Use of Gene Dosage Effects for a Whole-Genome Screen To Identify Mycobacterium marinum Macrophage Infection Loci

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We recently identified two loci, mel1 and mel2, that affect macrophage infection by Mycobacterium marinum. The ability of these loci to confer enhanced infection in trans is presumably due to gene dosage effects since their presence on plasmids increases expression from five- to eightfold. Reasoning that this phenomenon would allow identification of other mycobacterial genes involved in macrophage infection, we conducted a screen of an M. marinum DNA library that provides 2.6-fold coverage of the entire genome for clones that affect macrophage infection. Our preliminary screen identified 76 plasmids that carry loci affecting macrophage infection. We eliminated plasmids that do not confer the expected phenotype when retransformed (70%), that have identical physical maps (5%), or that carry either of the mel1 or mel2 loci (14%) from further consideration. Four loci that confer enhanced infection (mel) and four that confer repressed infection (mel) of macrophages were identified, and two of each group were chosen for detailed analysis. Saturating transposon mutagenesis was used to identify the loci responsible, and M. marinum mutants were constructed in the genes involved. We expect these genes to provide insight into how mycobacteria parasitize macrophages, an important component of innate immunity.

Mycobacterium marinum is a natural pathogen of humans (41, 49, 57), fish, and amphibians (18), causing more than 150 human infections each year in the United States alone (29). Although M. marinum causes primarily skin lesions on the extremities in humans (19), it causes a systemic tuberculous disease in fish and amphibians (30, 73, 101). M. marinum infections result in granuloma formation, whether in humans, mice, fish, or amphibians (18–20, 101). Granuloma formation occurs because macrophages become infected and allow growth of M. marinum during disease (19, 74) and in laboratory model systems (7, 33, 69, 81). These characteristics of infections, along with the relative ease of manipulation (3, 37, 82, 88), rapid growth rate compared to other pathogenic mycobacteria (18), and the presence of numerous useful virulence models (11, 20, 27, 33, 84, 89, 92), have aroused great interest in the molecular mechanisms of M. marinum pathogenesis. Significant progress has been made toward understanding M. marinum evolution (106), trafficking (7, 86, 98), secretion (1, 36), gene regulation (6, 82), phochromogenicity (35, 83), cell wall synthesis (3, 24, 37), granuloma formation (23, 27, 97), resistance to oxidative species (78, 79, 95, 96), and mechanisms of macrophage infection (32, 38, 66).

As a means to better understand the molecular mechanisms of macrophage infection by M. marinum, we recently screened a genomic library for loci that have the ability to confer enhanced macrophage infection to M. smegmatis (32), a non-pathogenic mycobacterial species that does not infect macrophages efficiently. We identified two M. marinum loci, mel1 and mel2, that confer enhanced macrophage infection to both M. smegmatis and M. marinum. The phenotypic effect of cosmids containing mel loci in M. marinum suggests that gene dosage effects, due to the resulting gene copy number, increase the expression of these genes above basal levels and thereby increase the efficiency of macrophage infection. This conclusion is consistent with our previous observations, and that of other groups, that many pathogenic bacteria, including other mycobacteria, are more virulent when grown in eukaryotic cells than when grown in laboratory medium (12–15, 65). If these hypotheses are correct, gene dosage effects could be used to identify additional genes that play a role in macrophage infection through screening a M. marinum genomic library in wild-type M. marinum for enhanced macrophage infection under standard laboratory growth conditions.

In the present study, we demonstrate the feasibility of this approach by evaluating the efficiency of macrophage infection under different stages of M. marinum growth when cultured in laboratory medium. We also confirm the ability of the mel1 and mel2 loci to confer enhanced infection of macrophages to M. marinum when carried on plasmids and evaluate the ability of gene dosage effects to increase levels of melF expression, the first gene in the mel2 locus. These observations led us to conduct a whole-genome screen in M. marinum for macrophage infection loci using gene dosage effects. We identified a total of eight loci: four that enhance and four that repress macrophage infection. Detailed characterization of four of these loci resulted in the identification of at least seven newly described genes that play a role in macrophage infection by M. marinum. These observations suggest, for the first time, that M.
MATERIALS AND METHODS

Strains and growth conditions. M. marinum strain M, a clinical isolate obtained from the skin of a patient (91), was used in these studies. M. marinum strains were grown at 33°C in 7H9 broth (Difco, Detroit, MI) supplemented with 0.5% glycerol, 10% albumin-dextrose complex (ADC), and 0.25% Tween 80 (M-ADC-TW) for 5 days. Cultures were grown to an optical density at 600 nm (OD600) of 0.5 (except where specifically indicated otherwise), and were used in subsequent experiments. E. coli (strain XL1-Blue [Stratagene] was grown in Luria-Bertani (LB) medium (Difco) at 37°C. When appropriate, kanamycin or chloramphenicol was added at a concentration of 25 µg/ml (E. coli) or 10 µg/ml (M. marinum). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at concentrations of 40 µg/ml in E. coli and 90 µg/ml in M. marinum, when needed.

Monocytic cell lines and culture conditions. The murine macrophage cell line RAW 264.7 (ATCC CRL-2278) and 7741.1 (ATCC TIB67) were maintained in 5% CO2 at 37°C in high-glucose Dulbecco modified Eagle medium (Gibco, Bethesda, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 2 µM l-glutamine. The human monocytic cell line THP-1 was maintained in RPMI medium supplemented with 2 µM l-glutamine at 37°C. Where appropriate, kanamycin or chloramphenicol was added at a concentration of 25 µg/ml (E. coli) or 10 µg/ml (M. marinum). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at concentrations of 40 µg/ml in E. coli and 90 µg/ml in M. marinum, when needed.

Determination of RNA transcript levels. Reverse transcription PCR (RT-PCR) was used to quantify levels of 16S rRNA, rRNA, melF, melM, melN, melO, and melf transcription in different mycobacterial strains within the linear amplification range, as described previously (96). Basically, 300 to 500 ng of total DNA were mixed with gene-specific reverse primers (Table 1) and 5U of Thermopol enzyme (NEB) in a total volume of 50 µl. The reaction was performed in an Applied Biosystems 7500 RT-PCR system in triplicate. Products were verified by gel agarose gel electrophoresis after amplification. Relative quantities of RNA transcripts were calculated by using the comparative Ct method with 16S rRNA as an internal standard. The fold increase in expression was calculated by the 2−ΔΔCT method, as described previously (9).

Construction of an arrayed M. marinum total DNA cosmid library in M. marinum. A contiguous fragment library was constructed in the cosmid pJDC16 from M. marinum total DNA as described previously (32). The average fragment size for DNA inserts in this library were between 40 and 50 kbp in length. Approximately 20,000 cosmid clones from this library in E. coli were pooled and plated for individual colonies on LB agar plus kanamycin. A total of 384 individual colonies were picked and grown in 100 µl of LB plus kanamycin in 96-well microtiter dishes. A replica was made of this arrayed library, and both samples were stocked in 50% glycerol and stored at −80°C until use. The randomness and integrity of the library was confirmed by restriction analysis of 20 random cosmids from the library. Of the 20 clones, 100% carried the appropriate size of insert and displayed unique PsI physical maps. Plasmid was prepared from each clone in the entire E. coli library and used to transform M. marinum by electroporation as described previously (16). One clone from each transformation was grown in M-ADC-TW plus kanamycin and stored in 50% glycerol at −80°C in 96-well microtiter dishes until use.

M. marinum whole-genome screen for loci affecting macrophage infection. The entire arrayed M. marinum genomic library carried by M. marinum was screened for the effects of gene dosage on macrophage infection in RAW 264.7 cells. Groups of between 5 and 12 clones from the library were grown separately and evaluated in all assays, along with wild-type M. marinum and M. marinum that carries the vector backbone pJDC16. All cultures for assays were grown in 1 ml
FIG. 1. Growth of *M. marinum* in M-ADC-TW broth as determined by OD_{600} (A) and CFU (B). The percentages of the *M. marinum* inoculum that enters human monocytic/macrophage THP-1 (C), murine macrophage J774A.1 (D), and RAW 264.7 (F) cell lines and the fish monocytic/macrophage CLC (E) cell line when grown to different optical densities are also shown. Each data point indicates the mean, and error bars indicate the standard deviation of triplicate samples from a representative experiment of two independent experiments. **, *P* < 0.01 compared to the means of data at OD_{600} = 0.3 to 0.5.
of M-ADC-TW plus kanamycin and without kanamycin for the wild type without vector. Monolayers of 10^6 RAW 264.7 cells were then infected with approximately 10^7 bacteria from each culture for 30 min at 37°C in 24-well tissue culture dishes, washed three times with warm PBS, and lysed with 0.1% Triton X-100, and dilutions were plated on M-ADC to determine cell-associated CFU. The percent cell association was then calculated as follows: (CFU_{intracellular}/CFU_{inoculum}) \times 100. In the initial screen, all assays were carried out in triplicate, and two independent assays were carried out with each of the 384 clones in the library. All data were normalized to the median cell association for all assays, arbitrarily set to 1. Positive clones were chosen based on their significantly (P < 0.05) different, either increased or decreased, cell association from the median. Cosmids were then purified from all 76 positive clones, transferred to E. coli, physically mapped with PstI and NotI, and screened for the presence of mel1 or mel2 genes (32) by PCR using the oligonucleotide pairs MelDF-MelDR or MelF2F-MelF2R, respectively. The cosmids were retransformed into M. marinum by electroporation and reassayed for the effects on RAW 264.7 cell association in three independent assays in triplicate. Cosmid clones that consistently conferred a difference in macrophage infection in cell association assays were chosen for determination of the genes involved.

Identification of mycobacterial enhanced and repressed macrophage infection loci. Four cosmids were chosen for detailed characterization as described previously (32): two that confer enhanced macrophage infection (mel) and two that confer repressed macrophage infection (mrl) to M. marinum. All four cosmids were mutagenized by saturating transposon mutagenesis with the chloramphenicol-resistant mini-Mu transposon as recommended by the manufacturer of the system (Finnzymes). Each mutagenized cosmid was transformed into M. marinum, and the resulting transformants were evaluated in three independent RAW 264.7 cell association assays in triplicate. Cosmids that no longer confer the phenotype of the original cosmid, due to the presence of the transposon in the locus involved, were purified and physically mapped with PstI and NotI, and the sequence was obtained from the junction next to the transposon to determine the gene interrupted using the SeqA and SeqB primers provided by the manufacturer.

In silico analysis of loci. Detailed analysis of the amino acid sequence of putative open reading frames (ORFs) within mel and mrl loci was carried out by using protein-protein National Center for Biotechnology Information BLAST (5) and Conserved Domain Search (64) as described previously (32).

Construction of M. marinum mutants by allelic exchange. In order to construct mutants in the M. marinum mel and mrl loci identified, we cloned each locus into the unique BamHI, EcoRI, or HindIII site of pYU174 for allelic exchange as described previously (32). Each locus was in vitro mutagenized randomly using a kanamycin-resistant mini-Mu transposon as recommended by the manufacturer of the system (Finnzymes). Plasmids that carried mutations in genes of interest were then transformed into M. marinum by electroporation, and recombinants were selected for by the presence of kanamycin-resistant blue colonies on X-Gal M-ADC plates. Individual colonies were then grown in the presence of kanamycin, plated for single colonies on the same plates, and screened visually for white colonies, which should be mutants resulting from allelic exchange. The presence of the appropriate mutation was confirmed in each case by PCR and Southern analyses.

Construction of complementing strains for each M. marinum mutant. Complementing constructs were made for each region by high-fidelity PCR and cloning into pIDC89 as described previously (66). Basically, the fragments were amplified by PCR directly from M. marinum or pIDC89 by using NheI and PacI (pIDC117) or PacI and Scal (pIDC119, pIDC120, and pIDC121). All constructs were confirmed by physical mapping with restriction enzymes, and the absence of mutations incorporated by PCR was demonstrated by sequencing.

Microscopic method to confirm efficiency of macrophage infection. Macrophage infection assays were conducted and evaluated by microscopy as described previously (33). Coverslips were seeded with macrophages in 24-well tissue culture plates. Bacteria were added to achieve a multiplicity of infection of 10. The infection was allowed to proceed for 30 min, after which the cells were washed twice with PBS. The cells were fixed with methanol, washed once with PBS, and stained by the Ziehl-Neelsen technique (47), using carbol-fuchsin and malachite green (Sigma). Cells were examined by using a Nikon TE300 light microscope with differential interference contrast optics. At least two independent coverslips were examined for each sample. The percentage of infected cells was determined for three counts of 25 fields, each with greater than 20 cells per field. The number of bacterial vacuoles per cell was determined for three counts of 50 cells each, and the number of bacteria per vacuole was determined for three counts of 50 cells each. These values were used to calculate an infectivity index for each mutant as the (percent infected cells \times the number of vacuoles per cell) \times 100. For each experiment, triplicate samples were performed, and the data were normalized to the median cell association for all assays, arbitrarily set to 1. Positive clones were chosen based on their significantly (P < 0.05) different, either increased or decreased, cell association from the median. Cosmids were then purified from all 76 positive clones, transferred to E. coli, physically mapped with PstI and NotI, and screened for the presence of mel1 or mel2 genes (32) by PCR using the oligonucleotide pairs MelDF-MelDR or MelF2F-MelF2R, respectively. The cosmids were retransformed into M. marinum by electroporation and reassayed for the effects on RAW 264.7 cell association in three independent assays in triplicate. Cosmid clones that consistently conferred a difference in macrophage infection in cell association assays were chosen for determination of the genes involved.

RESULTS

Macrophage infection by M. marinum is regulated. Genes involved in the virulence of bacterial pathogens are usually tightly regulated under different environmental conditions to allow economic use of available nutritional resources (28, 67, 68, 85). We examined the ability of M. marinum to infect macrophages during growth in laboratory medium to better understand how the genes involved might be regulated and their mechanism of action. M. marinum displays approximately 10- to 20-fold-higher levels of macrophage infection at an OD_{600} of between 0.8 and 1.2 compared to early or late stages of growth (Fig. 1). This difference is consistent in human, murine, and fish monocytic cells and macrophages, although there are some differences in the magnitude of the effect seen. Since all assays, irrespective of the growth state of the bacteria, were conducted with the same number of bacteria in the same fashion, it is unlikely that this difference is due to experimental conditions other than regulation of the M. marinum factors involved.

Gene dosage effects allow subtle upregulation of genes in M. marinum. Our previous studies allowed identification of two
loci, mel1 and mel2, from M. marinum that have the ability to confer enhanced infection of macrophages to M. smegmatis, a nonpathogenic mycobacterial species (32). Since mutations in these loci affect the ability of M. marinum to infect macrophages, their regulation may be at least partially responsible for the differences in efficiency of macrophage infection observed at different stages of growth in laboratory medium. Based on these observations, we hypothesized that M. marinum containing plasmids that carry the mel loci would upregulate these genes and display enhanced macrophage infection compared to wild-type bacteria. We found that cosmids containing the mel1 or mel2 loci confer ~10-fold-enhanced infection of macrophages to M. marinum (Fig. 2). When we examined the level of expression of mel2 in wild-type M. marinum and the strain carrying the cosmid that contains mel2, we found that expression of the melF gene within this locus increases at least fivefold (Fig. 3). These observations suggest that gene dosage effects can be used to increase the expression of genes involved in macrophage infection and raises the possibility that this technique could be used to identify additional macrophage infection genes.

Whole-genome screen for M. marinum genes involved in macrophage infection. In order to gain additional insight into the mechanisms of macrophage infection by M. marinum, we developed a strategy using gene dosage effects to screen the M. marinum genome. We first constructed a total DNA genomic cosmid library that can replicate in both E. coli and M. marinum (32). We arrayed 384 individual E. coli clones from this library in four 96-well plates, transformed each of these cosmids into M. marinum, and arrayed the resulting transformants in 96-well plates in the same manner. Since the average fragment size for the cosmid library is ~45 kbp and the M. marinum genome is ~6.6 Mbp, 384 clones provide ~2.6-fold coverage of the entire M. marinum genome or a 93% likelihood

FIG. 3. RT-PCR (A and B) and quantitative RT-PCR (C) studies to evaluate the effects of gene dosage on expression of melF in M. marinum. Equivalent amounts of RNA from M. marinum wild-type and M. marinum carrying cos31 (M. marinum::cos31) were reverse transcribed and subjected to PCR using specific oligonucleotides within melF and 16S rRNA. (A) The dilutions of cDNAs are, from left to right: 1:4, 1:2, and 1:1. Equal amounts of each PCR product were loaded on 0.8% agarose gels and compared to the 16S rRNA control RT-PCR for each strain (16S rRNA) carried out on the same samples in the same manner by ethidium bromide staining (A) and densitometry (B). (C) Quantitative RT-PCR analyses of increased expression levels of RNA transcript in M. marinum carrying cos31 compared to wild-type M. marinum confirmed conventional RT-PCR studies. In the case of quantitative RT-PCR, the sigA gene was used as a control that should not display changes in expression in the presence of the cosmids. All transcript levels were calculated by the comparative C_T method using 16S rRNA as a comparator. The data shown are representative of two independent experiments. **, P < 0.05 compared to sigA.
that the entire genome is represented in the library. Each of
the resulting M. marinum clones carrying a cosmid with a large
fragment of the M. marinum genome was then screened in two
independent assays in triplicate for the ability to infect macro-
phages (Fig. 4). We chose to avoid use of antibiotics in this
screen to eliminate the possibility that some of the differences
observed would be due to differences in antibiotic suscepti-
bility, rather than interactions with macrophages. To normalize
our data, the median level of macrophage infection for the
entire library was set to 1, and results from each assay were
evaluated for significant differences from the median (P <
0.05). Interestingly, an approximately equivalent number of
clones display decreased (repressed) and increased (enhanced)
macrophage infection in these assays. Since it is possible that
repression of host cell infection is equally as important as
enhancement of host cell infection during pathogenesis, we
selected both types of clones for further analyses. A total of 76
clones had significantly enhanced or repressed macrophage
infection in our initial screen.

Confirmation of the ability of cosmids to confer effects on
macrophage infection. Individual cosmids that we isolated
from the 76 M. marinum clones displaying enhanced or re-
pressed macrophage infection were screened for similar phys-
ical maps, the presence of previously identified loci, and the
ability to confer the expected phenotype when transformed
back into M. marinum. Restriction patterns with PstI and NotI
were examined for all 76 cosmids (data not shown). Four
plasmids with identical restriction patterns were identified and
eliminated from further analyses. In addition, we examined
each cosmid for the presence of either mel1 or mel2 by PCR
(data not shown), since these loci had been previously shown to
confer enhanced macrophage infection. We identified 11
clones out of 76 that carry either mel1 or mel2, and these clones
were not analyzed further. Transformation of cosmids back
into M. marinum was used to confirm their phenotype and
ensure that it is due to genes present on the cosmid, rather
than a mutation in the M. marinum chromosome. All 76 clones
resulting from transformation of these cosmids back into M.
marinum were assayed in triplicate in three independent assays
in the same manner as our initial screen (Fig. 5). We identified
eight cosmid clones that confer significantly enhanced or re-
pressed macrophage infection compared to the median for all
assays (P < 0.05). These cosmids have unique restriction pat-
terns and do not carry either the mel1 or mel2 locus. Four of
these cosmids, two that confer enhanced macrophage infection
(mel) and two that confer repressed macrophage infection
(mrl), were chosen for further analysis.

Identification of M. marinum mel and mrl loci. Physical maps
were constructed for all four cosmids, and the cosmids were in
vitro mutagenized with a chloramphenicol-resistant mini-Mu
transposon. Each cosmid displays a unique NotI physical map
and ranges in size from 44 to 52 kbp (Fig. 6). The exact
locations of transposon insertions in each cosmid were deter-
mined by sequencing out from the transposon. Cosmids that
carried unique transposon insertions in the insert M. marinum
DNA were transformed into M. marinum to evaluate whether
the mutagenized cosmid retained the ability to confer the ex-
pected phenotype for macrophage infection compared to wild-
type M. marinum and the nonmutated cosmid. These assays
were carried out three times independently in triplicate, and
mutagenized cosmids that display a significantly different phe-
notype compared to the original cosmid (P < 0.05) were con-
cluded to have a transposon insertion in the locus affecting
macrophage infection (Fig. 6). We identified one region from
each cosmid that is involved in either enhancing, designated
mel3 and mel4, or repressing, designated mrl1 and mrl2, mac-
rophage infection by M. marinum. Based on the positions of
each transposon insertion and their effect on the phenotype
conferred by the cosmid, we estimated the approximate size of

FIG. 4. Summary of all data from normalized cell association with the RAW 264.7 murine macrophage cell line for 384 individual clones of M.
marinum carrying an arrayed M. marinum total genomic DNA cosmid library assayed in two independent assays in triplicate. Each data point
represents the normalized mean of triplicate samples. All cell association data are normalized to the median of all library clones arbitrarily set to
1. Horizontal dashed lines indicate one standard deviation from the median, and the horizontal dotted lines indicate two standard deviations from
the median.
each locus. The mel2 and mel3 loci are somewhat smaller (3 to 6 kbp) than the mrl1 and mrl2 loci (8 to 10 kbp).

**In silico and expression analysis of mel and mrl loci.** Sequences obtained from each transposon insertion were compared to the M. marinum genome available from the M. marinum Sequencing Group at the Sanger Institute (http://www.sanger.ac.uk/Projects/M_marinum/). This allowed determination of the complete sequence of each locus and annotation of the regions involved (Fig. 7). Quantitative RT-PCR analyses of the transcript levels of the genes identified within the mel4 and mrl2 loci confirm that the cosmids 2-G2 and 4-H9 increase expression of the loci that they carry when present in M. marinum (Fig. 7), similar to our observations with the cosmid that carries melF. The loci identified are not clustered in an obvious fashion in the M. marinum genome and are unique with respect to previously identified M. marinum virulence determinants.

We analyzed the putative coding regions within these loci for similarity to other bacterial genes and motifs present within them to gain insight into their potential functions. Detailed in silico analysis of the putative ORFs identified in this manner is described in Table 2. Based on these analyses, the mel3 locus carries a single ORF that is similar to the mycobacterial PknD,
a “eukaryotic-like” serine/threonine kinase that appears to affect regulation through SigF (42, 44, 80). The mel4 locus encodes four putative ORFs, two of which are similar to zinc-dependent proteases and two with unknown functions. Interestingly, both of the mrl loci identified contain ORFs with potential involvement in synthesis of the mycobacterial cell wall. There are five putative ORFs within mrl1 that display similarity to cell wall biosynthetic proteins in other bacteria and ten putative ORFs within mrl2 that primarily display similarity to lipid biosynthetic proteins. Since lipids are an important component of the mycobacterial cell wall, it is possible that mrl1 and mrl2 are involved in synthesis of modified mycobacterial cell wall components, resulting in a composition that is less conducive to efficient phagocytosis by macrophages under certain growth conditions.

**Mutations in M. marinum mel loci affect infection of macrophages.** To eliminate the possibility that the phenotypes conferred by mel and mrl loci are due to artifacts resulting from overexpression, rather than the natural gene functions, we constructed mutations in them by allelic exchange and examined their effects on macrophage infection. Each locus was first cloned into a suicide plasmid for mycobacteria, pYUB174, and mutated by in vitro transposon mutagenesis. The exact location of each transposon insertion was determined by sequencing
TABLE 2. Characteristics of genes in mel and mrl loci

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Similar organism (gene)?</th>
<th>E value (score)?</th>
<th>% Identity (no. of aa)?</th>
<th>Other similarities or motifs?</th>
<th>Putative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mel3</td>
<td>M. smegmatis (pknD)</td>
<td>3e-94 (349)</td>
<td>66 (272)</td>
<td>S_TKc, DUF477</td>
<td>Serine/threonine kinase</td>
</tr>
<tr>
<td>mel4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>melM</td>
<td>M. tuberculosis (Rv2315c)</td>
<td>0.0 (859)</td>
<td>86 (505)</td>
<td>COG0312, Zn-dep. prot.</td>
<td>Protease</td>
</tr>
<tr>
<td>melN</td>
<td>M. tuberculosis (Rv2314c)</td>
<td>0.0 (711)</td>
<td>84 (457)</td>
<td>COG0312, Zn-dep. prot.</td>
<td>Protease</td>
</tr>
<tr>
<td>melO</td>
<td>M. tuberculosis (Rv2313)</td>
<td>5e-113 (410)</td>
<td>84 (283)</td>
<td>COG2128, unchar.cons.prot.</td>
<td>Unknown</td>
</tr>
<tr>
<td>melP</td>
<td>N. farcinica (nfa42450)</td>
<td>3e-13 (78.6)</td>
<td>52 (91)</td>
<td>None</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

| mnl1       |                          |                 |                         |                               |                   |
| mnlA       | Arthrobacter sp. (Arth4051)| 2e-47 (194)  | 46 (276)                | Cell wall biogenesis         |                   |
| mnlB       | M. avium subsp. paratuberculosis (MAP0964c) | 0.0 (704) | 71 (507)                | Cell wall biogenesis         |                   |
| mnlC       | M. avium subsp. paratuberculosis (MAP0963c) | 3e-150 (535) | 68 (488)                | Cell wall biogenesis         |                   |
| mnlD       | M. avium subsp. paratuberculosis (MAP0962c) | 0.0 (685) | 63 (663)                | Cell wall biogenesis         |                   |
| mnlE       | M. avium subsp. paratuberculosis (MAP0961c) | 8e-125 (449) | 72 (349)                | Cell wall biogenesis         |                   |

| mnl2       |                          |                 |                         |                               |                   |
| mnlF       | M. avium subsp. paratuberculosis (MAP3191) | 0.0 (646) | 87 (370)                | Phe and Tyr metabolism       |                   |
| mnlG       | M. avium subsp. paratuberculosis (MAP3192) | 0.0 (711) | 87 (392)                | Amino acid transport/metabolism |                   |
| mnlH       | M. avium subsp. paratuberculosis (MAP3193) | 0.0 (685) | 90 (396)                | Lipid transport/metabolism    |                   |
| mnlI       | M. avium subsp. paratuberculosis (MAP3194) | 2e-104 (381) | 81 (301)                | Metabolism                   |                   |
| mnlJ       | M. avium subsp. paratuberculosis (MAP3195) | 1e-87 (325) | 80 (213)                | Transcriptional regulation    |                   |
| mnlK       | M. avium subsp. paratuberculosis (FadE12–3) | 0.0 (734) | 87 (412)                | Lipid metabolism             |                   |
| mnlL       | M. avium subsp. paratuberculosis (MAP3197) | 3e-89 (322) | 75 (264)                | Metabolism                   |                   |
| mnlM       | M. avium subsp. paratuberculosis (MAP3198) | 5e-37 (156) | 62 (129)                | Metabolism                   |                   |
| mnlN       | M. avium subsp. paratuberculosis (MAP3199) | 2e-56 (221) | 64 (173)                | Unknown                      |                   |
| mnlO       | M. tuberculosis (Rv3143) | 7e-55 (216)    | 88 (130)                | C00156, sig.rec.             | Signal receiver  |

* That is, the organism that carries the protein most similar to that encoded by the M. marinum gene identified, along with the name of the corresponding gene in that organism in parentheses.
* The expectation frequency (E value) is a parameter that describes the significance of the sequence match and the score of the sequence match according to NCBI protein-protein BLAST when it is compared to the closest homologue.
* The percent identity at the amino acid (aa) level and the number of amino acids (in parentheses) that could be aligned to show this level of identity.

** This column indicates the placement of the putative protein product into a cluster of orthologues (COG or KOG [eukaryotic]) using the CD-Search at NCBI and SignalP v.1.1 at the Center for Biological Sequence Analysis website. COG, clusters of orthologous groups; KOG, clusters of orthologous groups for eukaryotic genomes; pfam, protein family; cd, conserved domain; S_TKc, serine/threonine protein kinase catalytic domain; DUF477, domain of unknown function found in Eukarya and Eubacteria; Zn-dep.prot., Zn-dependent proteases; unchar.cons.prot., uncharacterized conserved protein; teich.ac.syn., teichoic acid synthesis proteins; dip.diph.ep., predicted nucleoside-diphosphate sugar epimerase; polys.syn., polysaccharide biosynthesis protein; MviN, uncharacterized membrane protein thought to be involved in virulence; glyc.tran., predicted glycosyltransferases; glyc.tran1, glycosyltransferases group 1; hom.diox., homogentisate 1,2-dioxigenase involved in the metabolism of Phe and Tyr; dip.amin.pep., dipeptidyl aminopeptidases/acylaminoacyl- peptidases; TetR_N, TetR family bacterial regulatory proteins; AcR, AcR family of transcriptional regulators; ACAD, acyl-coenzyme A dehydrogenase; phosphotr., phosphotransferase enzyme family; ketost.isom., ketosteroid isomerase; YceI, E. coli YceI-like family of inducible periplasmic proteins; sig.rec., signal receiver domain.

from the transposon. We identified insertions in melL, melM, melN, mnlA, mnlB, mnlC, mnlD, mnlE, mnlF, mnlG, mnlH, mnlK, mnlL, mnlM, and mnlO using this technique (Fig. 7). Each of these mutations was then transferred to M. marinum by allelic exchange using the β-galactosidase gene carried by this pYUB174 to screen for single and double recombination events in a two-step allelic exchange. Each of the resulting mutants was compared for its ability to infect macrophages with wild-type M. marinum and M. marinum carrying the original cosmid (Fig. 8). As expected, the melL mutant displays a significant defect (P < 0.01) in macrophage infection compared to wild-type M. marinum. Although transposon insertions in these genes within the original mel4 cosmid affect the ability of the cosmids to enhance macrophage infection, melM, melN, and melO mutants do not display a significant defect in macrophage infection and may actually display enhanced macrophage infection. We hypothesized that the functional ORF within the mel4 locus might be melP, rather than the other three genes. Since we did not obtain a mutation in this gene, we could not test the role of melP directly, but we examined whether its transcription was
affected by the insertions in the other ORFs within mel4 (Fig. 9). We found that insertions in each of the mel4 ORFs affected all genes downstream, with the exception of melP, suggesting that the melP gene is within a different transcriptional unit from the other genes within mel4. The phenotype of mutants that affect macrophage infection was confirmed by microscopy using acid-fast stains (Table 3). Acid-fast stains revealed similar differences in the ability of these mutants and the original cosmids to infect macrophages, as observed in cell association assays. Since both viability-based assays, such as cell association assays, and microscopy, which is not affected by bacterial viability, display similar results, it is unlikely that survival in host cells is solely responsible for their phenotype. Rather, the observed phenotype is most likely due to differences in macrophage infection.

**Mutations within mrl loci enhance macrophage infection.** Since the presence of the mrl loci on cosmids represses infection of macrophages, most likely due to gene dosage effects, we expected that mutants in these genes could enhance macrophage infection. We found that M. marinum mrlA, mrlG, mrlH, mrlI, mrlL, and mrlN mutants display enhanced macrophage infection compared to the wild type (P < 0.01), whereas mrlF, mrlK, and mrlO mutants infect macrophages at levels similar to the wild type (Fig. 8). These observations suggest that mrlF and mrlO are not required for inhibition of macrophage infection. This may be the case for mrlK as well, although this mutation might not interfere with the activity of the protein product due to its carboxy-terminal position of insertion. However, the other predicted mrl genes—mrlA, mrlG, mrlH, mrlI, mrlL, and mrlN—play a role in reducing macrophage infection by wild-type M. marinum.

**Complementation restores wild-type macrophage infection to mel and mrl mutants.** In order to ensure that secondary mutations had not occurred elsewhere in the M. marinum chromosome during mutagenesis of the mel and mrl loci, we transformed each mutant with a plasmid carrying the appropriate wild-type chromosomal regions. The mutant, wild-type and complementing strains were then compared for their ability to infect macrophages, and we found that complementation restores the expected phenotype (Fig. 10). The mrlA mutation could be complemented with the mrlA through mrlC region alone, so we suspect that either mrlD and mrlE are in a different transcriptional unit or they are not required for reduction of macrophage infection. The mrlI mutation could be complemented by the entire mrl2 locus but not the mrlI through mrlO region alone, confirming the importance of mrlI for reduction of macrophage infection. These data suggest that the genes within the mel and mrl loci are responsible for the effects of each cosmid on macrophage infection by M. marinum and that gene dosage effects allowed identification of novel genes that are involved in interactions with macrophages by wild-type M. marinum.

**DISCUSSION**

Macrophage infection by bacteria involves a complex interplay between adherence, phagocytosis, and initial intracellular survival. Since mycobacteria are thought to replicate primarily within macrophages during disease, it is likely that proper interactions with macrophages are critical to pathogenesis. In-
Infection of macrophages by *M. marinum* is dependent upon the stage of growth in laboratory medium, suggesting that the genes involved in macrophage infection are regulated. This observation, combined with the ability of gene dosage to increase the expression of genes, was utilized in the present study to identify *M. marinum* genes involved in macrophage infection. This approach is advantageous because it allows the entire genome to be screened using relatively few clones, since cosmids can contain a large numbers of genes. The conclusion that this approach allowed identification of genes involved in the normal interactions of wild-type *M. marinum* with macrophages is supported by the fact that specific mutants in these genes display the expected phenotype and complementation results in recovery of the wild-type phenotype. In addition, the phenotype of each cosmid and mutant was confirmed by microscopy, which suggests that the effect is on macrophage infection rather than on survival either within the cells or during the experimental procedure. Furthermore, studies in *Salmo-

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**FIG. 9.** RT-PCR studies to evaluate the effects of insertion mutations on expression of *melM*, *melN*, *melO*, and *melP* in *M. marinum*. Approximately equivalent amounts of RNA from *M. marinum* wild-type and *M. marinum*, *melM*, *melN*, *melO*, and *melP* mutants were reverse transcribed and subjected to RT-PCR with specific oligonucleotides within *melM*, *melN*, *melO*, and *melP* and 16S rRNA. Equal amounts of each PCR product were loaded on 0.8% agarose gels and compared to the 16S rRNA control RT-PCR for each strain (16S rRNA) carried out on the same samples in the same manner by ethidium bromide staining (A) and densitometry (B). The data shown are representative of two independent experiments where similar results were obtained in both.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Gene</th>
<th>melM</th>
<th>melN</th>
<th>melO</th>
<th>melP</th>
<th>16S rRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><em>melM</em></td>
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<tr>
<td><em>melN</em></td>
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<td><em>melO</em></td>
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<tr>
<td><em>melP</em></td>
<td></td>
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</tbody>
</table>

**A.** Strain comparison of RT-PCR results for wild-type (*WT*) and *M. marinum* mutants.

**B.** Comparison of RT-PCR results for individual genes *melM*, *melN*, *melO*, and *melP* in wild-type and *melM* mutants.

Approximately equivalent amounts of RNA from *M. marinum* wild-type and *M. marinum* *melM*, *melN*, *melO*, and *melP* mutants were reverse transcribed and subjected to RT-PCR with specific oligonucleotides within *melM*, *melN*, *melO*, and *melP* and 16S rRNA. Equal amounts of each PCR product were loaded on 0.8% agarose gels and compared to the 16S rRNA control RT-PCR for each strain (16S rRNA) carried out on the same samples in the same manner by ethidium bromide staining (A) and densitometry (B). The data shown are representative of two independent experiments where similar results were obtained in both.
nella (54) and Legionella (17, 85) strains have yielded similarly promising results using comparable strategies to increase expression of virulence genes to allow their identification by functional assays.

Gene dosage has been used previously to control regulation of genes of interest (2, 8, 77) and has been used successfully by our own group for whole-genome screens for virulence genes in Legionella (17). One caveat of this approach could be that operator regions present on the cosmids might saturate regulators, resulting in down- or upregulation of unlinked loci in the same regulon (50, 53, 77). We have not previously found unlinked loci that were responsible for the observed phenotype, nor was this type of problem observed in the present study, since mutations in the loci identified display the expected phenotype. This problem may have been avoided because our studies have used relatively low-copy-number vectors. The mycobacterial origin of replication on our cosmid is derived from pAL5000, thought to be a low-copy-number plasmid (94). This fact most likely allowed relatively subtle enhancement of the expression of genes that this plasmid carries in our library, rather than extremely robust expression that might be detrimental to bacterial growth in vitro and lead to pleiotropic effects. The conclusion that this library only results in subtle increases in expression is supported by our observation that the presence of a cosmid containing mel2 in M. marinum increases the expression of melF approximately 5- to 10-fold. This level of expression allowed identification of loci that are involved in macrophage infection in wild-type M. marinum, rather than only affecting macrophage infection when overexpressed. These observations suggest that we are examining the natural function of these genes, rather than an indirect effect of overexpressing them.

Our initial screen identified 76 cosmids that had the ability to confer enhanced or repressed macrophage infection to M. marinum. Some of these were false positives, either because the cosmid did not confer the expected phenotype after transformation back into M. marinum or because they did not consistently display the same phenotype. A possible reason that some of the original cosmids did not confer the expected phenotype when transformed back into M. marinum is that a secondary mutation occurred in the bacterial chromosome, which cannot be transferred. However, it is equally possible that mutations or rearrangements occur in the cosmids themselves as they are passaged back through E. coli. This possibility could be examined by going back to the original cosmid clones in E. coli and comparing them to those from M. marinum. Cosmids that did not display a consistent phenotype may do so because they are impacted greatly by the phase of growth of the culture or their phenotype in macrophages is less obvious under laboratory conditions. Neither issue makes the genes that they carry of less interest, although it is equally possible that they are true negatives. The only clones that we carried through our entire analysis in the present study were those that display the expected phenotype in all assays, making it possible that not all genes that could affect macrophage infection have been identified, as yet. Further studies to evaluate such clones in additional assays are ongoing and may result in the identification of additional mel and mrl loci.

Most of the M. marinum mel and mrl mutants constructed had the expected phenotype, but some mutants did not display a difference in macrophage infection compared to the wild type. Mutations in the melM, melN, and mrlO genes do not affect macrophage infection, although mutations in this locus on the cosmid affect the enhancement of macrophage infection. Although this observation could be the result of genetic buffering or redundancy (46), the absence of an effect of these mutations on melP expression could also explain these observations. It appears that melP is expressed from a different RNA transcript, despite the close proximity to mrlO. These observations might suggest that melP is important for the enhanced macrophage infection phenotype conferred by cosmid 2-G2. However, it is possible that the melM-O genes are also involved, explaining their apparent impact on macrophage infection when present on the cosmid, although it is clear that they are not individually necessary for the function of melP in macrophage infection. If the prediction of protease activity for MelM and MelN is correct, other proteases might compensate for a mutation in a single protease, providing functional redundancy. In fact, MelN may compensate for MelM and vice versa. Construction of a double mutant or deletion of both genes would provide some of the reagents necessary to test this possibility. More detailed analysis of this region is needed to

### Table 3. Infection of murine macrophages

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Infected cells</th>
<th>No. of vacuoles/cell</th>
<th>No. of bacteria/vacuole</th>
<th>Infectivity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. marinum::1-B12</td>
<td>51 ± 4.7</td>
<td>1.2 ± 0.6</td>
<td>10 ± 7.8</td>
<td>1.0 ± 0.10</td>
</tr>
<tr>
<td>M. marinum (melL)</td>
<td>3.7 ± 0.9</td>
<td>1.1 ± 0.2</td>
<td>9.5 ± 6.8</td>
<td>9.6 ± 0.77†</td>
</tr>
<tr>
<td>M. marinum::2-G2</td>
<td>49 ± 5.2</td>
<td>2.1 ± 1.6</td>
<td>12 ± 8.3</td>
<td>0.2 ± 0.05*</td>
</tr>
<tr>
<td>M. marinum::4-B2</td>
<td>6.1 ± 2.3</td>
<td>1.0 ± 0.4</td>
<td>16 ± 13</td>
<td>0.6 ± 0.23†</td>
</tr>
<tr>
<td>M. marinum (mrlA)</td>
<td>30 ± 5.3</td>
<td>2.2 ± 1.3</td>
<td>11 ± 6.0</td>
<td>4.3 ± 0.76*</td>
</tr>
<tr>
<td>M. marinum::4-H9</td>
<td>7.2 ± 1.1</td>
<td>1.1 ± 0.3</td>
<td>13 ± 9.1</td>
<td>0.6 ± 0.09*</td>
</tr>
<tr>
<td>M. marinum (mrlO)</td>
<td>37 ± 2.1</td>
<td>1.9 ± 1.4</td>
<td>11 ± 8.3</td>
<td>4.6 ± 0.06*</td>
</tr>
<tr>
<td>M. marinum (mrlL)</td>
<td>28 ± 2.5</td>
<td>1.1 ± 0.6</td>
<td>14 ± 12</td>
<td>2.6 ± 0.23*</td>
</tr>
</tbody>
</table>

a That is, the percentage of cells containing at least one bacterium in a field. The results are the means ± standard deviations for three counts of 25 fields.
b That is, the number of bacterial vacuoles in each cell. Results are the means for three counts of 50 cells.
c That is, the number of bacteria in each vacuole. Results are means ± the standard deviations for three counts of 50 cells.
d The infectivity index was calculated as follows: (% infected cells × the number of vacuoles per cell × the number of bacteria per vacuole for the strain)/(% infected cells × the number of vacuoles per cell × the number of bacteria per vacuole for M. marinum). * Significantly different from wild type M. marinum (P < 0.01); †, significantly different from wild-type M. marinum (P < 0.05).
better understand the interactions of MelM-O with MelP, particularly because our bioinformatic analyses provided little information regarding the predicted functions of these genes.

Mutations in three of the genes—mrlF, mrlK, and mrlL—within mrl2 did not affect macrophage infection. These observations may be for reasons similar to those for the melM-O genes. However, since two of these genes are on the periphery of the locus, these analyses may have simply allowed a more refined definition of the functional locus. The need for the entire region, rather than just the mrlJ-O region, to complement a mrlI mutation suggest that at least mrlI is required for repression of macrophage infection. In the case of mrlK, the mutant may not display a phenotype due to the production of a functional protein product, since the mutation is very near the predicted carboxy terminus. Thus, we cannot, at this point, rule out a role for mrlK in macrophage infection, and the entire region from mrlG to mrlN may be involved. Construction of additional mutations and complementing constructs, as well as more detailed analysis of the biochemical activities of the product of each gene, should improve our understanding of the role of this locus in macrophage infection.

Interestingly, we identified cosmid clones, now designated mrl for “mycobacterial repressed macrophage infection loci,” that consistently confer repressed macrophage infection to M. marinum. This observation may be due to increased expression of repressors that control macrophage infection or genes that are involved in antiphagocytic activities. Antiphagocytic activity is often attributed to capsule on pathogenic organisms (4, 10, 25, 31, 39, 48, 58, 60, 90, 91, 105), which fits well with the predicted role of the mrl genes in cell wall and lipid biosynthetic pathways. Since tuberculosis and other mycobacteria can produce capsule (34, 55, 75, 76, 87, 93), it is tempting to speculate that these genes are involved in the synthesis of a capsule-like structure during certain stages of infection. However, it is equally likely that modification to the mycobacterial cell wall can modulate phagocytosis similar to the cell wall of M. leprae (70–72). Since mycobacteria are normally considered intracellular pathogens, the fact that there are stages of mycobacterial infection where the bacteria are primarily found extracellular (26, 56, 61, 99, 100) and may be expressing antiphagocytic activities is only rarely examined. Examples of stages where extracellular bacteria are found during tuberculosis include within liquefied lesions, on the periphery of hypoxic lesions, and possibly during dissemination (21, 22, 26, 56, 59, 61, 62). Environmental mycobacteria are likely to require the ability to persist extracellularly for an extended period of time, particularly in water environments where nutrients are usually dilute and protozoa that allow intracellular growth (15, 45, 51, 52) may be sparse (40, 43, 63, 102–104). Therefore, it seems likely that the ability to express an antiphagocytic activity at specific stages of disease would provide an advantage for pathogenic and environmental mycobacteria. These observations emphasize the importance of further studies to better understand the importance of the genes we have identified during pathogenesis.

These observations, along with our earlier studies designed to elucidate the mechanisms used by M. marinum to control infection of macrophages (32, 66), suggest that interactions of M. marinum with macrophages are complex and multifaceted. Although it seems likely that we do not yet have a comprehen-

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**FIG. 10.** Cell association of M. marinum wild type, M. marinum mutants, and mutants carrying complementing constructs with RAW 264.7 cells. All data are expressed relative to the cell association of wild-type M. marinum that was included as an internal control in all experiments. The data and error bars represent the means and standard deviations, respectively, of three independent experiments carried out in triplicate. ***, P < 0.01 compared to wild-type M. marinum.**
sive picture of how _M. marinum_ parasitizes macrophages, we did recover the _mel1_ and _mel2_ loci previously identified (32), confirming that these loci are important for interactions with macrophages and that our screen was relatively comprehensive. Four additional cosmids, beyond those characterized here in detail, were identified that display unique restriction maps and do not carry previously recognized macrophage infection loci. Studies are ongoing to better characterize these cosmids, which should provide additional insight into the molecular mechanisms of macrophage infection and may identify overlap with our earlier screen by transposon mutagenesis (66). Continued analysis of the molecular mechanisms of action of these macrophage infection loci should provide us with a much better understanding of this complex, but critical, aspect of mycobacterial pathogenesis.

ACKNOWLEDGMENT

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REFERENCES


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