Transposon Mutagenesis of *Mycobacterium marinum* Identifies a Locus Linking Pigmentation and Intracellular Survival

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Pathogenic mycobacteria survive and replicate within host macrophages, but the molecular mechanisms involved in this necessary step in the pathogenesis of infection are not completely understood. *Mycobacterium marinum* has recently been used as a model for aspects of the pathogenesis of tuberculosis because of its close genetic relationship to *Mycobacterium tuberculosis* and because of similarities in the pathology and course of infection caused by this organism in its natural hosts, fish and frogs, with tuberculosis in humans. In order to advance the utility of the *M. marinum* model, we have developed efficient transposon mutagenesis of the organism by using a *Drosophila melanogaster mariner*-based transposon. To determine the efficiency of transposition, we have analyzed pigmentation mutants from the transposon mutant library. In addition to insertions in four known genes in the pathway of pigment biosynthesis, two insertions in novel genes were identified in our mutant library. One of these is in a putative inhibitor of the carotenoid biosynthesis pathway. The second unexpected insertion is in an intergenic region between two genes homologous to *Rv2603c* and *Rv2604c* of *M. tuberculosis*. In addition to a pigmentation defect, this mutant showed increased susceptibility to singlet oxygen and grew poorly in murine macrophages. Complementation with *M. tuberculosis* genomic DNA encompassing *Rv2603c* to *Rv2606c* corrected the pigmentation and growth defects of the mutant. These data demonstrate the utility of *mariner*-based transposon mutagenesis of *M. marinum* and that *M. marinum* can be used to study the function of *M. tuberculosis* genes involved in intracellular survival and replication.

The global burden of *Mycobacterium tuberculosis* infection is overwhelming, with approximately one-third of the world's population infected and 2 million deaths each year (9). As an intracellular obligate human pathogen, *M. tuberculosis* has evolved a complex parasitic lifestyle and sophisticated mechanisms to combat host defense machinery. It modulates a number of host cell processes, including inhibition of acidification of the bacterium-containing phagosome and its maturation to a phagolysosome (7, 34), inhibition of host cell apoptosis to benefit intracellular bacterial growth and to evade detection and destruction by the host immune system (12, 17), and inhibition of the macrophage response to gamma interferon, thus altering antigen presentation (20, 24, 42). All of these mechanisms are thought to lead ultimately to enhanced survival of *M. tuberculosis* within macrophages. Despite this understanding of the exploitation of host cell processes by mycobacteria, the bacterial components responsible for mediating these processes are largely unknown. In addition to these basic issues in host-microbe interaction, efforts to understand the molecular bases for mycobacterial subversion of host defense has value for the development of new antibiotics that target these bacterial mediators. One direct and effective approach to this goal is to generate, isolate, and characterize defined mutations that affect steps in the pathogenesis of infection.

Transposon mutagenesis is a powerful technique for the identification of bacterial virulence factors which uses a traceable mobile element to randomly disrupt genes in the chromosome. Several transposable elements have been identified and isolated from *Mycobacterium* species, such as IS*1096* (from *Mycobacterium smegmatis*) and IS*6110* (from *M. tuberculosis*), and used to generate transposon mutants in mycobacteria (15, 21). However, these transposons do not function in all mycobacteria (22, 41) and seem to require recognition of relatively large sequences for insertion (21). Therefore, the development of a transposon that functions in a broad host range, recognizes a short sequence for insertion, and segregates from transposase upon chromosomal insertion should provide an extremely useful and potentially more-general tool for genetic studies of mycobacteria as well as other microorganisms. Such potentially valuable transposon systems have been identified, initially in insects, and include members of the *mariner*/*Tc1* transposon superfamily (16, 27). *Mos1* (from *Drosophila melanogaster* [32]) and *Himar1* (from horn fly *Haematobia irritans* [19]) are among the best-studied *mariner* transposons that target insertion at a TA dinucleotide recognition site (27). *mariner* transposons are able to insert into diverse genomes of distantly related organisms, including both eukaryotes and prokaryotes (3, 11, 14, 19, 33, 36).

Mycobacterium marinum, a fish, amphibian, and opportunistic human pathogen, has recently been developed as a model system for studying *M. tuberculosis* pathogenesis (6, 28, 29, 31, 40). *M. marinum* is phylogenetically closest to members of the *M. tuberculosis* complex (43), and the infection it causes in its natural hosts manifests pathological hallmarks of tuberculosis, including granulomas (6, 31, 40). These observations suggest

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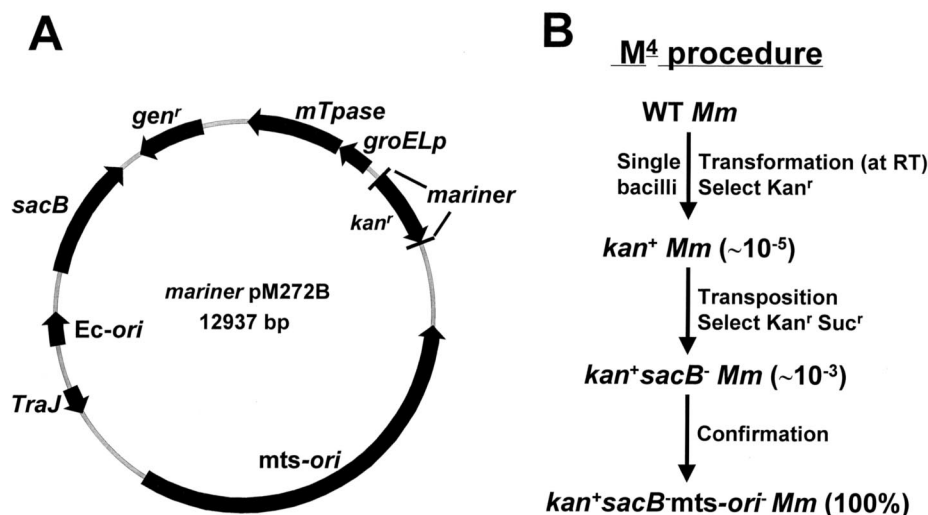
FIG. 1. Map of the *mariner* transposon mutagenesis vector pM272B (A) and the M⁴ procedure (B). (B) Transposon insertion mutants were isolated by a two-step selection procedure, first for Kan^r and second for Kan^r and Suc^r. The numbers in parentheses indicate the frequencies of the occurrence of resistant colonies. Mm, *M. marinum*; Ec-*ori*, *E. coli* origin of replication; WT, wild type; RT, room temperature; *sacB*^−, *sacB* deficient; mts-*ori*^−, mts-*ori* deficient.

that many of the basic mechanisms of disease initiation are conserved between *M. marinum* and *M. tuberculosis*. *M. marinum* grows significantly faster than *M. tuberculosis* and other slow-growing mycobacteria, with a generation time of 4 h compared to 20 h for *M. tuberculosis* (28). Therefore, *M. marinum* offers an attractive and potentially valuable model for studying the molecular biology of mycobacterial pathogenesis. In the present work, we demonstrate that a *mariner*-based transposon system can be used for facile generation of random mutations in *M. marinum* (*M. marinum mariner*-based mutagenesis [M⁴]) and that these mutants can be used to identify and study genes involved in intracellular survival.

MATERIALS AND METHODS

Bacterial strains and media. *M. marinum* strain M (the kind gift of Lalita Ramakrishnan, University of Washington, Seattle) was cultured either on Middlebrook 7H9 (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase enrichment (7H9 broth) or on Middlebrook 7H10 agar (Difco), supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase enrichment. The initial inoculum was cultured at 32°C in 7H9 broth to mid-log phase with slow shaking (100 rpm), and the culture was concentrated in 7H9 broth containing 30% glycerol, aliquoted, and frozen at −80°C (passage 1). Subsequent cultures were carried out by thawing frozen aliquots of passage 1 on ice and growing them in 50 ml of 7H9 broth for 4 days to reach an optical density at 600 nm (OD_{600}) of ~1.5 (passage 2). For infection or transformation experiments, passage 2 cultures were diluted 50 times and cultured for an additional 48 h to reach an OD_{600} of ~1.8 to 1.9 (post-log phase) (passage 3). When required, the Middlebrook media were supplemented with the antibiotic kanamycin (KAN) or Zeocin (ZEO) (Invitrogen) at a concentration of 50 or 100 µg/ml, respectively. To test strains of *M. marinum* for sensitivity to light induction of methylene blue, passage 3 cultures were centrifuged at 1,000 × g for 5 min. The supernatants containing single bacilli were diluted, and small aliquots were spotted onto 7H10 plates containing 25 µM methylene blue and incubated in the presence of light. *Escherichia coli* DH5α (Invitrogen) was used for DNA cloning procedures. *E. coli* was grown in Luria-Bertani broth or on agar (Difco) at 37°C. When required, Luria-Bertani media were supplemented with the following antibiotics: carbenicillin at 50 µg/ml, KAN at 30 µg/ml, gentamicin at 15 µg/ml, or ZEO at 25 µg/ml. Standard molecular biology techniques involving cloning in *E. coli* were performed as previously described (35).

Construction of *mariner*-based transposon mutagenesis vectors. A series of *mariner*-based transposon mutagenesis vectors were constructed by inserting different *mariner* transposon cassettes into the plasmid backbone of pPR27 (26), a mycobacterial shuttle vector that contains the *sacB* gene from *Bacillus subtilis* (38), the Gen^r marker, an *E. coli* origin of replication, and a thermosensitive *Mycobacterium ori* (mts-*ori*). These *mariner* transposon cassettes contained the following elements constructed in different orientations: the 5′ and 3′ inverted repeats of *Mos1* surrounding the Kan^r cassette of Tn*903* (from *MosK*ΔCD [44]) and the *Mos1* transposase (*mTpase* from pET3a-Tpase [44]), which was under the control of the mycobacterial *groEL* promoter (*groELp* from pSMT3 [13]). These *mariner*-based transposon vectors had four different orientations of the *mariner* and the *groEL* promoter-*mariner* transposase cassettes, namely, pM271A, pM271B, pM272A, and pM272B. The map of one of these vectors, pM272B, is shown in Fig. 1A.

Transformation of *M. marinum* with *mariner*-based transposon mutagenesis vectors and selection for transposition mutations. A passage 3 culture of the wild-type *M. marinum* strain M was washed three times in a wash buffer containing 10% glycerol and 0.05% Tween 80 and resuspended in an appropriate volume of wash buffer to an OD_{600} of ~1.5 (competent bacteria) prior to electroporation. In preliminary experiments, we examined growth stage, bacterial clumping, electroporation conditions, and temperature as variables that could affect the efficiency of transformation. In addition, we tested one-step selection against sequential positive and negative selections for transposition of the mini-transposon into the bacterial chromosome. Finally, we compared the four transposon mutagenesis vectors described above for transformation and transposition efficiencies. Our final M⁴ protocol for efficient transformation and transposition is illustrated in Fig. 1B. *M. marinum* bacteria grown to an OD_{600} of ~1.9 were repeatedly passed through a 26-gauge needle and then centrifuged at 500 × g to remove clumps. All procedures were carried out at room temperature. Competent bacteria (200 µl) were mixed with ~2 µg of plasmid pM272B, incubated for 10 min to facilitate DNA adsorption, transferred into a 0.2-cm-gap electroporation cuvette, and subjected to a pulse of 2.5 kV, 25 µF, and 1,000 Ω (Gene Pulser II; Bio-Rad). Electroporated bacteria were incubated in 10 ml of 7H9 broth at 30°C for 6 h or overnight before being plated onto 7H10 agar containing KAN (positive selection) at ~10^8 bacteria/plate. Transformants were observed after 6 to 7 days of incubation at 32°C. As a negative control to estimate the level of spontaneous resistance to KAN, competent bacteria were electroporated with the transposon-free vector pPR27 and further handled in the same way. The transformation efficiency for pM272B was ~1.2 × 10^4 CFU/µg of DNA/10^9 mycobacteria electroporated compared to an extremely low background of ~10 CFU/µg of DNA/10^9 mycobacteria electroporated for pPR27. To enrich and select for transposition mutations, the transformants obtained after 7 to 10 days of incubation were pooled (~100 colonies/pool) and cultured in 50 ml of 7H9 broth containing KAN to an OD_{600} of ~1.5. Single bacteria were obtained as described above and plated onto multiple 7H10 plates containing KAN and 10% sucrose (Suc) (negative selection) at ~10^5 bacteria/plate. Transposon

insertion mutants were obtained after 7 to 10 days of incubation at 32°C. The transposition efficiency was approximately 3×10^2 Kan^r and Suc^r bacteria/10^5 Kan^r bacteria. To ensure that each colony arose from a single mutant, bacteria were resuspended, passed through a 26-gauge needle, recentrifuged, and replated. Individual mutant colonies were cultured in 1 ml of 7H9 broth containing KAN to late-log phase, mixed with glycerol (30%), transferred to 96-well plates, and frozen at −80°C (passage 1).

Detection of *mariner*-based transposon insertions by PCR. Transposon insertion mutants selected on 7H10 agar containing KAN and Suc were initially characterized by PCR designed to amplify elements that define transposon insertion into the chromosome, including Kan^r, *sacB*, and mts-*ori*. The primers used for detecting Kan^r were KN-15′ (5′ GAGGCAGTTCCATAGGATGG) and KN-23′ (5′ TCAGGTGCGACAATCTATCG). The primers used for detecting *sacB* were SACB-15′ (5′ ACCCATCACATATACCTGCC) and SACB-23′ (5′ ATCGTTAGACGAAATGCCGT). The primers used for detecting essential open reading frames (ORFs) 1 and 2 of mts-*ori* were MORI-15′ (5′ AGGACCGCAAAGATGTTCCG) and MORI-23′ (5′ GATCTATGCCGAGT GCCACG). Transposon insertion mutant bacteria were resuspended in H$_2$O, and ~10^5 bacteria were added to each PCR as a source of template DNA. The PCR products were examined by agarose gel electrophoresis.

Southern blot analysis. Passage 3 cultures (OD$_{600}$ of ~1.0, 50 ml) of strains of *M. marinum* were used for isolation of genomic DNA by the cetyltrimethylammonium bromide method (21, 23) with minor modifications. After delipidation with 1 ml of chloroform-methanol (2:1) for 5 min, the bacteria were resuspended in 10 mM Tris–1 mM EDTA (pH 8.0), digested with lysozyme (10 mg/ml) at 37°C for 5 h, and further digested with proteinase K (200 μg/ml) in 1% sodium dodecyl sulfate at 55°C for an additional 3 h. Fully digested bacteria were extracted once with cetyltrimethylammonium bromide-NaCl and then twice with phenol-chloroform-isoamyl alcohol (25:24:1) before precipitation of genomic DNA with isopropanol. Approximately 1 μg of Tris-HCl-dissolved genomic DNA was digested with *Pst*I or *Bam*HI and subjected to Southern blotting by following standard procedures (32). The probe was labeled and hybridization was detected by using an enhanced chemiluminescence random prime labeling and detection system (Amersham Pharmacia, Piscataway, N.J.) according to the manufacturer's instructions.

Recovery of *mariner* transposon insertion sites from mutants and sequencing of the insertion junction. Genomic DNA (~200 ng) of transposon insertion mutants was digested with *Pst*I or *Bam*HI, and the restriction fragments were self-ligated. The genomic sequence surrounding the transposon was amplified by performing inverse PCR with the outward primers 821A (5′ CGCATCTTCCC GACAACGCAGACCGTTCC) and 822A (5′ TAATCGCGGCCTCGAGCAA GACGTTTCCCG). Inverse PCR products were confirmed for the appropriate sizes by agarose gel electrophoresis or verified by Southern blotting and then sequenced with either primer 821A or primer 822A.

Isolation of pigmentation mutants of *M. marinum* and localization of the transposon insertions. A pool of ~15,000 transposon insertion mutants was screened for the isolation of pigmentation mutants. After growth of the mutant colonies to a visible size in the dark, the agar plates were exposed to light on the bench top. Wild-type *M. marinum* turned gold upon exposure to light in approximately 48 h. Pigmentation variants were identified that either did not produce colored pigment or were red rather than gold. The chromosomal location of the transposon insertion for mutant MmR03 relative to those of other pigment mutants was determined by PCR with genomic DNA from an individual mutant as a template and a combination of primer R03F1 (5′ GTGCCACCGTTCCA GTGGTTTGGGTGCCC) or R03R1 (5′ GGCGCACCCAAACCACTGGAACG GTGGCAC) with 821A or 822A. The R03F1 and R03R1 primers were designed based on the genomic sequences surrounding the transposon insertion in MmR03.

Construction of mycobacterial expression vector pLYG204.*Zeo*. A mycobacterial expression vector containing a Zeo^r determinant was constructed based on pMV261.*kan* (39). The Zeo^r gene was PCR amplified from pEM7/*Zeo* (Invitrogen) and ligated into *Nhe*I- and *Spe*I-digested pMV261.*kan* to generate pLYG204.*Zeo*, replacing the Kan^r gene of pMV261 with the Zeo^r gene.

Complementation of *M. marinum* pigmentation mutants. To complement the *crtB* defect of mutant MmW01, the wild-type *crtB* gene coding sequence (30) was PCR amplified with *M. marinum* genomic DNA as the template and primers mcrtBF1 (5′ GCTACGTATTCGCAGTGAATTGGACGCCGCCG, with an engineered *Sna*BI site underlined) and mcrtBR1 (5′ GGAATTCGCTCATGCGG CACCGTACTCGGAG, with an engineered *Eco*RI site underlined). The PCR product was cloned into *Eco*RV-digested pBluescript II KS(+) to generate pBSm*crtB*. The *Sna*BI-to-*Eco*RI fragment of pBSm*crtB* was subsequently cloned into *Msc*I- and *Eco*RI-digested pLYG204.*Zeo* to create pLYG601, in which the 5′ end of the *crtB* coding sequence was placed in frame with the plasmid-derived start codon following the *groEL* promoter. pLYG601 was electroporated into

MmW01 and MmR02, and the transformants were selected on 7H10 plates containing ZEO. The empty vector pLYG204.*Zeo* was used as a negative control for complementation.

To restore the defects of mutant MmW04, the *M. tuberculosis* genomic fragment encompassing *Rv2603c* to *Rv2606c* (corresponding to bases 2930803 to 2934068 of the published H37Rv sequence [8]) was PCR amplified from genomic DNA of the Erdman strain and ultimately cloned into pLYG204.*Zeo* to create pLYG602. The electroporation of pLYG602 into MmW04 and the selection of transformants were performed as described above.

Infection of J774 macrophages by *M. marinum*. The mouse macrophage-like cell line J774 (ATCC TIB67) was maintained at 37°C in 5% CO$_2$ in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; HyClone). Immediately prior to infection, J774 monolayers were washed once with FBS-free DMEM. Single disaggregated bacilli of passage 3 cultures of wild-type, mutant, or complemented *M. marinum* (OD$_{600}$, ~1.9) were added to J774 monolayers at a multiplicity of infection of 3 and incubated for 2 h at 32°C and 5% CO$_2$. At the end of the 2-h incubation period, infected monolayers were washed twice with DMEM and further incubated in DMEM containing 2% FBS and 200 μg of amikacin/ml for 2 h to kill extracellular bacteria. At the end of the antibiotic treatment, monolayers were washed twice with DMEM and incubated in DMEM containing 1% FBS at 32°C and 5% CO$_2$. This was designated time zero. The incubation medium was changed at 48 h. At 96 h after infection, bacteria were enumerated by combining supernatants and hypotonically lysed cell monolayers for quantitation of CFU. *M. marinum* grows poorly in tissue culture medium containing small amounts of FBS (data not shown), and at 96 h following infection, the cells infected with wild-type *M. marinum* showed some cytotoxicity. Therefore, the bacteria in the supernatant (always <10% of the total) represent bacteria that have escaped from macrophages, probably after lysis.

Similarity searches. Similarity searches were performed by using BLASTp and BLASTn (2), as appropriate, at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/). Similarity searches against the incomplete *M. marinum* genome sequences were performed at the Sanger Center website (http://www.sanger.ac.uk/Projects/M_marinum/). Domain similarities were found by using the conserved domain search at the National Center for Biotechnology Information.

RESULTS

Molecular characterization of transposon insertion into the chromosome of *M. marinum*. *M. marinum* strain M was transformed with the *mariner*-based transposon mutagenesis vector pM272B (Fig. 1A), and the transposition mutants were selected by using the M^4 protocol described in Materials and Methods and illustrated in Fig. 1B. Since the vector contains the *mariner* transposon with a Kan^r gene for positive selection and a *sacB* gene for counterselection, the Kan^r and Suc^r bacteria potentially had a chromosomal insertion(s) by *mariner*, with loss of the delivery vector. To test this, we examined the presence of the Kan^r gene, *sacB*, and mts-*ori* by PCR in 15 randomly picked Kan^r and Suc^r colonies (data not shown). All contained the Kan^r gene but had lost mts-*ori* and *sacB*, suggesting the integration of the transposon to the chromosome. Southern blots of *Pst*I-digested or *Bam*HI-digested genomic DNA probed with the transposon sequences yielded a single hybridization fragment for each of the colonies, suggesting a single transposition event for each colony, and each hybridization fragment had a unique size, indicating that the transposon had inserted at different sites of the chromosome (data not shown). There was no hybridization with sequences from the plasmid backbone (data not shown), indicating loss of the delivery vector sequence. In subsequent Southern blot analyses of over 50 colonies, only 2 have shown evidence of more than one insertion, suggesting that the vast majority of colonies arose from single transposition events. Additional proof for transposon insertion into the chromosome was obtained by
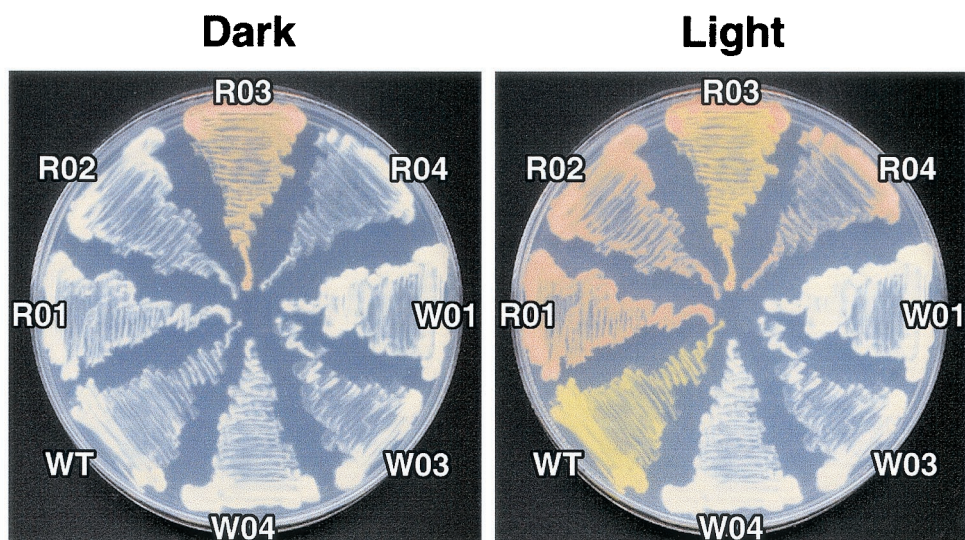
FIG. 2. Pigmentation of *M. marinum* mutants in the presence or absence of light. *M. marinum* colonies were grown in the dark (left) and then exposed to light for 48 h (right). Wild-type (WT) *M. marinum* is pigmented only after light exposure. R01, MmR01; R02, MmR02; R03, MmR03; R04, MmR04; W01, MmW01; W03, MmW03; W04, MmW04.

hybridization of undigested genomic DNA from the Kan^r and Suc^r bacteria with the Kan^r gene probe: hybridization was detected only at the size range for genomic DNA (data not shown). Definitive proof for transposon insertion into the chromosome was obtained by sequencing across the transposon insertion junctions outwards from *mariner*. All sequences (now from 40 independent colonies) have a TA dinucleotide at the transposon-chromosome junction, followed by diverse GC-rich genomic sequences. Taken together, these results prove that M^4 leads to integration of the *mariner* transposon into the chromosome of *M. marinum* at random sites, usually as a single copy per genome, and that the delivery vector is completely lost upon transposition.

Identification and characterization of transposon insertion within genes of the carotenoid biosynthesis pathway. Using the M^4 protocol, we obtained a *M. marinum* mutant library consisting of ~15,000 individual transposon insertion mutants from a single electroporation. To evaluate the randomness of *mariner* transposition, we screened for pigmentation mutants. Assuming that *M. marinum* has about the same number of genes as *M. tuberculosis* (~4,000), if transposition is random, the Poisson statistic predicts that the likelihood of failure to find in this library at least one insertion in any gene involved in pigmentation is approximately 2%. Normally, *M. marinum* is gold when exposed to light because of the production of carotenoid pigments (30, 45). We identified eight colonies that demonstrated pigmentation variations: four of them lost pigment, resulting in white colonies (MmW01 to MmW04); three others showed a red colony color upon exposure to light (MmR01, MmR02, and MmR04); and one appeared red in the dark and developed a gold color after exposure to light (MmR03) (Fig. 2). The sequences of the transposon insertion junctions for all eight mutants showed that seven of them were unique insertions. MmW01 and MmW02 showed the same sequence, which could be due either to duplication of siblings or to independent insertions at the same site.

BLAST searches performed for these seven unique insertion junction sequences revealed that five of them matched segments of four previously identified contiguous ORFs of *M. marinum* in a carotenoid biosynthesis locus (30). As shown in Fig. 3A, MmW01 and MmW02 had an insertion in the *crtB* gene and MmW03 had an insertion in the *crtI* gene. MmR01, MmR02, and MmR04 had independent insertions in two previously identified (30) but uncharacterized ORFs (ORF-3 and ORF-4) of this *M. marinum* locus. We found that ORF-3 and ORF-4 have significant homology to *crtYc* and *crtYd*, respectively, of *Mycobacterium aurum* and *Brevibacterium linens* (18, 30, 45). Both the sequence and genomic organization of *crtI*, *crtB*, *crtYc*, and *crtYd* are highly conserved among *M. marinum*, *M. aurum*, and *B. linens* (18, 30, 45). As diagrammed in Fig. 3A, our transposon mutant library contained at least one mutation in each of these ORFs identified in the *M. marinum* carotenoid biosynthesis gene locus. Although only one locus was analyzed, this frequency is consistent with the generation of random mutations throughout the *M. marinum* genome by *mariner*.

CrtB (phytoene synthase) and CrtI (phytoene desaturase) are thought to catalyze two consecutive biochemical reactions leading to the synthesis of lycopene, which has a red pigment (Fig. 3A) (45). Mutations in either of these genes in MmW01/MmW02 and MmW03 produced no colored pigments (intermediates before lycopene in the pathway are not bright colored) (Fig. 2). MmR01, MmR02, and MmR04 had mutations in the *crtY* locus, which controls the conversion of lycopene (red) to beta carotene (gold) (18, 45). As predicted, these mutants accumulated lycopene and were therefore red (Fig. 2).

In addition to these five insertions within the locus known to be involved in carotene biosynthesis, we identified two mutants, MmR03 and MmW04, that had insertions within novel genes. The sequence at the transposon insertion junction of MmR03 was used in a BLAST search of the *M. marinum* database at the Sanger Center (http://www.sanger.ac.uk /Projects/M_marinum/). The insertion junction sequence was
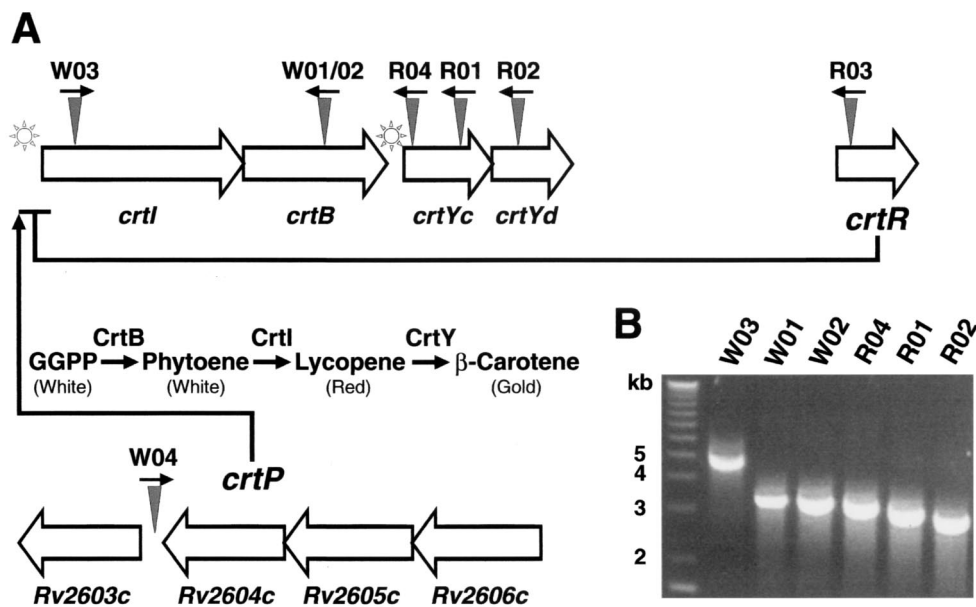
FIG. 3. *M. marinum* carotenoid biosynthesis and regulatory genes, their genomic organization, and location of transposon insertions. (A) The known carotenoid biosynthesis locus in *M. marinum* is shown as a gene cluster from *crtI* to ORF-4. Below these genes the biosynthetic pathway is depicted; GGPP represents the initial substrate, geranylgeranylpyrophosphate. The novel *crtR* and *crtP* genes encode a putative repressor and a positive regulator, respectively, for the carotenoid biosynthesis locus. *crtP* indicates the locus containing the *Rv2606c* to *Rv2603c* homologues. The star symbols represent putative light-inducible promoters. Triangles indicate sites of transposon insertion, and arrows represent the orientation of the Kan[r] gene in the chromosome. R01, MmR01; R02, MmR02; R03, MmR03; R04, MmR04; W01, MmW01; W02, MmW02; W03, MmW03; W04, MmW04. (B) The location of transposon insertion in mutant MmR03 is shown relative to those in other pigmentation mutants as detected by a PCR that amplifies the genomic sequence between insertions. The distance of the transposon insertion in MmW04 from others in the carotenoid biosynthesis locus is insufficiently detected by PCR, and the results were not shown.

identical in reverse orientation to bases 77208 to 77843 of the assembled contig sequence mar502f07.plk. This sequence is within a hypothetical ORF that contains a domain homologous to the helix-turn-helix MarR family of transcription repressors (1). Thus, the gene interrupted by the transposon in MmR03 (*crtR*) is likely a repressor of the *crt* locus, consistent with production of red pigment in the mutant in the absence of light induction. We examined the chromosomal location of the MmR03 transposon insertion relative to the known carotenoid biosynthesis genes by a PCR protocol that amplified sequences between the transposon inserts. As shown in Fig. 3B, the MmR03 insertion is approximately 2.8 kb downstream from that of MmR02.

The second unanticipated transposon insertion (in MmW04) was in an intergenic sequence immediately downstream of a coding sequence homologous to *Rv2604c* of *M. tuberculosis* (8) (Fig. 3A). In the *M. tuberculosis* genome, *Rv2604c* and the two upstream genes, *Rv2605c* and *Rv2606c*, are likely in a single operon (Fig. 3A). *Rv2603c* is 130 bp downstream of *Rv2604c* and thus might also be part of the putative operon. Both *Rv2603c* and *Rv2604c* are highly conserved but without known function. *Rv2605c* is homologous to acyl coenzyme A thioesterase II genes in bacteria, and *Rv2606c* is homologous to a family of genes conserved among prokaryotes and eukaryotes that confer resistance to singlet oxygen generated by light induction of photosensitizers such as methylene blue. Consistent with the possibility that these genes have a similar function in mycobacteria, MmW04 showed a fivefold increase in sensitivity to singlet oxygen produced by light induction of methylene blue (Fig. 4A). The increased sensitivity did not result from the

loss of pigment because the other white mutants were equivalent to the wild type in this assay. Unlike the other pigment mutants, MmW04 also showed reduced intracellular growth in J774 cells, with an eightfold-lower CFU count than the wild type at 4 days after infection (Fig. 4B). Thus, in addition to the lack of pigmentation, this mutant showed a general decrease in the ability to survive environmental stress. The transposon insertion in MmW04 is not sufficiently close to the known carotenoid biosynthesis genes to be linked by PCR or to be on the same *Bam*HI fragment (~10 kb) as the pigmentation locus, as assessed by Southern blot analysis (data not shown).

Complementation of the pigmentation mutants. To prove that the mutant phenotypes were specifically caused by the transposon insertions, we carried out complementation studies by expressing the corresponding wild-type coding sequence within an appropriate mutant. We first developed a mycobacterial expression vector, pLYG204.*Zeo*, which carries a Zeo[r] gene immediately downstream from a mycobacterial *groEL* promoter and multiple cloning sites for the insertion of a gene of interest. The *M. marinum crtB* gene coding sequence (30) was cloned into the multiple cloning site of pLYG204.*Zeo* and transformed into MmW01, and the Zeo[r] colonies were examined for complementation of pigmentation. As shown in Fig. 5A, the mutant MmW01 was fully complemented by episomal expression of *crtB* to produce wild-type pigment. Complementation of MmW01 by *crtB* was specific, since the empty vector did not complement (data not shown), and the expression of *crtB* did not complement the MmR02 mutant (Fig. 5A).

The homology of the insertion site in MmW04 with an *M. tuberculosis* locus provided an opportunity to assess the ability
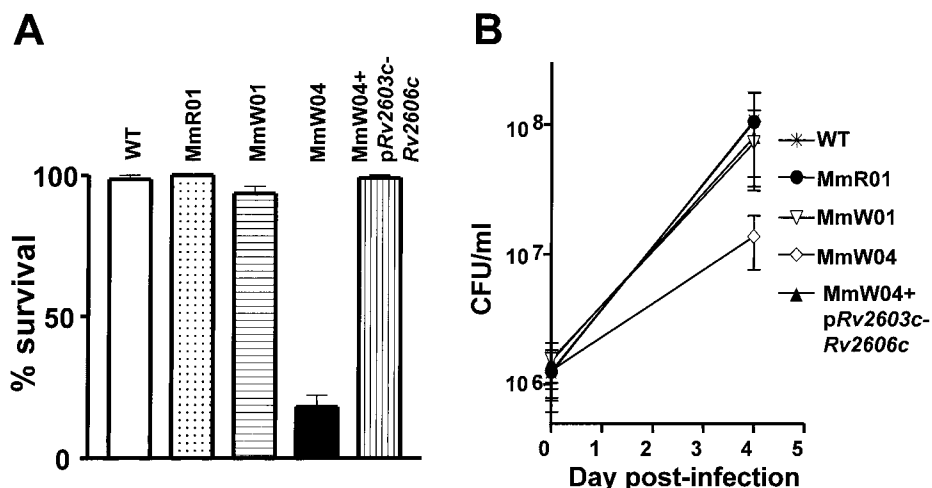
FIG. 4. Examination of resistance to light induction of methylene blue (A) and intracellular replication within J774 macrophages (B) for *M. marinum* pigmentation mutants and the complemented strain. (A) Dilutions of *M. marinum* strains were plated onto 7H10 plates containing 25 μM methylene blue and incubated in the dark or in ambient light for 7 days. The data are percentages of CFU in the dark divided by CFU in ambient light for each strain. Data for the wild type (WT), three pigmentation mutants, and the complemented MmW04 strain are graphed. The data are means of results from two experiments under the same conditions. (B) CFU associated with J774 cells were enumerated at time zero and after 96 h of intracellular growth. Data for the same strains as those used for panel A are graphed. The means and standard deviations of results from quadruplicate experiments performed on two different days are depicted. pRv2603c-Rv2606c indicates complementation with *M. tuberculosis* genes encompassing *Rv2603c* to *Rv2606c*. Differences between MmW04 and the WT or the complemented strain are statistically significant ($P < 0.05$); the levels of growth of the WT and the complemented mutant are not different.

of *M. tuberculosis* genes to function in *M. marinum*. Because of the possibilities that (i) several of the genes in the region from *Rv2603c* to *2606c* are likely in an operon, (ii) the direction of transcription of the transposon insertion is opposite to the direction of transcription of these genes (Fig. 3A), leading to the possibility of antisense transcripts arising from the transposon, and (iii) the insertion was in a regulatory region for one or more of these genes, we attempted to complement the MmW04 phenotypes by using an *M. tuberculosis* genomic fragment encompassing *Rv2603c* to *Rv2606c*. An ~3.3-kb genomic

fragment containing these genes was PCR amplified from the *M. tuberculosis* Erdman strain and cloned into pLYG204.*Zeo*. The expression plasmid was transformed into MmW04, and the phenotypes of the transformants were determined. The defects of the MmW04 mutant in pigmentation, resistance to singlet oxygen, and intracellular growth were completely restored to wild-type levels by expression of this *M. tuberculosis* genomic fragment (Fig. 4 and 5B). Thus, an *M. tuberculosis* operon was able to complement the defects of the MmW04 mutant.
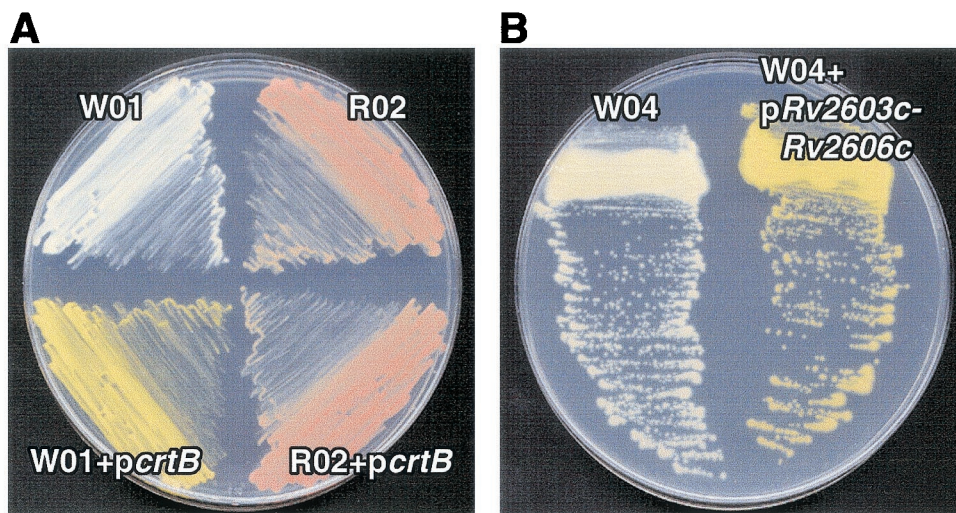

FIG. 5. Complementation of carotenoid biosynthesis for *M. marinum* mutants. (A) A plasmid expressing the *M. marinum crtB* gene was introduced into both MmW01 (W01) and MmR02 (R02), but it restored pigment synthesis only in MmW01. (B) A plasmid expressing *M. tuberculosis* genes *Rv2603c* to *Rv2606c* was used to complement the MmW04 (W04) mutant to produce wild-type pigment. Both plates were exposed to light for 48 h prior to imaging.

DISCUSSION

We have developed a method for M⁴ that is efficient and simple. The *mariner* transposon inserts at a TA recognition site in *M. marinum*, without any other apparent consensus sequence requirement, as is the case in other organisms. This property allowed for the construction of a transposon mutant library containing remarkably random insertions. There are ~70,000 possible *mariner* insertion sites in a typical mycobacterial chromosome. Since there are ~4,000 genes, this suggests that there are many potential sites of transposition in each gene. Indeed, in our library of ~15,000 individual mutants, we found mutations in multiple expected genes of *M. marinum* in the carotenoid biosynthesis pathway, suggesting that with M⁴ it will be possible to generate mutations within most genes compatible with survival in vitro. The ~15,000 mutants that we screened were generated from a single electroporation, and a mutant library with a larger size can be easily obtained by plating out more Kanʳ bacteria on Kan- and Suc-containing plates, which could potentially saturate all possible TA sites. High-efficiency transposition by a slightly different *mariner* transposon has been reported for *E. coli* and several mycobacterial species (33). Together, these studies demonstrate the utility of the *mariner* transposon in random bacterial mutagenesis.

Our first mutant screen has enhanced the understanding of the regulation of carotenoid biosynthesis in *M. marinum*. We found one mutant (MmR03) that produces a red pigment in the dark, unlike wild-type *M. marinum*, which is white in the absence of light. Since the MmR03 mutant accumulated lycopene in the dark and produced beta carotene upon exposure to light, the normal gene product (CrtR) likely has an inhibitory activity on *crtI* and *crtB* that is released upon light induction. Consistent with this, the ORF interrupted by the transposon in MmR03 shows homology to the MarR family of transcription repressors, which bind DNA through a helix-turn-helix motif. Release of repression by MarR is thought to occur by direct binding of inducers to the repressor, leading to loss of DNA binding. Based on this, we hypothesize that CrtR is a constitutive inhibitor of *crtI* and *crtB* that is released when *M. marinum* is exposed to light. It will be interesting to determine the nature of the light-activated inducer of the *crtI/crtB* pathway in *M. marinum*. Expression of *crtY* also apparently requires induction by light, since the MmR03 mutant accumulates red, but not gold, pigment in the dark, yet it does develop a gold color when exposed to light. It is possible that there is a common inducer for both steps in carotenoid production.

Our screen also identified one nonpigmented mutant (MmW04) that had a transposon insertion outside the known carotenoid biosynthesis locus within a region that exhibits significant sequence homology to *M. tuberculosis* genes. Since the mutation also caused decreased resistance of *M. marinum* to singlet oxygen and compromised intracellular growth, the normal gene product (CrtP) is likely involved in the positive regulation of a general stress response in mycobacteria. *M. tuberculosis* does not produce colored pigments, yet its homologous locus complemented the defects of MmW04 in pigmentation, resistance to singlet oxygen, and intracellular growth. Thus, the role of the locus in regulating stress response is conserved among *Mycobacterium* species. In fact, genes homologous to

those in this locus, such as *SOR* 1 (singlet oxygen resistance) (10), *SNZ* 1 (stationary-phase gene) (4), *pyroA* (pyridoxine biosynthesis) (25), and *HEVER* (Hevea ethylene responsive) (37), are conserved in distantly related organisms. All of these genes have been implicated in responses to a variety of stresses. As intracellular pathogens, mycobacteria must have mechanisms for protection from the stresses of the intraphagosomal environment; it is likely that the *crtP* locus (*Rv2606c* to *Rv2603c*) has a role in this response in *M. tuberculosis*, as its homologue does in *M. marinum*. In *M. marinum*, this locus has an additional role in regulating carotenoid biosynthesis. Since no other nonpigmented mutants have defects in intracellular survival or resistance to singlet oxygen, carotenoids apparently are not directly involved in protection against either of these stresses. This implies that the *crtP* locus activates stress responses in addition to pigmentation that more directly affect bacterial survival in the intracellular environment. We are currently investigating which gene(s) of the *crtP* locus is responsible for mediating the stress response in *M. marinum*.

The potential for using *M. marinum* as a model to study molecular mechanisms of *M. tuberculosis* pathogenesis has been explored systematically by Chan et al. (5) and Ramakrishnan et al. (29) as well as others (40). We have extended the potential utility of this model by showing that for genes with *M. tuberculosis* homologues, the *M. tuberculosis* genes can be used to complement the mutant *M. marinum* phenotypes. While this has been done to some extent in *M. smegmatis*, this organism does not persist in macrophages or other host cells and therefore cannot be used to study genes involved in intracellular growth and/or persistence. In contrast, *M. marinum* is able to replicate within macrophages, making it possible to screen for mutants defective in intracellular survival or growth by using the random transposon mutagenesis technique we describe here. Indeed, we have screened ~1,000 mutants from this library for the ability to survive in macrophages and have identified 23 different genes, in addition to the MmW04 gene, that are involved in intracellular survival. Significantly, each of these genes has an *M. tuberculosis* homologue, and in all 10 mutants in which we have tried, we have successfully corrected the intracellular survival defect of the mutant with the *M. tuberculosis* homologue. Thus, *M. marinum* is a model organism well suited not only for discovery of mycobacterial genes involved in intracellular survival but also for functional analysis of *M. tuberculosis* genes required for this aspect of pathogenesis.

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