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A *crtB* Homolog Essential for Photochromogenicity in *Mycobacterium marinum*: Isolation, Characterization, and Gene Disruption via Homologous Recombination

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A gene essential for light-induced pigment production was isolated from the photochromogen *Mycobacterium marinum* by heterologous complementation of an *M. marinum* cosmid library in the nonchromogen *Mycobacterium smegmatis*. This gene is part of an operon and homologous to the *Streptomyces griseus* and *Myxococcus xanthus crtB* genes encoding phytoene synthase. Gene replacement at this locus was achieved via homologous recombination, demonstrating that its expression is essential for photochromogenicity. The ease of targeted gene disruption in this pathogenic *Mycobacterium* allows for the dissection of the molecular basis of mycobacterial pathogenesis.

Mycobacterium marinum is a relatively rapidly growing animal- and human-pathogenic organism that is photochromogenic (4, 6, 10, 22, 30, 42a, 44, 46). When grown in the dark, *M. marinum* colonies are white, but upon exposure to visible light, they turn bright yellow (42a). The principal pigment responsible for this coloration is a beta-carotene, and the photoreceptor for the photoinduction process is likely a porphyrin (6, 36). Carotenoids are thought to protect cells against lethal photooxidative damage by quenching singlet oxygen and harmful radicals formed upon illumination (14, 54). Indeed, this has been shown to be the case for *M. marinum*, which is protected from lethal photooxidation by the presence of its pigment (36).

There is evidence that the color is the result of a photoinduced synthesis of pigments in living cells rather than the photooxidation of a preformed compound (4). Unpigmented *M. marinum* killed by heat or UV light is incapable of developing pigment upon light exposure. Similarly, alcohol extracts from *M. marinum* grown in the dark do not turn yellow upon exposure to light. Also, the addition of chloramphenicol (an inhibitor of protein synthesis) to live cells inhibits light-induced pigment synthesis, suggesting that these pigments are not preformed (46, 47). Taken together, these data suggest that interaction of the photoreceptor with light causes either the activation or the derepression of the gene(s) encoding the enzymatic machinery involved in carotenoid synthesis.

The studies described above, coupled with those demonstrating that the light-induced carotenoid pigments of *Myxococcus xanthus* are controlled by a light-induced promoter (20, 39, 49), led us to examine the genetic basis of pigment formation in *M. marinum*. The isolation of the locus responsible for photochromogenicity in *M. marinum* was facilitated by the ability to transfer this phenotype to *Mycobacterium smegmatis*, a nonchromogenic mycobacterial species. A gene encoding a phytoene synthase homolog was found to be necessary for pigment formation. The easily discernible phenotype resulting from the expression of this gene made it an ideal locus to develop and test the feasibility of gene replacement via homologous recombination in this pathogenic *Mycobacterium* species.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. marinum* M (43) and *M. smegmatis* mc^{2}155 (52) were used. *M. marinum* obtained from frozen stocks was grown at 33°C in 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose complex (OADC), 0.01% cycloheximide, and 0.25% Tween 80 (OADC-Tw broth) for 7 to 9 days. Cultures of *M. smegmatis* mc^{2}155 were grown in the same medium from a frozen aliquot at 37°C for 1 to 2 days. Both *M. marinum* and *M. smegmatis* mc^{2}155 were plated on 7H9 agar supplemented with 0.5% glycerol, 10% OADC, and 0.01% cycloheximide (OADC agar). *Escherichia coli* DH12S and HB101 were used for manipulations of the various plasmids.

Mycobacterial transformation. One hundred-milliliter cultures of *M. marinum* were grown to mid-log phase without shaking to approximately 0.6 optical density unit (A_{600}), washed three times in ice-cold 10% glycerol, and resuspended in 1 ml of ice-cold glycerol. Approximately 1 μg of plasmid DNA mixed with 100 μl of washed bacteria was placed in a 0.2-cm-gap electroporation cuvette on ice for 5 min and subjected to a pulse of 2.5 kV, 25 mF, and 800 W (Bio-Rad electroporator); 1 ml of OADC-Tw broth was added to the cells in the cuvette, which was incubated at 33°C for 3 to 5 h before plating the bacteria on OADC agar supplemented with 25 μg of kanamycin per ml. Transformants were observed 5 to 7 days after incubation at 33°C. *M. smegmatis* mc^{2}155 was transformed in the same way, with the following modifications: the culture was grown with shaking and incubated on ice for 2 h prior to the glycerol washes. The cultures were resuspended in 5 ml of glycerol, and 400 μl was used for transformation. After electroporation, the cells were moved to tubes with 5 ml of OADC-Tw broth and incubated at 37°C with rotation for 2 h prior to plating. Transformants were observed 2 to 3 days after incubation at 37°C.

Molecular and genetic manipulations. All manipulations involving *E. coli* were performed as described previously (32). *E. coli* DH12S carrying the the cosmid of interest was mutagenized with Tn*5*-B21 (51). For the Tn*3* mutagenesis, the protocol using a mini-Tn*3*-Km vector was performed as described elsewhere (29).

Construction of pYUB213Δkm. pYUB213 is a *Mycobacterium-E. coli* shuttle plasmid that contains both kanamycin and bleomycin resistance genes. It was constructed as follows (9a). The bleomycin resistance gene of plasmid pLTV3 (9) was obtained by PCR using primers with *Bam*HI flanking sites and inserted into the *Bam*HI site of pMV262, so that it could be expressed from the 5′ mycobacterial *HSP60* promoter. pMV262 differs from pMV261 (53) by the addition of a single nucleotide (G) just 5′ of the *Bam*HI site. pYUB213Δkm was constructed by removing the kanamycin resistance gene contained within the *Nhe*I and *Spe*I sites of pYUB213. To accomplish this, pYUB213 was digested with *Nhe*I and *Spe*I, ligated, transformed into *E. coli* DH12S, and plated on Luria-Bartani (LB) plates containing 2 μg of bleomycin per ml. The resultant colonies were screened for kanamycin sensitivity. Plasmids from the kanamycin-sensitive colonies were confirmed to have lost the 1.2-kb *Nhe*I-*Spe*I fragment by restriction analysis with various enzymes.

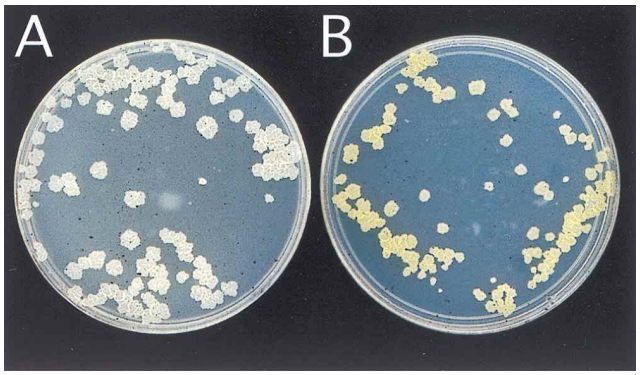

FIG. 1. Expression of the *M. marinum crtB* gene in *M. smegmatis*. *M. smegmatis* transformed with the original *M. marinum* cosmid containing the *crtB* gene was grown in the dark (A) and exposed to light for several days (B).

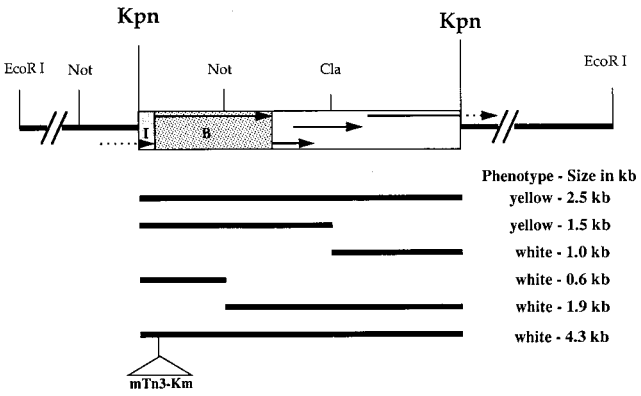

FIG. 2. Functional analysis of the photochromogenicity locus. A restriction map of the *M. marinum crtB* locus is shown (not to scale). The 2.5-kb *Kpn*I fragment was sequenced, and the 9.2-kb *Eco*RI fragment was used for the homologous recombination experiments. The arrows represent ORFs; the dotted lines at the two ends of the *Kpn*I fragment represent truncated ORFs. I, truncated *crtI* (nucleotides 1 to 101); B, *crtB* (nucleotides 115 to 1074). The remaining ORFs denoted by arrows extend from nucleotides 1071 to 1394, 1109 to 1765, and 1759 to 2521 (truncated). The relative positions of the arrows denote their translational reading frames with respect to *crtB*, which is arbitrarily designated +1. Thus, the position of the frame of *crtI* is +3, and those of the remaining ORFs are, from 5′ to 3′, +3, +2, and +1. The dark lines beneath the restriction map denote the fragments used for expression in *M. smegmatis*, and the site of the 1.8-kb mini-Tn*3*-Km (mTn3-Km) insertion is indicated.

Construction of plasmids expressing *sacB*. The *sacB* gene was obtained by PCR (32) from plasmid pCVD442 (15), using the primers 5′ GCTCTAGAGG CATTTTCTTTTGCG 3′ and 5′ GCTCTAGACCTGCCGTTCACTATTATTT 3′ (*Xba*I recognition sequences are underlined), and the resulting 1.8-kb PCR product was inserted into the *Xba*I site of pBluescript SK+. Individual plasmids were transformed into *E. coli* DH12, which was tested for sucrose sensitivity on LB agar plates containing 5% sucrose. The *sacB* gene from the plasmid of a sucrose-sensitive *E. coli* clone was then inserted into pYUB213, and the resultant plasmid was transformed into *M. marinum*. Individual *M. marinum* transformants expressing *sacB* were confirmed to be sucrose sensitive by plating on OADC agar supplemented with 10% sucrose before the *sacB* fragment from the corresponding plasmid was inserted into the *Xba*I site of the p127.10 plasmid for use in the homologous recombination experiments.

Southern blot analysis. Genomic DNA was prepared from 5-ml cultures of *M. marinum* grown to approximately 0.6 optical density unit (A_{600}) by the cetyltrimethylammonium bromide method (37). The DNA obtained was used for one or two Southern blots after digestion with various restriction enzymes, electrophoresis on 0.7% agarose, and transfer to nylon membranes (Nytran or Hybond) (32). Hybridization was performed with plasmid p127.10S labeled with [α-^{32}P]dCTP as a probe, and washes were performed as described previously (32).

DNA sequencing and sequence analysis. DNA sequencing was performed on ABI 373 and 377 DNA Sequencers, using fluorescent dye primer cycle sequencing chemistry with TaqFS (PE/Applied Biosystems, Foster City, Calif.) and with ThermoSequenase (Amersham Life Science, Cleveland, Ohio), and using fluorescent dye terminator chemistry with TaqFS, all with conditions as recommended by the supplier. The sequences were assembled by using the new phred-Phrap software (copyright 1992-1996 by Phil Green and Brent Ewing, University of Washington) and edited by using Consed (developed by David Gordon, University of Washington).

Protein and DNA homologies with sequences in the available databases were determined by the program BLAST (National Center for Biotechnology Information at the National Library of Medicine) (2). Alignments were done with the programs in the Genetics Computer Group (Madison, Wis.) package (version 9.0).

Nucleotide sequence accession number. Nucleotide sequences described in this report have been deposited in GenBank (accession no. U92075).

RESULTS

Isolation of the DNA fragment encoding the *M. marinum* photochromogenicity gene(s). An *M. marinum* cosmid library was made by ligating 30- to 35-kb fragments of *M. marinum* genomic DNA partially digested with *Sau*3AI into the *Bam*HI site of the mycobacterial expression vector pYUB18 (24). The cosmid library DNA, derived from a pool of 5,000 *E. coli* HB101 transformants, was used to transform the rapidly growing, nonphotochromogenic species *M. smegmatis* mc^{2}155 (52), and colonies were screened for the light-inducible yellow phenotype. Seven of the 1,200 *M. smegmatis* transformants turned yellow when exposed to light (Fig. 1). Cosmid DNA was isolated from all seven yellow *M. smegmatis* colonies, and one of these cosmids was subsequently used to transform *Mycobacterium bovis* BCG, where its presence conferred a light-inducible yellow color, although the intensity was much lower than in *M. smegmatis* (data not shown). None of the seven cosmids produced any pigment in *E. coli* DH12S. Restriction analysis revealed a 9.2-kb *Eco*RI fragment common to all seven cosmids (Fig. 2). Tn*5* transposon mutagenesis was performed on one of the cosmids after transformation into *E. coli* DH12, and DNA from a pool of approximately 10,000 transposon mutants was used to transform *M. smegmatis* mc^{2}155. Two of the 140 transformants obtained were no longer photochromogenic, and restriction analysis of the cosmid DNA from both nonchromogenic colonies revealed the same shift in the size of the 9.2-kb *Eco*RI fragment, suggesting that the transposon insertion into this fragment was responsible for the inactivation of photochromogenicity. The native 9.2-kb *Eco*RI fragment from one of the cosmids was inserted into the *E. coli-Mycobacterium* shuttle vector pYUB213 (see Materials and Methods) to create pLAP9.2. Transformation of *M. smegmatis* mc^{2}155 with the resultant plasmid resulted in brilliant yellow colonies, confirming that the gene(s) essential for chromogenicity was present on this fragment. Further subcloning analysis revealed that a 2.5-kb *Kpn*I fragment was sufficient to confer light-induced yellow color on *M. smegmatis* (Fig. 2); the plasmid containing this fragment will be referred to as pLAP2.5.

To further narrow the location of the gene, the internal *Kpn*I-*Cla*I fragments and *Kpn*I-*Not*I fragments were inserted into pYUB213 (Fig. 2). *M. smegmatis* mc^{2}155 was transformed with these plasmids, pLAP1.5, pLAP1.0, pLAP1.9, and pLAP0.6, and colonies were screened for the yellow phenotype. pLAP1.5 conferred a yellow phenotype, whereas pLAP1.0, pLAP1.9, and pLAP 0.6 did not (Fig. 2).

Sequence analysis of the 2.5-kb *Kpn*I fragment reveals genes homologous to the *crtB* and *crtI* genes involved in carotenoid biosynthesis. The complete nucleotide sequence of the 2,527-bp *Kpn*I fragment was determined, and computer analysis identified three complete open reading frames (ORFs) flanked by two truncated ORFs, all in the same transcriptional orientation (Fig. 2). Comparison of both DNA and amino acid sequences with those in the database revealed strong similarity to genes involved in carotenoid biosynthesis from other organ-

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                Domain I                                    Domain II

M. marinum   127 LTITDYPDRDALNTYMRGSAEAIGLQLLPILG 158...165 AMPYAAALGRAFQLTNFIRDVDEDLARNRIYLPADELAAYGVDRDVL 211

                 LT+  Y   + L  YM GSA  IGLQ+LP+LG           A P+AAALG AFQLTNF+RDV EDL R R+YLP + LAA+G DRD
S. griseus   128 LTVGGYASYEELGRYMHGSAAVIGLQMLPVLG 159...167 AAPHAAALGVAFQLTNFLRDVGEDLDRGRLYLPTELLAAHGADRDRF 213

                 LT   Y   + L+ Y    A  +GL L P+LG           A+  AA LGRA QLTN +RDV EDL R R+YLPA+ELAA+G+  D L
M. xanthus   144 LTKHRYATWEELDLYCYRVAGVVGLMLTPVLG 175...181 AVEPAADLGRAMQLTNILRDVREDLERGRVYLPAEELAAFGLSEDDL 227

                 +  T   D D L  Y R  A  IG   L I G              YA  LG A Q TN +RDV ED    RIYLP DEL   GV R  L
M. tuber.    110 IDWTGCRDFDELIVYCRRGAGTIGKLCLSIFG 141...147 TSRYAEQLGIALQQTNILRDVREDFLNGRIYLPRDELDRLGV.RLRL 192

                 L  + Y + D L  Y    A  +GL  +P++G              A ALG A QLTN +RDV ED  R RIYLP DELA  G+ ++
N. pseudon.  233 LKKSRYKNFDELYLYCYYVAGTVGLMSVPVMG 264...276 VYNAALALGIANQLTNILRDVGEDARRGRIYLPQDELAEAGLSDEDD 321

                 L    Y   + L TY    A  +GL   P++G              A ALG A QLTN +RDV ED  R RIYLP +ELA +      L
Scoccus sp.  125 LLQNRYSTFEDLYTYCYRVAGTVGLMSQPVMG 155...173 PTQEALALGIANQLTNILRDVGEDARRGRIYLPQEELAQFNYSEQDL 219

                 L  + Y   + L+ Y    A  +GL    +LG              A ALG A QLTN +RDV ED+ R RIYLP ++L  +      L
Scystis sp.  153 LYRSRYQTFEELDLYCYRVAGTVGLMSSAVLG 184...202 PQEEAIALGVANQLTNILRDVGEDVERGRIYLPLEDLERFNYSEQDL 248

Consensus        L  + Y   + L  Y    A  +GL  +P+LG              A ALG A QLTN +RDV ED+ R R+YLP ++LA +G+ ++ L
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FIG. 3. *M. marinum* has two conserved domains in the enzyme phytoene synthase. The amino acid sequences of the two conserved domains of the enzyme phytoene synthase are found in several different organisms. The consensus includes conserved residues in at least three of the sequences. Plus signs represent similarity in amino acid residues based on polarity as well as presence of similar functional groups. *M. tuber.*, *M. tuberculosis*; *N. pseudon.*, *Narcissus pseudonarcissus*; *Scoccus* sp., *Synechococcus* sp., *Scystis* sp., *Synechocystis* sp.

isms, particularly those from *Streptomyces griseus* and *M. xanthus* (7, 50).

The ORF designated B (Fig. 2) was deduced to encode a 319-residue peptide that has a high DNA and peptide sequence similarity to the phytoene synthases of several organisms, particularly those of *S. griseus* and *M. xanthus* (7, 50). Phytoene synthase is an enzyme in the carotenoid biosynthetic pathway catalyzing the conversion of geranylgeranyl-pyrophosphate to phytoene, the first C_{40} carotene (7, 8). This is the first committed step in the biosynthesis of carotenoids. The overall identities of ORF1 at the DNA level were 56% to *S. griseus* and 48% to *M. xanthus*. At the peptide level, there were 51% identity and 58% similarity, and 35% identity and 43% similarity, respectively, to the phytoene synthases of *S. griseus* and *M. xanthus*. For both organisms, the homology was separated into two distinct regions which correspond to two particularly well conserved domains suggested to be involved in the catalytic reaction of phytoene synthase (7, 25, 35, 48) (Fig. 3). Homology was also found to a phytoene synthase homolog of *Mycobacterium tuberculosis*, with 45% identity at the DNA level and 30% identity and 39% similarity at the peptide level (42).

The truncated ORF ending 13 bp upstream of *crtB* likely encodes the *M. marinum crtI* gene, since it displays homology to the carboxy-terminal region of the phytoene dehydrogenases (CrtI) of *S. griseus* and *Streptomyces setonii*. Despite the short length of the sequence, there were 85% identity and 95% similarity over 21 residues with *S. setonii*. Phytoene dehydrogenase is responsible for the second committed step in carotenoid biosynthesis, catalyzing the conversion of phytoene to lycopene.

The remaining three ORFs 3′ of the *crtB* region did not display significant homology to known peptides in the database. The close spacing (and, in some cases, overlap) of the ORFs and the apparent lack of regulatory elements within the fragment suggested that these comprise an operon with a promoter region that lies upstream of the *crtI* gene. Ongoing sequence analysis indicates that the *M. marinum crtI* coding region extends beyond the 5′ end of the 9.2-kb *Eco*RI fragment. Our finding that the 2.5-kb *Kpn*I fragment when inserted into pYUB213 and expressed in *M. smegmatis* mc^2155 conferred a yellow color indicates that the *crtB* gene in pLAP2.5 and pLAP9.2 is probably expressed by readthrough transcription from a constitutive promoter in pYUB213.

Insertional inactivation of the photochromogenicity phenotype maps to the *crtB* locus. The easily discernible phenotype conferred by the *crtB* gene made it ideal for testing homologous recombination in *M. marinum*. To this end, the entire 9.2-kb *Eco*RI fragment was inserted into the suicide plasmid pILL570 (29). An *E. coli* strain bearing the resultant plasmid was mutagenized with a mini-Tn*3*-Km cassette (29), and the resulting transposition events were initially screened for the presence of insertions in the internal 2.5-kb *Kpn*I fragment. Five such plasmids were found, and their *Kpn*I fragments were inserted into pYUB213Δkm for transformation of *M. smegmatis* mc^2155. Only one of the five plasmids with Tn*3* insertions had abrogated the yellow phenotype (Fig. 2). The insertion in the corresponding parent plasmid, p127.10, mapped to the 5′ *Kpn*I-*Not*I fragment which contains part of *crtB* (ORF1) (Fig. 2). Sequencing analysis of p127.10 showed that the Tn*3* insertion was 11 bp upstream of the translational start site of *crtB* in the region between *crtI* and *crtB*. Coupled with the data on the heterologous expression of *crtB*, the finding that the plasmid containing the inactivated *crtB* no longer conferred the yellow phenotype on *M. smegmatis* demonstrated that *crtB* is both necessary and sufficient for yellow pigment formation in *M. smegmatis*.

Homologous recombination at the *crtB* locus of *M. marinum*. To enable selection of *M. marinum* that had undergone gene replacement events, we used *sacB* as a negative counterselectable marker (40, 41). *sacB* expression confers sucrose sensitivity so that gene replacement events with looping out of intervening plasmid sequences result in the loss of *sacB* and render the organism resistant to sucrose again. The *sacB* gene was inserted into p127.10 (see Materials and Methods) to generate plasmid p127.10S (Fig. 4A). p127.10S was used to transform *M. marinum*, and the kanamycin-resistant colonies obtained were initially screened by plating on OADC agar with 10% sucrose to distinguish between integration events and spontaneous kanamycin resistance arising after electroporation. Southern

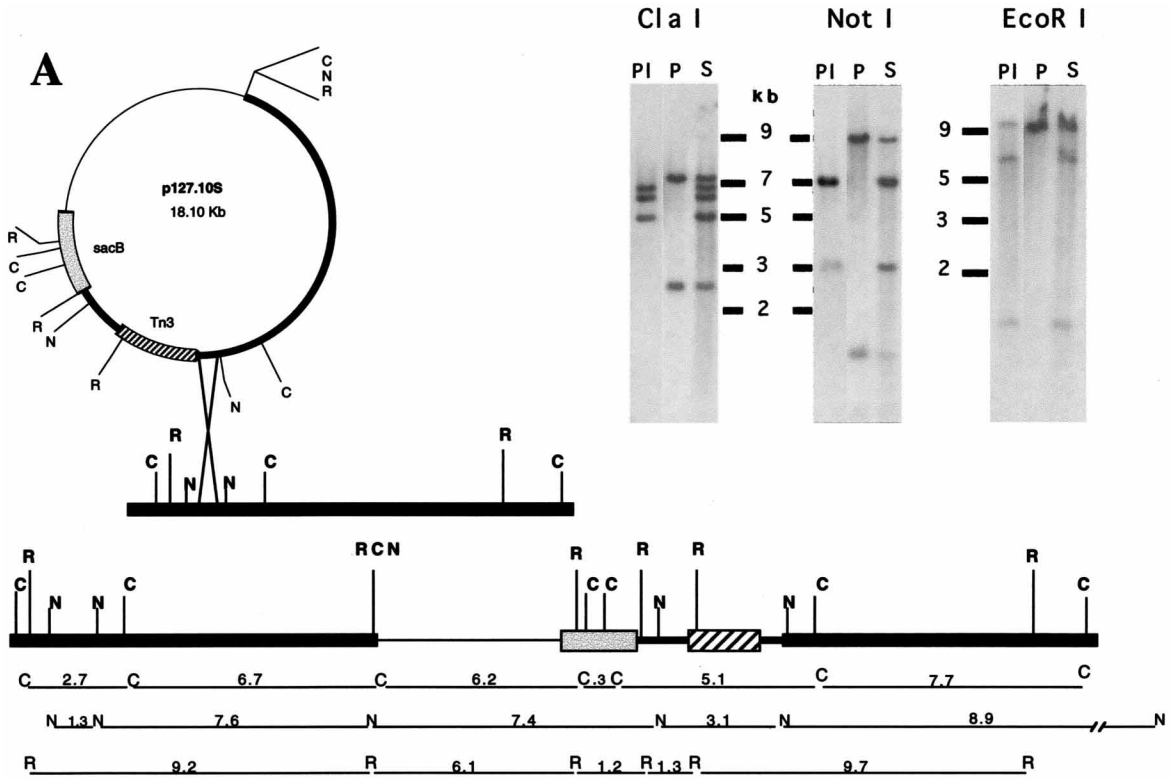

FIG. 4. Homologous recombination at the *crtB* locus. (A) Schematic representation (not to scale) of the single-crossover event of p127.10S into the *M. marinum crtB* locus, with the resultant merodiploid shown on the left. The expected sizes of the resulting fragments are shown below (C, *Cla*I; N, *Not*I; R, *Eco*RI). On the right are Southern blots of plasmid p127.10S (lanes Pl), *M. marinum* parent (lanes P), and a merodiploid (lanes S) digested with *Cla*I, *Not*I, and *Eco*RI. The entire p127.10S plasmid was used as a probe. Lanes Pl are a shorter exposure of the same Southern blot to compensate for the varying intensity of the signal in the different lanes. (B) A schematic representation (not to scale) of the gene replacement event is shown at the top. Below is a Southern blot with a merodiploid and disrupted *crtB* genes digested with *Cla*I. The entire p127.10S plasmid was used as a probe. Lanes: P, parent; S, single crossover (merodiploid); D, white colonies with double-crossover (gene replacement) events. The 2.7-kb *Cla*I fragment in the parent (lane P) has been replaced by a 4.5-kb fragment containing the insertionally inactivated gene in lanes D. Lanes P and S are a shorter exposure of the same Southern blot as lanes D to compensate for the varying amounts of DNA loaded on the gel.

blot analysis of the genomic DNA from the sucrose-sensitive *M. marinum* clones revealed that a recombination event at the homologous locus had occurred in all 35 of the sucrose-sensitive colonies (Table 1; Fig. 4A).

Southern blot analysis using *Cla*I-digested DNA demonstrated that in all 35 clones, the single-crossover event had occurred in the region 3′ to the Tn*3* insertion site (Fig. 4A and data not shown). This finding was not surprising, as the 3′ region of homology flanking the insertion is 8.1 kb whereas the 5′ flanking region homology is only 1.1 kb. Restriction analysis with multiple enzymes was then performed on selected clones to confirm that recombination had occurred at the homologous locus (Fig. 4A) and that no episomal form of the introduced plasmid remained (data not shown).

Gene replacement at the *crtB* locus confers a nonchromogenic phenotype on *M. marinum*. Several of the transformants that had undergone single-crossover events were plated on 10% sucrose to assess the frequency of second recombination events leading to gene replacement. Resolution of the merodiploid should lead to the retention of either the wt (wild-type) allele or the mutated allele. Initially, we selected only for those events leading to the retention of the mutated allele by including kanamycin in the selection. In each of two independently derived merodiploids, the recombination frequencies were 1.8×10^{-4} and 2.4×10^{-4}, very similar to those observed for the single-crossover events (Table 1). Reversion to sucrose resistance can also be a result of mutations in *sacB* rather than

TABLE 1. Recombination frequency at the *crtB* locus of *M. marinum*

Expt	Transformation frequency[a] (per μg of DNA)	No. of recombinants[b]	Recombination frequency[c]
1	1.0×10^3	15	7.5×10^{-3}
2	4.7×10^4	14	1.5×10^{-4}
3	6.0×10^4	7	5.8×10^{-5}

[a] Number of kanamycin-resistant colonies obtained by introducing a replicating plasmid into electrocompetent *M. marinum.*

[b] Total number of recombinants obtained from two transformations of 1 μg each of plasmid p127.10S.

[c] Ratio between the number of integration events and the number of transformants obtained with the replicating plasmid, each with 1 μg of DNA. The recombinants were initially identified by their sensitivity to 10% sucrose, and the occurrence of a single crossover was confirmed by Southern blot analysis in 35 of the 36 recombinants.

crossover events leading to its deletion (40). The relative ratio of these two events was easily discerned by determining whether the sucrose-resistant colonies were yellow or white after exposure to light. In the two merodiploids analyzed, 3% (18 of 590) and 24% (52 of 212) of the colonies were found to be yellow, suggesting wide variations in the frequency of mutation of *sacB.*

To assess the relative frequency of resolution of the merodiploid to the wt and mutated configurations of the gene, one of the transformants was plated on sucrose plates that did not contain kanamycin. Only 19.2% (201 of 1,047) of the colonies were white, indicating that resolution to the wt gene is heavily favored. However, this may be an overestimation; among the yellow colonies are merodiploids that have acquired sucrose resistance by mutating the *sacB* gene, an event deduced to occur at some frequency by our previous analysis.

In a representative merodiploid, the 2.7-kb parental *Cla*I fragment had been replaced by a 4.5-kb fragment containing the Tn*3*-Km insertion (Fig. 4B). An example of a merodiploid and of bacteria with a gene replacement at *crtB* is shown in Fig. 5.

FIG. 5. Gene replacement at the *crtB* locus results in a nonchromogenic phenotype. An OADC agar plate with a merodiploid on the left and a colony with a subsequent gene replacement event on the right is shown after exposure to light for several days.

DISCUSSION

We have demonstrated that a *crtB* homolog in *M. marinum* is necessary for the organism's photochromogenicity. In *M. xanthus* and other bacteria, *crtB* encodes phytoene synthase, which constitutes the first committed step in carotenoid biosynthesis. The *M. marinum crtB* gene is part of an operon, a configuration similar to that of the carotenoid genes from other species (7, 50). Our preliminary sequence analysis shows that just 13 bp 5′ of the *crtB* gene is the *crtI* gene encoding phytoene dehydrogenase, which catalyzes the desaturation of phytoene. This juxtaposition of the *crtI* and *crtB* genes in an operon is similar to the organization of these genes in other species such as *S. griseus*, *Rhodobacter capsulatus*, *Erwinia herbicola*, and *Erwinia uredovora* (3, 50). In *M. xanthus*, ORF2, which is homologous to other genes encoding phytoene dehydrogenase, is also present just upstream of *crtB* (7). However, the functional gene (*crtI*) encoding phytoene dehydrogenase is unlinked to the *crtB* gene (18, 34). Experiments using S1 RNase protection show that the *M. marinum crtB* RNA extends upstream of the 5′ *Eco*RI site (data not shown), suggesting that *crtB* and *crtI* share a promoter.

Our finding that the cosmid containing the *M. marinum crtB* gene confers light-induced yellow color on both *M. smegmatis* and *M. bovis* BCG suggests that these mycobacteria possess functional copies of the other genes involved in carotenoid biosynthesis. *M. tuberculosis* has been found to have an ORF homologous to *crtB* (42) (Fig. 3), suggesting that it may harbor a mutation in the gene abrogating pigment expression. Phytoene itself is colorless and has to be converted to pigmented carotenoids by other enzymes in the pathway. Our data show that the addition of a functional *crtB* to *M. smegmatis* can activate the synthesis of yellow color, suggesting that many of the enzymes involved in the later steps of pigmented carotenoids are functional in this organism. Thus, our results are at variance with the inference made by previous studies (21) that the *M. smegmatis crtB* gene must be intact. In that study, various small fragments from a *Mycobacterium aurum* cosmid that conferred color on *M. smegmatis* were found to be associated with different carotenoid pigments when expressed in *M. smegmatis. M. smegmatis* itself expresses some yellow-orange pigments upon exposure to light, suggesting that low levels of *crtB* may be expressed. It is interesting that *M. bovis* BCG expressing the cosmid displays a much lighter yellow color than *M. smegmatis* bearing the cosmid or *M. marinum*. This could result from lower levels of expression or differences in the carotenoids encoded by the downstream genes of the pathway in these organisms. Our finding that the promoterless 2.5-kb *M. marinum crtB* fragment also confers light-induced yellow color on *M. smegmatis* suggests that a functional light-activated promoter controlling genes downstream in the carotenoid biosynthesis pathway is present in *M. smegmatis*. Alternatively, the pigment may be the result of a chemical change induced by light. We are currently attempting to dissect the mechanism of light-induced pigment formation by examining transcriptional fusions of gene fragments containing the putative promoter region to a promoterless *lacZ* gene.

We are currently analyzing the region upstream of *crtI* to identify the promoter and directly test it for light inducibility. Mycobacterial promoters are as yet not well characterized (28), and only a few genes with inducible promoters have been reported to date (17, 31). Hence, an inducible promoter that is active in members of the *M. tuberculosis* complex may well

provide a useful reporter system. In *M. marinum*, this gene can be activated by exposing the bacteria either to light or to antimycin A, which has been shown to mimic light-induced carotenogenesis (36). In addition to providing an easy visual screen for gene expression, there is potential for its use as a selectable marker. Toluidine blue, which acts as a photosensitizer, has been shown to kill unpigmented *M. marinum* exposed to light, whereas pigmented *M. marinum* survives (4). We are currently testing whether *crtB* confers toluidine blue resistance when expressed in *M. smegmatis* and conversely whether the *ctrB* mutant *M. marinum* is more susceptible to toluidene blue.

The homology of the *M. marinum crtB* and *crtI* genes to the *S. griseus* and *M. xanthus* carotenoid gene cluster begs the question of how these genes are regulated in *M. marinum*. The *S. griseus* carotenoid gene cluster appears to be regulated by *crtS*, which shows homology to genes encoding alternative sigma factors (27). The *M. xanthus crtB* and *crtI* genes are regulated by CarS and CarQ, respectively (19). The *M. xanthus carQ*, *carR*, and *carS* genes are transcribed from a single operon where CarQ, an RpoE (σ^E) (also an alternative sigma factor) homolog, upregulates the transcription of its own (*carQRS*) operon and that of the *crtI* gene. CarR is an inner membrane protein which sequesters CarQ and thus prevents transcriptional activation of *carQ* and thereby of *carS*, *crtB*, and *crtI*. Light causes the breakdown of *carR*, releasing *carQ* and allowing the activation of pigment gene synthesis. A *carQ* homolog has been identified in *M. tuberculosis* (6a, 55) as well as in *M. marinum* (43a). We are currently trying to isolate the *M. marinum carQ* gene to determine its role, if any, in regulating photochromogenicity.

While homologous recombination has been easily achieved in the rapidly growing nonpathogen *M. smegmatis*, it has been a difficult problem in the slowly growing pathogenic species (38) where it is most needed for the molecular analysis of the determinants of pathogenesis (16). Initial studies suggested that when linearized suicide plasmid DNA containing homologous sequences was transformed into *M. bovis* BCG and *M. tuberculosis*, illegitimate recombination occurred at a high frequency, possibly masking homologous recombination events (1, 26). Recently, homologous recombination and gene replacement were demonstrated at the urease locus of *M. bovis* BCG (45). The reason for the differences in the success of these experiments is not clear. Gene replacement has also been reported for *M. tuberculosis* with the use of long (40- to 50-kb) linear recombination substrates. In all of these cases, the frequency of recombination is much lower than that for *M. smegmatis*. It has been postulated that the fact that the *M. tuberculosis* (but not the *M. smegmatis*) RecA has to undergo a protein splicing event for its maturation (11, 12) may alter the RecA levels in the cell and reduce the frequency of homologous recombination (38). It is interesting that homologous recombination has been demonstrated in another slowly growing species, *Mycobacterium intracellulare*, with a RecA that is not spliced (13, 33). In this case, however, it was not possible to assess the frequency of recombination. In *M. marinum*, the RecA is also not processed (13). While other studies have reported that linear recombination substrates lead to higher levels of recombination in mycobacteria, we obtained virtually no transformants when p127.10 linearized at the *Xba*I site was used.

We have used *M. marinum* as a model to study pathogenesis and have developed an in vitro model to assess bacterial persistence and subcellular trafficking (5, 43) and a chronic and acute disease model in *Rana pipiens*, a natural host species for this organism (44). For the pursuit of our studies of molecular pathogenesis in *M. marinum*, it was critical to determine the feasibility of gene replacement via homologous recombination. We chose the *crtB* locus not only because of its easily discernible phenotype but also because inactivation of this gene was unlikely to be lethal to the organism. We have not had difficulty achieving gene replacement at a reasonable frequency. Furthermore, we have now obtained gene replacement at a second locus, the *ura* locus of *M. marinum*, by using essentially the strategy described in this report. The 7-kb fragment containing the *ura* locus on a suicide plasmid was inactivated by mini-Tn*3*-Km insertion transformed into *M. marinum. M. marinum* colonies with gene replacement events were selected from the resultant merodiploids by resistance to 5-fluoroorotic acid, which results from the inability to synthesize uracil (23).

The facility of gene replacement in *M. marinum* will now make it possible to test the role of candidate virulence genes both in tissue culture infection models and in disease in frogs.

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REFERENCES

1. **Aldovini, A., R. N. Husson, and R. A. Young.** 1993. The *uraA* locus and homologous recombination in *Mycobacterium bovis* BCG. J. Bacteriol. **175:** 7282–7289.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Armstrong, G. A.** 1994. Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. J. Bacteriol. **176:**4795–4802.
4. **Baker, J. A.** 1938. Light as a factor in the production of pigment by certain bacteria. J. Bacteriol. **35:**625.
5. **Barker, L. P., K. George, S. Falkow, and P. L. C. Small.** 1997. Differential trafficking of live and dead *Mycobacterium marinum* organisms in macrophages. Infect. Immun. **65:**1497–1504.
6. **Batra, P. P., and H. C. Rilling.** 1964. On the mechanism of photoinduced carotenoid synthesis. Arch. Biochem. Biophys. **107:**485–492.

6a. **Bishai, W. R.** Personal communication.

7. **Botella, J. A., F. J. Murillo, and R. Ruiz-Vazquez.** 1995. A cluster of structural and regulatory genes for light-induced carotenogenesis in *Myxococcus xanthus*. Eur. J. Biochem. **223:**238–248.
8. **Bramley, P. M., and A. MacKenzie.** 1988. Regulation of carotenoid biosynthesis. Curr. Top. Cell. Regul. **29:**291–343.
9. **Camilli, A., D. A. Portnoy, and P. Youngman.** 1990. Insertional mutagenesis of *Listeria monocytogenes* with a novel Tn*917* derivative that allows direct cloning of DNA flanking transposon insertions. J. Bacteriol. **172:**3738–3744.

9a. **Cirillo, J. D.** Personal communication.

10. **Clark, H. F., and C. C. Shepard.** 1963. Effect of environmental temperatures on infection with *Mycobacterium marinum* (Balnei) of mice and a number of poikilothermic species. J. Bacteriol. **86:**1057–1069.
11. **Davis, E. O., P. J. Jenner, P. C. Brooks, M. J. Colston, and S. G. Sedgwick.** 1992. Protein splicing in the maturation of *M. tuberculosis Rec A* protein: a mechanism for tolerating a novel class of intervening sequences. Cell **71:** 201–210.
12. **Davis, E. O., S. G. Sedgwick, and M. J. Colston.** 1991. Novel structure of the *recA* locus of *Mycobacterium tuberculosis* implies processing of the gene product. J. Bacteriol. **173:**5653–5662.
13. **Davis, E. O., H. S. Thangaraj, P. C. Brooks, and M. J. Colston.** 1994. Evidence of selection for protein introns in the RecAs of pathogenic mycobacteria. EMBO J. **13:**699–703.

14. **Di Mascio, P., T. P. A. Devasagayam, S. Kaiser, and H. Sies.** 1990. Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers. Biochem. Soc. Trans. **18:**1054–1056.
15. **Donnenberg, M. S., and J. B. Kaper.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive selection suicide vector. Infect. Immun. **59:**4310–4317.
16. **Falkow, S.** 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev. Infect. Dis. **10:**S274–S276.
17. **Fiss, E., S. Yu, and W. J. Jacobs.** 1994. Identification of genes involved in the sequestration of iron in mycobacteria: the ferric exochelin biosynthetic and uptake pathways. Mol. Microbiol. **3:**557–569.
18. **Fontes, M., R. Ruiz-Vazquez, and F. J. Murillo.** 1993. Growth phase dependence of the activation of a bacterial gene for carotenoid synthesis by blue light. EMBO J. **12:**1265–1275.
19. **Gorham, H. C., S. J. McGowan, P. R. H. Robson, and D. A. Hodgson.** 1996. Light-induced carotenogenesis in *Myxococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. Mol. Microbiol. **19:**171–186.
20. **Hodgson, D. A.** 1993. Light-induced carotenogenesis in *Myxococcus xanthus*: genetic analysis of the *carR* region. Mol. Microbiol. **7:**471–488.
21. **Houssaini-Iraqui, M., H. L. David, S. Clavel-Seres, F. Hilali, and N. Rastogi.** 1993. Characterization of *car* α, *car Lep*, and *Crt I* genes controlling the biosynthesis of carotenes in *Mycobacterium aurum*. Curr. Microbiol. **27:**317–322.
22. **Huminer, D., S. D. Pitlik, C. Block, L. Kaufman, S. Amit, and J. B. Rosenfeld.** 1986. Aquarium-borne Mycobacterium marinum skin infection. Report of a case and review of the literature. Arch. Dermatol. **122:**698–703.
23. **Husson, R. N., B. E. James, and R. A. Young.** 1990. Gene replacement and expression of foreign DNA in mycobacteria. J. Bacteriol. **172:**519–524.
24. **Jacobs, W. R., Jr., G. V. Kalpana, J. D. Cirillo, L. Pascopella, R. A. Udani, W. D. Jones, Jr., R. G. Barletta, and B. R. Bloom.** 1991. Genetic systems for mycobacteria. Methods Enzymol. **204:**537–555.
25. **Jennings, S. M., Y. H. Tsay, T. M. Fisch, and G. W. Robinson.** 1991. Molecular cloning and characterization of the yeast gene for squalene synthetase. Proc. Natl. Acad. Sci. USA **88:**6038–6042.
26. **Kalpana, V. G., B. R. Bloom, and W. R. Jacobs, Jr.** 1991. Insertional mutagenesis and illegitimate recombination in mycobacteria. Proc. Natl. Acad. Sci. USA **88:**5433–5437.
27. **Kato, F., A. Nakaji, M. Tanaka, and Y. Koyama.** 1995. Carotenoid synthesis in *Streptomyces setonii* ISP5395 is induced by the gene *crtS*, whose product is similar to a sigma factor. Mol. Gen. Genet. **247:**387–390.
28. **Kremer, L., A. Baulard, J. Estaquier, J. Content, A. Capron, and C. Locht.** 1995. Analysis of the *Mycobacterium tuberculosis* 85A antigen promoter region. J. Bacteriol. **177:**642–653.
29. **Labigne, A., P. Courcoux, and L. Tompkins.** 1992. Cloning of *Campylobacter jejuni* genes required for leucine biosynthesis, and construction of *leu*-negative mutant of *C. jejuni* by shuttle transposon mutagenesis. Res. Microbiol. **143:**15–26.
30. **Linell, F., and A. Norden.** 1954. *Mycobacterium balnei*, a new acid fast bacillus occurring in swimming pools and capable of producing skin lesions in humans. Acta Tuberc. Scand. Suppl. **33:**1–84.
31. **Mahenthiralingam, E., P. Draper, E. O. Davis, and M. J. Colston.** 1993. Cloning and sequencing of the gene which encodes the highly inducible acetamidase of *Mycobacterium smegmatis*. J. Gen. Microbiol. **139:**575–583.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Marklund, B. I., D. P. Speert, and R. W. Stokes.** 1995. Gene replacement through homologous recombination in *Mycobacterium intracellulare*. J. Bacteriol. **177:**6100–6105.
34. **Martinez-Laborda, A., M. Elias, R. M. Ruiz-Vasquez, and F. J. Murillo.** 1986. Insertions of Tn5 linked to mutations affecting carotenoid synthesis in *Myxococcus xanthus*. Mol. Gen. Genet. **205:**107–114.
35. **Math, S. K., J. E. Hearst, and C. D. Poulter.** 1992. The *crtE* gene in *Erwinia herbicola* encodes geranyl-geranyl diphosphate synthase. Proc. Natl. Acad. Sci. USA **89:**6761–6764.
36. **Mathews, M. M.** 1963. Studies on the localization, function, and formation of the carotenoid pigments of a strain of *Mycobacterium marinum*. Photochem. Photobiol. **2:**1–8.
37. **McAdam, R. A., T. R. Weisbrod, J. Martin, J. D. Scuderi, A. M. Brown, J. D. Cirillo, B. R. Bloom, and W. R. Jacobs, Jr.** 1995. In vivo characteristics of leucine and methionine auxotrophic mutants of *Mycobacterium bovis* BCG generated by transposon mutagenesis. Infect. Immun. **63:**1004–1012.
38. **McFadden, J.** 1996. Recombination in mycobacteria. Mol. Microbiol. **21:**205–211.
39. **McGowan, S. J., H. C. Gorham, and D. A. Hodgson.** 1993. Light-induced carotenogenesis in *Myxococcus xanthus*: DNA sequence analysis of the *CarR* region. Mol. Microbiol. **10:**713–735.
40. **Pelicic, V., J.-M. Reyrat, and B. Gicquel.** 1996. Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. Mol. Microbiol. **20:**919–925.
41. **Pelicic, V., J.-M. Reyrat, and B. Giquel.** 1996. Expression of the *Bacillus subtilis sacB* gene confers sucrose sensitivity on mycobacteria. J. Bacteriol. **178:**1197–1199.
42. **Phillip, W. J., S. Poulet, K. Eiglmeier, L. Pascopella, V. Balasubamanian, B. Heym, S. Bergh, B. R. Bloom, W. R. Jacobs, Jr., and S. T. Cole.** 1996. An integrated map of the genome of the tubercle bacillus, *Mycobacterium tuberculosis* H37Rv, and comparison with *Mycobacterium leprae*. Proc. Natl. Acad. Sci. USA **93:**3132–3137.

42a. **Ramakrishnan, L.** 1997. *Mycobacterium marinum* infection of the hand. N. Engl. J. Med. **337:**612.

43. **Ramakrishnan, L., and S. Falkow.** 1994. *Mycobacterium marinum* persists in cultured mammalian cells in a temperature-restricted fashion. Infect. Immun. **62:**3222–3229.

43a. **Ramakrishnan, L., H. T. Tran, and W. R. Bishai.** Unpublished data.

44. **Ramakrishnan, L., R. H. Valdivia, J. H. McKerrow, and S. Falkow.** 1997. *Mycobacterium marinum* causes both long-term subclinical infection and acute disease in the leopard frog (*Rana pipiens*). Infect. Immun. **65:**767–773.
45. **Reyrat, J.-M., F.-X. Berthet, and B. Gicquel.** 1995. The urease locus of *Mycobacterium tuberculosis* and its utilization for the demonstration of allelic exchange in *Mycobacterium bovis* bacillus Calmette-Guerin. Proc. Natl. Acad. Sci. USA **92:**8768–8772.
46. **Rilling, H. C.** 1962. Photoinduction of carotenoid synthesis of a *Mycobacterium* sp. Biochim. Biophys. Acta **60:**548–556.
47. **Rilling, H. C.** 1964. On the mechanism of photoinduction of carotenoid synthesis. Biochim. Biophys. Acta **79:**464–475.
48. **Romer, S.** 1993. Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*. Biochem. Biophys. Res. Commun. **196:**1414–1421.
49. **Ruiz-Vasquez, R., M. Fontes, and F. J. Murillo.** 1993. Clustering and coordinated activation of carotenoid genes in *Myxococcus xanthus* by blue light. Mol. Microbiol. **10:**25–34.
50. **Schumann, G., H. Nurnberger, G. Sandmann, and H. Krugel.** 1996. Activation and analysis of cryptic *crt* genes for carotenoid biosynthesis from *Streptomyces griseus*. Mol. Gen. Genet. **252:**658–666.
51. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn5 suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
52. **Snapper, S. B., R. E. Melton, S. Mustafa, T. Kieser, and W. R. Jacobs, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol. Microbiol. **4:**1911–1919.
53. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennet, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature **351:**456–460.
54. **Will, O. H., III, and C. A. Scovel.** 1989. Photoprotective functions of carotenoids, p. 229–236. *In* N. I. Krinsky, M. M. Mathews-Roth, and R. F. Taylor (ed.), Carotenoids: chemistry and biochemistry. Plenum Press, New York, N.Y.
55. **Wu, Q.-L., D. Kong, K. Lam, and R. N. Husson.** 1997. A mycobacterial extracytoplasmic function sigma factor involved in survival following stress. J. Bacteriol. **179:**2922–2929.