species of this genus, infecting over one-third of the world's population and causing the death of more people than any other single infectious agent (8), other mycobacteria also cause serious disease. For example, the *Mycobacterium avium* complex (MAC) organisms are nontuberculosis mycobacteria associated with life-threatening infections in people with underlying lung pathology (e.g., chronic obstructive lung disease) or immune system defects (e.g., AIDS) and, on occasion, in those without apparent predisposing conditions. MAC consists of two closely related but genetically distinct species (*M. avium* and *Mycobacterium intracellulare*) that have been grouped into 21 serotypes, with serotypes 1 to 6, 8 to 11, and 21 designated *M. avium* and serotypes 7 and 12 to 20 designated *M. intracellulare* (2, 9, 33).

M. avium and *M. intracellulare* have both been isolated from AIDS patients but differ in the frequency of isolation, the route of infection, and the type of disease caused. Both organisms have been isolated from the sputa of AIDS patients with active pulmonary disease (24, 39); however, *M. avium* is by far the more common of the two organisms to infect this population (4, 39). In AIDS patients, *M. avium* is acquired predominantly via the gastrointestinal tract, where it is able to invade and translocate the intestinal mucosa, infect and multiply within submucosal macrophages, and cause bacteremia leading to the dissemination of the organism to the liver, spleen, and bone marrow (13). It is not clear if *M. intracellulare* can also infect the gastrointestinal tracts of AIDS patients, but to date no report of this route of infection has been documented. To elucidate the nature of this phenotypic difference, we examined the abilities of both species to survive the acidic conditions encountered in the stomach, to resist cationic peptides associated with the antimicrobial defense of the gastrointestinal tract, and to invade intestinal epithelial cells in vitro and in vivo. We also compared the genomes of these species via genomic DNA subtractive hybridization (1, 10, 15, 28) and identified several possible virulence factors unique to *M. avium*.

MATERIALS AND METHODS

Bacterial strains, epithelial cell lines, plasmids, and growth conditions. *M. avium* strains 101, 104, and 109 were isolated from the blood of AIDS patients. *M. intracellulare* strains 83-8705, 84-8739, and 86-8953 were clinical isolates provided by Robert Good (Centers for Disease Control and Prevention, Atlanta, Ga.). *M. intracellulare* ATCC 13950 was acquired from the American Type Culture Collection (Manassas, Va.). *Mycobacterium smegmatis* mc^2155 was the kind gift of W. Jacobs. HEp-2 human pharyngeal epithelial cells and HT-29 human intestinal epithelial cells were obtained from the American Type Culture Collection and grown as described previously (6). Mycobacteria were cultured on Middlebrook 7H11 agar supplemented with oleic acid, albumin, dextrose, and catalase (OADC) (Difco, Detroit, Mich.) at 37°C. For invasion assays, transparent colonies of *M. avium* or *M. intracellular* were transferred to Middlebook 7H9 broth supplemented with OADC, grown to log phase, washed in Hanks' buffered salt solution (HBSS), and vortexed and agitated for 2 min to disperse any clumps. The bacterial suspension was allowed to sit for 5 min, and the top 1 ml was

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removed and stained by the Ziehl-Neelsen method to confirm that the suspensions contained dispersed bacterial cells. Bacteria were plated in triplicate onto agar plates and enumerated for each experiment. *Escherichia coli* XL1Blue MRF′ (Stratagene, La Jolla, Calif.) was cultured onto Luria broth supplemented with kanamycin (50 μg/ml) or ampicillin (25 μg/ml) when appropriate. DNA fragments obtained by subtractive hybridization were cloned into pCR2.1 (Invitrogen, Carlsbad, Calif.).

Acid tolerance assay. Mycobacteria were grown to a cell density of approximately 10^5 bacteria/ml in 7H9 broth (pH 6.8) supplemented with OADC. One milliliter of this culture was centrifuged, and the pellet was suspended in 7H9 broth adjusted to either pH 2.2 or 6.8 with 1 N HCl. The suspensions at pH 2.2 were incubated at 37°C for 2 or 24 h and serially diluted in HBSS, and viable bacteria were quantified by heterotrophic plate counts. The suspensions at pH 6.8 were immediately quantified to obtain the number of viable bacteria at the beginning of the experiment. The percent survival was determined by dividing the number of bacteria present at the end of the experiment by the number of bacteria present at the beginning of the experiment and multiplying by 100.

Polymyxin B resistance assay. The MICs of polymyxin B for *M. avium*, *M. intracellulare*, and *M. smegmatis* were identified by inoculating ca. 10,000 bacteria into 3 ml of 7H9 broth supplemented with OADC and polymyxin B, ranging in concentration from 0.5 to 500 μg/ml in twofold increments. The cultures were incubated at 37°C for 10 days and examined for growth by visual turbidity. The lowest concentration of polymyxin B that did not support growth was considered the MIC.

In vitro invasion assay. To evaluate the ability of *M. avium* and *M. intracellulare* to invade epithelial cells in vitro, we carried out invasion assays using HEp-2 (pharyngeal) and HT-29 (intestinal) epithelial cell lines as described previously (6).

Intestinal invasion assay. To determine the relative abilities of *M. avium* and *M. intracellulare* to invade the intestinal mucosa, we used a mouse intestinal loop model. C57BL/6 bg^+/bg^- black mice (female, 6 to 8 weeks old, weighing an average of 20 g) were obtained from Jackson Laboratories (Bar Harbor, Maine) and used after 1 to 2 weeks of quarantine. Mice were anesthetized using intraperitoneally administered phenobarbital and halothane by inhalation, which maintained them under anesthesia throughout the procedure. An incision in the abdominal cavity was made, and a segment of the small intestine ca. 3 cm in length above the ileo-cecal area was tied at both ends with a suture tight enough to close the intestinal lumen but not tight enough to restrict blood flow. Approximately 10^5 bacteria in HBSS were injected into the proximal portion of the segment, and the animals were maintained for 1, 1.5, and 2 h before the segment was removed, opened longitudinally, and washed extensively with HBSS to remove unbound bacteria. Washed intestines were placed in 5 ml of 7H9 broth, homogenized, serially diluted, plated onto 7H11 agar supplemented with antibiotics inhibitory to the intestinal biota but not the mycobacteria (polymyxin B, 5 μg/ml; amphotericin B, 4.5 μg/ml; carbenicillin, 20 μg/ml; and trimethoprim, 2.0 μg/ml), and quantified for mycobacteria via heterotrophic plate counts.

General molecular techniques. Plasmid DNA was prepared using the Quigen Plasmid Mini Kit (Qiagen, Valencia, Calif.). Genomic DNA was prepared as described previously (31). Digoxigenin (DIG)-labeled probes were generated using the DIG Chem-Link System (Boehringer Mannheim, Indianapolis, Ind.) as per the manufacturer's instructions. Southern blot hybridization was performed as described previously (31). PCR was performed using the GC Rich Kit (Roche Diagnostics, Indianapolis, Ind.). Nucleotide sequence analysis was performed at the University of California at San Francisco Biomolecular Resource Center.

Genomic DNA subtractive hybridization. *M. intracellulare* genomic DNA was sheared to an average size of 2 to 5 kbp using a sonicator (Heat Systems-Ultrasonics Inc., Plainview, N.Y.) and DIG labeled as described above. *M. avium* strain 104 genomic DNA was digested with *Sau*3AI to produced fragments of ca. 200 to 500 bp. One microgram of *M. avium* DNA was mixed with 10 μg of *M. intracellulare* DNA in subtraction buffer (50 mM HEPES [pH 7.5], 0.5 M NaCl, 0.1% sodium dodecyl sulfate, 1 mM EDTA) and denatured in a thermal cycler (Hybaid, Middlesex, United Kingdom) at 95°C for 45 min. The temperature was lowered at a rate of 4°C/h until the mixture reached 40°C. The mixture was removed and allowed to cool to room temperature. Anti-DIG antibodies bound to magnetic particles (Boehringer Mannheim) were added, and the *M. intracellulare* DNA fragments, along with any *M. avium* fragments annealed to them, were removed using a magnet. The subtraction protocol was repeated five more times, after which any remaining *M. avium* sequences were ligated into M13 reverse *Sau*3AI adapters consisting of equimolar amounts of GATCCAGG AAACAGCTATGAC and GTCATAGCTGTTTCCTG. The ligation products were then ethanol precipitated to remove unligated adapters and PCR amplified using the M13R primer. PCR products were ligated into pCR2.1 and transformed into *E. coli* XL1Blue MRF′. Genomic DNA fragments generated by the subtraction protocol were used as probes in Southern hybridization experiments against *M. avium* and *M. intracellulare* genomic DNAs to ensure that they hybridized with *M. avium* but not *M. intracellulare*.

TABLE 1. Mycobacterial acid tolerance and polymyxin B resistance

Species	Acid tolerance[a] (mean % survival ± SD)		Polymyxin B resistance[b] (MIC, μg/ml)
	2 h	24 h	
M. avium	91 ± 0.7	34 ± 9.1	>500
M. intracellulare	48 ± 2.8	17 ± 6.3	>500
M. smegmatis	40 ± 2.9	<1 ± 0.6	32

[a] Bacteria were exposed to pH 2.2 for 2 and 24 h as described in Materials and Methods.
[b] The MIC of polymyxin B was assayed as described in Materials and Methods.

Electron microscopy. Portions of the inoculated and control mouse ileal loops described above were cut into 1-mm pieces and fixed in ice-cold 1% glutaraldehyde in phosphate buffer for 1 h. The segments were immersed in 1% OsO_4 for 1 h at room temperature, dehydrated through a graded ethanol series, embedded in L. R. White resin, and polymerized at 52°C. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined with an electron microscope.

Nucleotide sequence accession numbers. The nucleotide sequences of subtracted *M. avium* DNA products can be obtained from GenBank under accession numbers AF320114, AF320115, AF320116, AF320117, AF320118, AF320119, AF320120, AF320121, AF320122, AF320123, and AF321121.

RESULTS

Acid tolerance. To simulate the acidic conditions encountered in the stomach, we exposed cultures of *M. avium*, *M. intracellulare*, and *M. smegmatis* to pH 2.2 for 2 and 24 h (Table 1). All cultures exposed to pH 2.2 showed a decrease in viable cell numbers at both 2 and 24 h. *M. avium* was the most acid tolerant species, showing 91 and 34% survival after 2 and 24 h, respectively. *M. intracellulare* was intermediately acid tolerant, showing 48 and 17% survival, and *M. smegmatis* was the most acid sensitive, showing only 40 and <1% survival after 2 and 24 h, respectively.

Resistance to polymyxin B. Polymyxin B is a small cationic lipoprotein that has been used to model the membrane-disrupting activities of many cationic peptides produced by intestinal epithelial cells (5). The level of resistance to polymyxin B was determined for *M. avium*, *M. intracellulare*, and *M. smegmatis* at concentrations ranging from 0.5 to 500 μg/ml (Table 1). *M. smegmatis* was most susceptible to polymyxin B, which had an MIC of 32 μg/ml. *M. avium* and *M. intracellulare* were both resistant to polymyxin B and grew in medium containing >500 μg of polymyxin B per ml.

Invasion of cultured epithelial cells. The ability of *M. avium* and *M. intracellulare* to invade cultured human epithelial cells was assayed using pharyngeal (HEp-2) and intestinal (HT-29) epithelial cells (Table 2). The percentages of *M. avium* and *M. intracellulare* that were able to invade the pharyngeal cells after 1 h were not significantly different (within twofold). However, when we compared the abilities of these strains to invade intestinal epithelial cells, we observed that *M. avium* was able to invade significantly more efficiently after 1 h (>5-fold).

Invasion of the intestinal mucosa. The ability of *M. avium* and *M. intracellulare* to interact with the intestinal mucosa was assayed in a mouse intestinal loop model. Approximately 10^5 bacteria were injected into a 3-cm segment of the intestine above the ileo-cecal area that was closed via two suture lines that blocked flow through the intestinal lumen but did not

TABLE 2. In vitro invasion assays

Species	Strain	Mean inoculum (10^6) ± SD	No. of repetitions	Invasion (mean % inoculum ± SD)[a]	
				HEp-2 cells	HT-29 cells[b]
M. avium	101	2.4 ± 0.3	10	3.6 ± 0.4	2.9 ± 0.2
	104	4.1 ± 0.2	10	3.2 ± 0.3	3.3 ± 0.1
	109	3.4 ± 0.2	10	3.1 ± 0.4	2.8 ± 0.4
M. intracellulare	83-8705	5.7 ± 0.3	3	2.5 ± 0.4	0.5 ± 0.07
	84-8739	3.1 ± 0.3	5	1.6 ± 0.2	0.4 ± 0.08
	86-8953	4.1 ± 0.2	5	2.7 ± 0.4	0.3 ± 0.03

[a] The assay was performed as described in Materials and Methods. Invasion was allowed to occur for 1 h.
[b] $P < 0.05$ for all comparisons between *M. avium* and *M. intracellulare* invasion of HT-29 intestinal cells.

hinder blood circulation. *M. avium* was able to invade the intestinal mucosa and reached $>6 \times 10^3$ bacteria/g of intestinal tissue after 1 h (Fig. 1). The number of bacteria in the intestinal tissues rapidly increased and reached $>3.9 \times 10^4$/g of intestinal tissue after 2 h. *M. intracellulare* was substantially less efficient in invading the intestinal mucosa and reached only 1.4×10^2 bacteria/g of intestinal tissue after 1 h (>40-fold fewer than *M. avium*) and only 1.7×10^3 bacteria/g of intestinal tissue after 2 h (>22-fold fewer than *M. avium*). To ensure that *M. avium* was invading the mucosal cells and not just adhering to them, we performed transmission electron microscopy of representative samples after 1 h and observed that *M. avium* was either in the process of invading the cells or was already intracellular (Fig. 2). Although we believe that the vast majority of the bacteria counted in these assays were intracellular, we cannot exclude the possibility that some were extracellular.

Subtraction of *M. avium* genomic DNA with DNA from *M. intracellulare*. We hypothesized that *M. avium* contains genes, absent in *M. intracellulare*, that allow it to efficiently invade the intestinal mucosa. To identify these genes, we performed genomic DNA subtractive hybridization of *M. avium* strain 104 genomic DNA with *M. intracellulare* ATCC 13950 genomic DNA as described in Materials and Methods and diagrammed in Fig. 3. To ensure that DNA fragments obtained via this protocol were not present in *M. intracellulare*, we performed Southern analysis under conditions of low stringency with both *M. avium* and *M. intracellulare* genomic DNAs, which confirmed that the fragments were unique to *M. avium* (data not shown). *M. avium* genomic DNA was also subtracted against itself and PCR amplified, but no products were produced (data not shown).

Identification of *M. avium*-specific genes. To identify the genes that the subtracted DNA fragments came from, we compared their sequences to the published *M. avium* genome sequence using the BLAST program (http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism=m_avium). In all, we identified 21 genes present in *M. avium* that were absent in *M. intracellulare* (Table 3). The JAM1 and JAM2 products are homologous to the products of the *M. tuberculosis* genes Rv0227 and Rv0226, respectively. These genes are predicted to be in an operon but have no known function. The JAM3, JAM6, and JAM14 products are homologous to *M. tuberculosis*

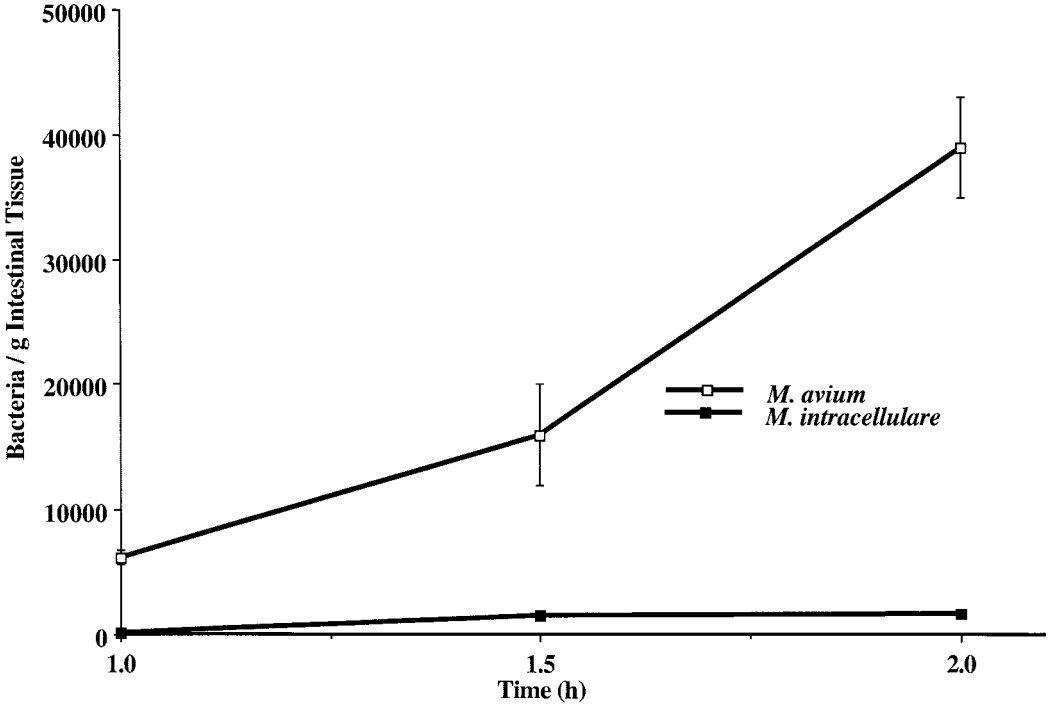

FIG. 1. Invasion of the mouse intestinal mucosa by *M. avium* or *M. intracellulare*. Ligated ileal loops were inoculated with ca. 10^5 CFU of *M. avium* or *M. intracellulare* and left for 1, 1.5, or 2 h, after which the loops were removed and quantified for mycobacteria as described in Materials and Methods. The experiment was repeated five times, and the data shown are the average numbers of bacteria per gram of intestine. For the comparison between *M. avium* and *M. intracellulare*, the *P* value was <0.001. Error bars represent standard deviations from five experiments; the error bars for *M. intracellulare* are too small to be visible.

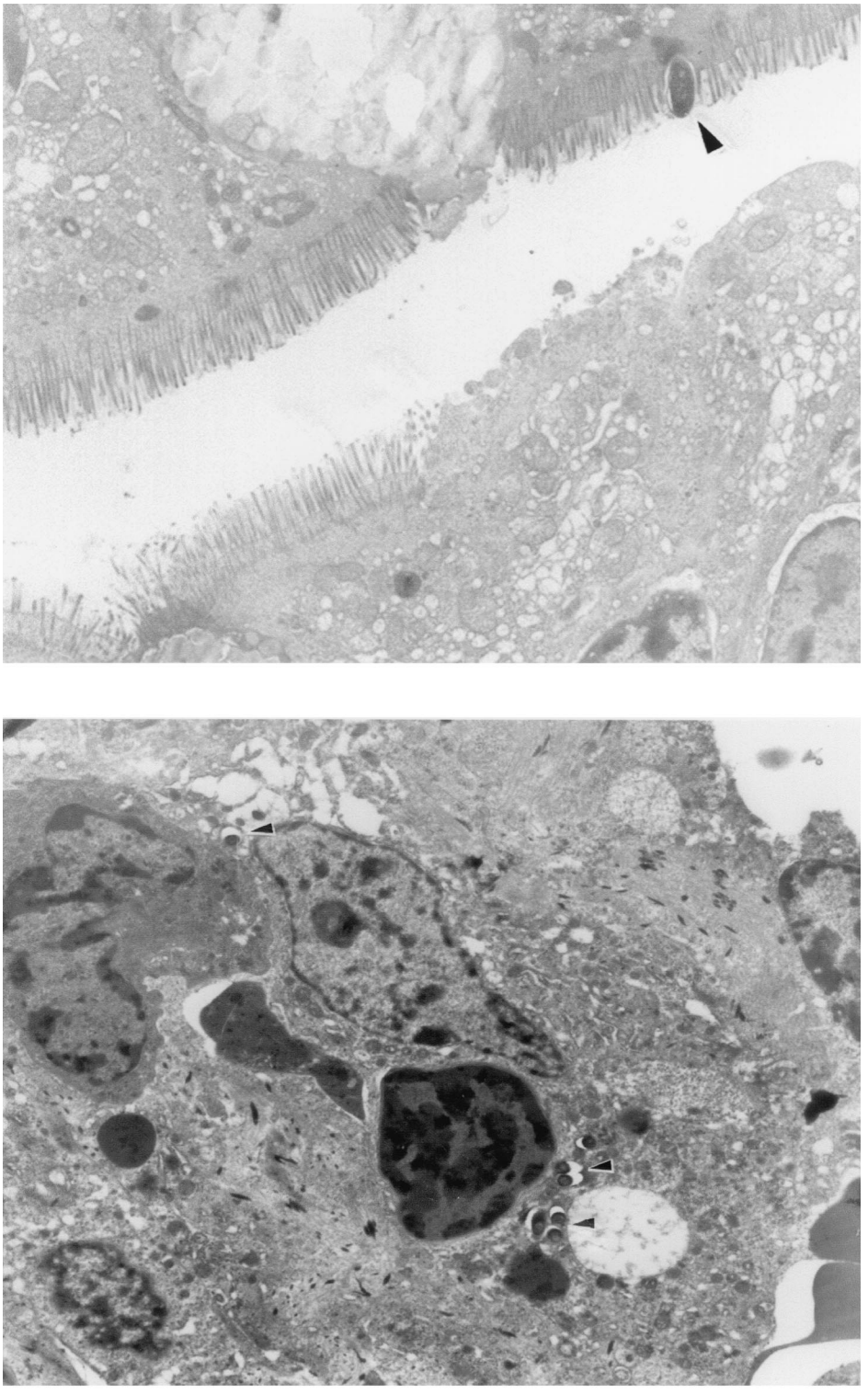

FIG. 2. Invasion of mouse intestinal tissues by *M. avium*. Mouse ligated ileal loops were inoculated with ca. 10^5 CFU of *M. avium* and left for 1 h, after which the ileal loops were removed, washed extensively, and prepared for electron microscopy as described in Materials and Methods. (Top) *M. avium* in the brush border of an enterocyte. (Bottom) *M. avium* inside an enterocyte. Arrowheads indicate bacterial cells.

PPE proteins that have no known function. The JAM4 product has homology with Zwf of *M. tuberculosis*, one of three glucose-6-phosphate dehydrogenase (G6PD) isoenzymes. The JAM5 product has homology with the *M. tuberculosis* protein encoded by Rv0106, which has no known function, and YciC, a membrane-bound protein that is involved in zinc uptake in *Bacillus subtilis* (18). The JAM7 product has homology with LipP from *M. tuberculosis* and EstA from *Streptomyces chrysomallus*.

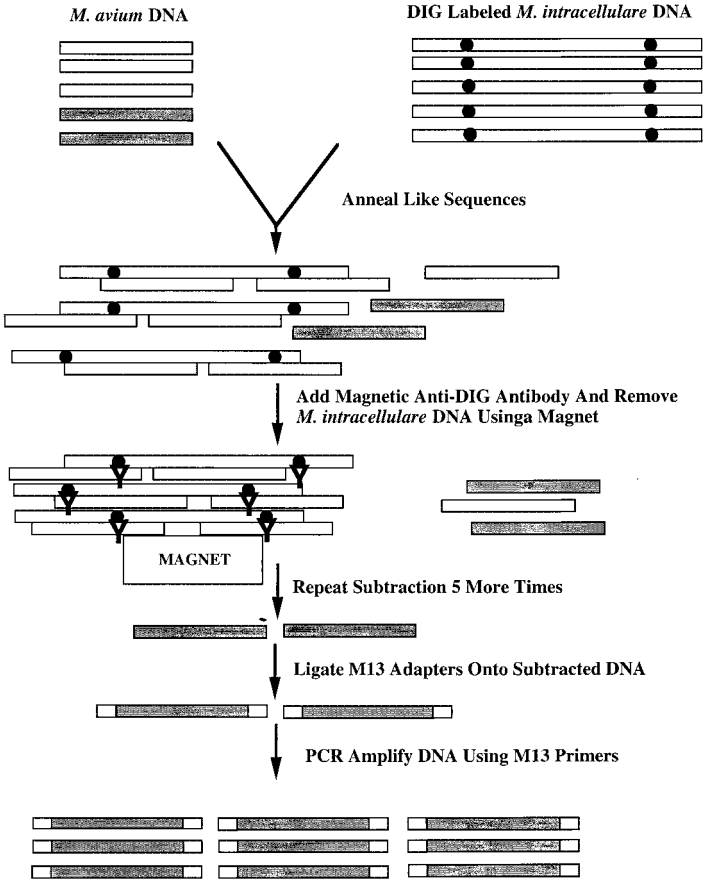

FIG. 3. Schematic diagram of the genomic DNA subtractive hybridization technique used to identify genes present in *M. avium* strain 104 but absent in *M. intracellulare*. Shaded rectangles, *M. avium* unique sequences; black circles, DIG label; Y, anti-DIG antibody; hatched rectangles, M13R-*Sau*3AI adapters.

The JAM8 product has homology with the *M. tuberculosis* and *Streptomyces coelicolor* protein Tig, a chaperon/prolylisomerase. The JAM9 product is a homolog of an ABC-type transporter ATP-binding protein from *M. tuberculosis* (Rv1463) and *Mycobacterium leprae* (CAB16169). The JAM10 product is a NifS-like protein that is likely involved in the assembly of iron-sulfur clusters. The JAM11 product has homology to the *M. tuberculosis* Rv1871c gene product of unknown function. The JAM12 product has a high degree of homology with LonA, an ATP-dependent protease. The JAM13 product has homology to the membrane-bound lipoproteins LprL and LprK of *M. tuberculosis*. The JAM15 product has homology with the *S. coelicolor* regulatory protein CAB88970, a putative repressor of secondary metabolism and a member of the LuxR family of transcriptional regulators. The JAM16 product has homology to the *M. tuberculosis* protein encoded by Rv3254, which has no know function. The JAM17 product has homology with a peptidase of *S. coelicolor* and hydrolases from *Pseudomonas aeruginosa* and *Campylobacter jejuni.* The JAM18 product has homology to an *S. coelicolor* Na/H exchanger (CAB5180). The JAM19 product has homology to an *M. leprae* oxidoreductase. The JAM20 product has homology to an *M. tuberculosis* probable methyltransferase, and the JAM21 product has homology with an *S. lividans* protein of unknown function.

DISCUSSION

MAC is composed of two closely related species, *M. avium* and *M. intracellulare* (2). However, there are significant differences in the pathologies caused by these organisms and the type of hosts they infect. *M. avium* infections are more common in AIDS patients, are acquired primarily via the gastrointestinal tract, and often result in disseminated disease (4, 39). *M. intracellulare* infections are more common in people with underlying lung pathology, are acquired via the respiratory tract, and usually remain limited to the pulmonary tissue (4, 39). Because of this information, we decided to compare the abilities of *M. avium* and *M. intracellulare* to evade the gastrointestinal defense barriers, such as the acidic conditions encountered in the stomach and the membrane-disrupting activities of cationic peptides in the intestine. We also examined the ability of the bacteria to invade intestinal epithelial cells in vitro and in vivo.

Enteropathogenic bacteria must be able to survive the acidic pH encountered in the stomach (pH of <3 for 2 h) (20, 21). We examined the abilities of *M. avium*, *M. intracellulare*, and *M. smegmatis* to survive pH 2.2 for 2 or 24 h and found that the order of acid tolerance was *M. avium* $>$ *M. intracellulare* $>$ *M. smegmatis*. Although there were differences in the level of acid

TABLE 3. Gene products unique to *M. avium* identified via subtractive hybridization

Gene product	Homolog	% Identity/% similarity/ no. of amino acids	Description/function
JAM1	*M. tuberculosis* Rv0227c	74/80/389	Possible membrane protein/unknown
	M. leprae CAA18554.1	67/76/446	Putative membrane protein/unknown
JAM2	*M. tuberculosis* Rv0226c	58/61/259	Probable membrane protein/unknown
	M. leprae CAA18553.1	50/55/276	Putative membrane protein/unknown
JAM3	*M. tuberculosis* Rv1779c	35/41/289	PPE family of proteins/unknown
JAM4	*M. tuberculosis* Zwf	91/95/483	GGPD/oxidative stress
	S. coelicolor CAB50762.1	69/79/483	G6PD/oxidative stress
JAM5	*M. tuberculosis* Rv0160	65/75/378	Unknown/unknown
	B. subtilis YciC	25/41/359	Zn Uptake/Zn homeostasis
JAM6	*M. tuberculosis* Rv0286	32/40/148	PPE family of protens/unknown
JAM7	*M. tuberculosis* LipP	78/83/320	Probable esterase/lipid biosynthesis
	S. chrysomallus EstA	45/55/317	Esterase/unknown
JAM8	*M. tuberculosis* Tig	96/97/59	Chaperone-proylisomerase/protein folding and secretion
	S. coelicolor Tig	54/74/59	Chaperone-proylisomerase/protein folding and secretion
JAM9	*M. tuberculosis* Rv1463	67/72/253	ABC transporter/ATP-binding protein
	M. leprae CAB16169.1	64/69/253	ABC transporter/ATP-binding protein
JAM10	*M. tuberculosis* NifS	88/91/100	Protein modification/Fe-S cluster formation
	M. leprae NifS	82/94/100	Protein modification/Fe-S cluster formation
JAM11	*M. tuberculosis* Rv1871c	77/81/109	Unknown/unknown
	S. coelicolor CAB88434.1	56/66/112	Unknown/unknown
JAM12	*M. smegmatis* LonA	85/92/227	ATP-dependent protease/protein degradation
	M. xanthus LonA	49/64/276	ATP-dependent protease/protein degradation
JAM13	*M. tuberculosis* LprL	28/47/374	Membrane lipoprotein/unknown
	M. tuberculosis LprK	25/42/261	Membrane lipoprotein/unknown
JAM14	*M. tuberculosis* Rv0453	30/37/147	PPE family of proteins/unknown
JAM15	*S. coelicolor* CAB88970	34/52/292	Regulatory protein/regulation of secondary metabolites
	M. tuberculosis Rv1204	25/36/526	Possible secreted protein/unknown
JAM16	*M. tuberculosis* Rv3254	40/45/344	Unknown/unknown
	S. coelicolor CAA20504	36/50/357	Possible secreted protein/unknown
JAM17	*S. coelicolor* CAB56384	57/67/403	Probable peptidase/protein degradation
	P. aeruginosa AAG06310	38/51/367	Probable hydrolase/unknown
JAM18	*S. coelicolor* CAB51980	26/37/412	Putative Na/H exchanger/unknown
	M. tuberculosis YjcE	24/37/538	Putative Na/H exchanger/unknown
JAM19	*M. leprae* CAB09639	81/83/285	Oxidoreductase/unknown
	D. radiodurans AAF11444.1	57/72/280	Oxidoreductase/unknown
JAM20	*M. tuberculosis* Rv0560c	68/87/250	Probable methyltransferase/unknown
	M. tuberculosis Rv3699	52/64/108	Probable methyltransferase/unknown
JAM21	*S. lividans* AAC25767	61/72/145	Unknown/unknown
	M. tuberculosis Rv1558	56/71/139	Unknown/unknown

tolerance among the mycobacteria, all three organisms were able to tolerate the acidic conditions encountered in the stomach better than many common enteropathogenic bacteria, including *Salmonella enterica* serovar Typhimurium (3), *Listeria monocytogenes* (26), and *Vibrio cholerae* (27). Thus, it does not appear that the level of acid tolerance of *M. avium* versus *M. intracellulare* is a limiting factor for gastrointestinal disease, although the difference observed may be clinically significant with low cell numbers.

The intestinal tracts of mammals are constantly exposed to potentially pathogenic microorganisms, but they remain disease free due in part to antimicrobial cationic peptides re-

leased from intestinal epithelial cells (7, 19, 30). The ability of the mycobacterial species to resist the membrane-disrupting activities of cationic peptides was modeled using polymyxin B (5). *M. smegmatis* was most susceptible to polymyxin B, which had a MIC of 32 μg/ml. Both *M. avium* and *M. intracellulare* were highly resistant and grew at concentrations of >500 μg of polymyxin B per ml. However, the polymyxin B MICs for many enteric bacteria, including *E. coli* (37), *S. enterica* serovar Typhimurium (37), and *S. enterica* (40), are less than 1 μg/ml. Thus, it does not seem likely that resistance to cationic peptides, as measured by resistance to polymyxin B, is a factor that limits the ability of *M. avium* or *M. intracellulare* to colonize the intestinal mucosa.

When assayed in a mouse intestinal loop model, *M. avium* invaded the intestinal mucosa >40-fold more efficiently than *M. intracellulare* after 1 h. To ensure that the bacteria were invading the mucosal cells, we performed transmission electron microscopy and observed that the bacteria either were in the process of invasion or were already internalized; however, we cannot exclude the possibility that some bacteria were only adherent. Because *M. avium* and *M. intracellulare* multiply so slowly (ca. one division per 20 h) (12), the percentage of bacteria that exit the lumen and invade the intestinal mucosa over short periods of time can be calculated by quantifying the number of bacteria in the intestinal tissues. In intestinal segments inoculated with *M. avium*, approximately 39% of the bacteria entered the intestinal mucosa after 2 h, compared with only 1.7% for *M. intracellulare*. These data are consistent with the in vitro data obtained using cultured intestinal epithelial cells and multiple strains of *M. avium* and *M. intracellulare* (Table 2) and confirm our hypothesis that only *M. avium* is able to efficiently invade the intestinal mucosa.

Genomic DNA subtractive hybridization of *M. avium* strain 104 genomic DNA with *M. intracellulare* chromosomal DNA (Fig. 3) revealed several genes present in *M. avium* that were absent in *M. intracellulare*. This list of genes should not be considered complete, as the subtractive hybridization protocol likely missed several genes which may play important roles in invasion; furthermore, we cannot conclude that all or any of the genes identified are absolutely necessary for invasion. Many of these genes that we identified encode proteins with no known or predicted function, including PPE proteins (JAM3, JAM6, and JAM14), membrane proteins of unknown function (JAM1 and JAM2), and other unknown proteins (JAM11, JAM16, and JAM21) (Table 3). However, some of the identified genes have the potential to participate in intestinal pathogenesis. One such gene encodes a protein with predicted G6PD activity. G6PD catalyzes the first step in the pentose phosphate pathway, which produces ribose for DNA synthesis and reducing equivalents in the form of NADPH that can be used for anabolic reactions and oxidative damage repair (16, 35). In *M. avium* there are at least three G6PD isoenzymes; one is homologous to the developmentally regulated DevB of *Anabaena* sp. (C. C. Bauer, unpublished data [GenBank accession no. U14553]), and the other two have various amounts of homology to Zwf of *E. coli*. In *M. avium* it is likely that each of the isoenzymes is regulated independently in response to factors such as growth rate, physiological state, and level of oxidative stress. Although nothing is known about the role that *zwf* plays in the pathogenicity of *M. avium*, *zwf* mutants of *S. enterica* serovar Typhimurium are avirulent (25). In *P. aeruginosa* the *soxR* regulon, including *zwf*, is required for the colonization of burn wounds, and *soxR* mutants which do not express multiple genes, including *zwf*, are unable to cause bacteremia and systemic disease in a burned mouse model (22).

Another gene that is possibly involved in intestinal epithelial cell invasion has homology with the chaperone/peptidyl-prolyl-*cis-trans*-isomerase (PPIase) Tig. This protein likely functions in the folding and secretion of proteins (11, 14). Enzymes with PPIase activity have been implicated as virulence factors in several pathogens, including *Legionella pneumophila* (17, 38), *Mycoplasma pneumoniae* (32), and *S. enterica* serovar Typhimurium (36). In *S. enterica* SurA functions as a PPIase and plays a role in the organism's ability to adhere to and invade HEp-2 cells (36). *S. enterica surA* mutants are attenuated for virulence in mice and can be used as an effective oral vaccine against virulent salmonellae (36).

An ABC-type transporter ATP-binding protein and a NifS homolog have also been identified and may play a role in intestinal invasion. ABC-type transporters are associated with a variety of processes that involve the translocation of small molecules across membranes (23), and NifS homologs are involved in the assembly of iron-sulfur clusters in proteins (41, 42). These genes have been implicated as *M. smegmatis* stress response genes and are upregulated during anaerobic stationary phase (29). Interestingly, these are the same conditions encountered in the human host after ingestion, and we have previously demonstrated that these conditions stimulate *M. avium* to invade intestinal epithelial cells with increased efficiency. It is plausible that these proteins are part of an anaerobic regulon required for gastrointestinal invasion. We are currently investigating the roles that these and other anaerobically induced proteins play in intestinal invasion.

Homologs of the *M. smegmatis* protease LonA and an *S. coelicolor* probable peptidase were also identified. Proteases mediate the degradation of damaged and short-lived proteins and provide amino acids for new protein synthesis. It is likely that the rapid elimination of key metabolic and regulatory proteins and the production of others are essential for *M. avium* to alternate from the saprophytic to the pathogenic lifestyle in the intestine. It is also likely that *M. avium* in the environment is in stationary phase and produces these and other stationary-phase-induced proteins, such as NifS and the ABC-type transporter ATP-binding protein mentioned above, and that this arsenal may be important for gastrointestinal invasion.

The ability to invade the gastrointestinal tract and cause disseminated disease is a complex trait and likely requires the coordinate production of multiple virulence factors in response to environmental cues. We have demonstrated that the invasion of the intestinal mucosa is an important phenotypic difference between virulent strains of *M. avium* and *M. intracellulare.* Although the subtractive hybridization protocol likely missed several genes present in *M. avium* but absent in *M. intracellulare*, it did identify several potential virulence factors that are unique to *M. avium* that may allow it to invade the intestinal mucosa more efficiently than *M. intracellulare*. Unfortunately to date there is no methodology to create site-specific mutations in *M. avium*, and thus we cannot examine

the roles that individual genes play in intestinal pathogenesis. However, we are currently working towards developing such techniques in order to delineate the roles that these genes play in invasion and dissemination.

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