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The Nonmevalonate Pathway of Isoprenoid Biosynthesis in *Mycobacterium tuberculosis* Is Essential and Transcriptionally Regulated by Dxs▽†

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Mycobacterium tuberculosis **synthesizes isoprenoids via the nonmevalonate or DOXP pathway. Previous work demonstrated that three enzymes in the pathway (Dxr, IspD, and IspF) are all required for growth *in vitro*. We demonstrate the essentiality of the key genes *dxs1* and *gcpE*, confirming that the pathway is of central importance and that the second homolog of the synthase (*dxs2*) cannot compensate for the loss of *dxs1*. We looked at the effect of overexpression of Dxr, Dxs1, Dxs2, and GcpE on viability and on growth in *M. tuberculosis*. Overexpression of *dxs1* or *dxs2* was inhibitory to growth, whereas overexpression of *dxr* or *gcpE* was not. Toxicity is likely to be, at least partially, due to depletion of pyruvate from the cells. Overexpression of *dxs1* or *gcpE* resulted in increased flux through the pathway, as measured by accumulation of the metabolite 4-hydroxy-3-methyl-but-2-enyl pyrophosphate. We identified the functional translational start site and promoter region for *dxr* and demonstrated that it is expressed as part of a polycistronic mRNA with *gcpE* and two other genes. Increased expression of this operon was seen in cells overexpressing Dxs1, indicating that transcriptional control is effected by the first enzyme of the pathway via an unknown regulator.**

Mycobacterium tuberculosis, the causative agent of human tuberculosis, poses an increasing threat to health. The escalating incidence of drug resistant strains provides a new impetus to understand this complex pathogen and to identify metabolic targets for novel therapeutics. As a first step toward this aim, we have been directing our efforts to identifying metabolic pathways essential for bacterial viability.

Several of the current antibiotics active against *M. tuberculosis* target cell wall biosynthesis, in particular the synthesis of mycolic acids (inhibited by isoniazid and ethionamide) or arabinogalactan and lipoarabinomannan (inhibited by ethambutol). Isoprenoid biosynthesis is a key synthetic pathway required for the generation of many cellular components, including cell wall components. *M. tuberculosis* synthesizes the isoprenoid precursor, isopentenyl diphosphate (IPP), via the nonmevalonate or 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway, in contrast to the human mevalonate pathway. Therefore, the bacterial enzymes can be specifically targeted without interfering with eukaryotic isoprenoid biosynthesis, making this an attractive pathway in the search for novel drugs.

The DOXP pathway has been characterized in several bacterial species; all of the genes can be identified by homology in the *M. tuberculosis* genome, and the proposed pathway can be reconstructed (Fig. 1). Recombinant proteins have been produced, and enzymatic activities have been confirmed for Dxs1 (2), Dxr (11, 18), IspD (14), and IspF (7). In addition, previous work demonstrated that Dxr, IspD, and IspF are all essential for the *in vitro* growth of *M. tuberculosis* (6, 7, 14). *M. tuberculosis* genome has two homologs of *dxs* (*dxs1*, Rv2682c; and *dxs2*, Rv3379c) and *lytB* (*lytB1*, Rv3382c; and *lytB2*, Rv1110). The presence of two homologs of Dxs is also seen in the closely related actinomycete *Streptomyces coelicolor* (8), where both enzymes are functional. In contrast, in *M. tuberculosis* only Dxs1 (Rv2682c) is a functional DOXP synthase (2).

The DOXP pathway has been implicated in the pathogenic potential of mycobacterial species. Disruption of the *lytB1-dxs2* operon in *M. tuberculosis* abolishes the bacterial ability to prevent acidification of the phagosome and results in attenuated intracellular survival (30). In addition, an *M. avium* subsp. *paratuberculosis gcpE* mutant is less able to colonize tissue during infection of mice or calves (35, 36), confirming that this pathway is required for virulence. However, in both cases, it is yet to be determined whether this is a direct link between isoprenoid biosynthesis and the phenotypic consequences, since no measure of IPP synthesis was made.

In contrast to the biochemical characterization, little is known about the regulation of expression of the DOXP pathway genes or the control of flux through the pathway in *M. tuberculosis*. In *M. tuberculosis*, as with other organisms, DOXP pathway genes are distributed throughout the genome, with no apparent motif for any recognized global regulator. Few clues as to the regulation of this pathway are found in other organisms, and no transcriptional regulators have been identified. Several of the DOXP genes appear to be located in operons:

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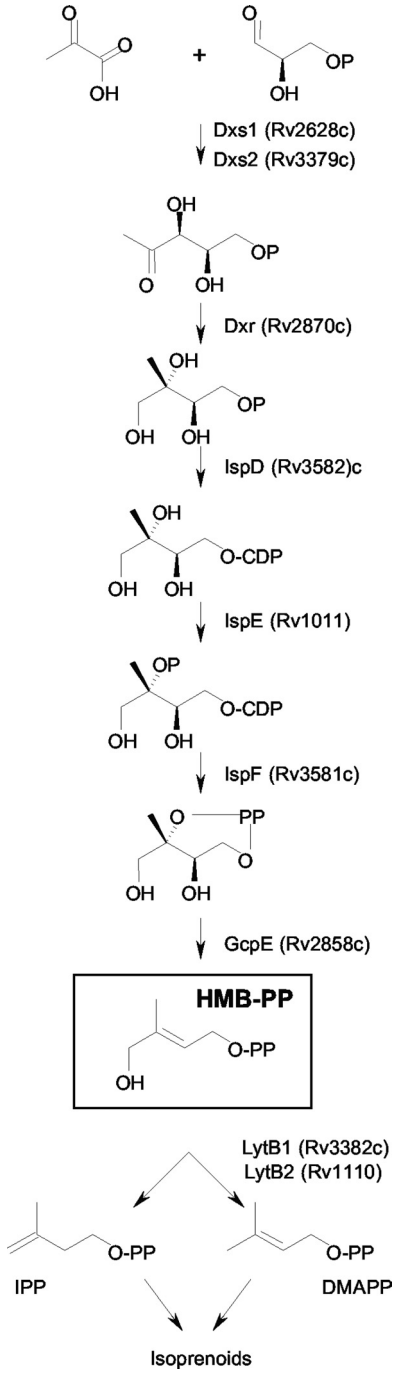


FIG. 1. DOXP pathway of isoprenoid biosynthesis in *M. tuberculosis*. The metabolic pathway from Dxs to LytB is shown. Enzyme names are given with the corresponding H37Rv gene numbers in parentheses. IPP, isopentenyl phosphate; DMAPP, dimethylallyl pyrophosphate HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate is boxed.

dxr is located in an apparent operon with *gcpE* and two other genes (*rip* and *gcn5*), *ispD* and *ispF* appear to be part of a bicistronic operon, and *lytB1* appears to be in an operon with *idsB* and possibly *dxs2*. The remaining genes (*ispF*, *dxs2*, and *lytB2*) are expressed as monocistronic units (Fig. 2). Changes in gene expression have been observed after antibiotic exposure (4) and LytB is downregulated in a cytochrome bc_1 mutant of *Mycobacterium smegmatis* (25).

In the present study we investigated the essentiality and regulation of key members of the DOXP pathway in *M. tuberculosis* (Dxs, Dxr, and GcpE). We confirmed that Dxs1 and GcpE are essential and that Dxs2 cannot complement for the functional loss of Dxs1. Since essential genes by definition cannot be deleted from the cell, we looked at the effect of overexpression of these enzymes on the viability and phenotypic characteristics of the bacilli. We also investigated the organization and expression of the *dxr* operon.

MATERIALS AND METHODS

For primer sequences and plasmid descriptions, see Tables S1 and S2 in the supplemental material.

Culture of mycobacteria. *M. tuberculosis* H37Rv was cultured in Middlebrook 7H9 liquid medium supplemented with 10% (vol/vol) OADC (oleic acid, bovine serum albumin, D-glucose, catalase; Becton Dickinson) and 0.05% (wt/vol) Tween 80 or on solid Middlebrook 7H10 agar supplemented with 10% (vol/vol) OADC at 37°C. For overexpression studies using the acetamidase promoter, 7H9 medium was supplemented with 0.2% (wt/vol) succinate, 0.5% (wt/vol) bovine serum albumin (BSA) (uninduced) or with 0.2% succinate, 0.2% (wt/vol) acetamide, and 0.5% (wt/vol) BSA (induced) instead of OADC. *M. smegmatis* mc^2155 was grown on Lemco medium (33). X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at 50 μg/ml, IPTG (isopropyl-β-D-thiogalactopyranoside) at 0.5 mM, kanamycin at 20 μg/ml, hygromycin B at 100 μg/ml, gentamicin at 10 μg/ml, and sucrose at 2% (wt/vol) where required. *M. tuberculosis* growth curves were conducted in 16-mm glass tubes containing an 8-mm magnetic stirrer bar with stirring at 150 rpm.

Determination of essentiality. Deletion delivery vectors for *dxs1*, *dxr*, and *gcpE* were constructed as follows. First, ~1 kb of the flanking regions surrounding each gene was amplified from *M. tuberculosis* genomic DNA using primer pairs (US/DS). Fragments were cloned into p2NIL using the PstI-HindIII restriction sites (28). The 6.3-kb PacI cassette from pGOAL19 (28) (*hyg*, *sacB*, and *lacZ*) was cloned into the sole PacI site. Vectors were verified by restriction digest and sequencing. 5 μg of UV pretreated plasmid DNA (27) was electroporated into competent *M. tuberculosis* (15), and single-crossover (SCO) transformants were selected on medium containing kanamycin, hygromycin, and X-Gal. Complementing vectors carrying a functional copy of the gene were made for *dxs1* (under the control of Ag85A promoter in pAPA3 [26]) and for *dxr* and *gcpE* (the complete operon together with the native promoter [6]). Merodiploid strains were constructed by electroporating the SCO strains with the appropriate complementing plasmids and isolating kanamycin-, hygromycin-, and gentamicin-resistant transformants. Double crossovers (DCOs) were isolated in the wild-type and merodiploid background by streaking cells onto plates lacking antibiotics and selected/screened on medium containing sucrose, X-Gal, and gentamicin where required as previously described (28). PCR screening to determine which allele (wild-type or deletion) was present in the chromosomal location was carried out using gene-specific screening primers. The genotype of selected strains were confirmed by Southern analysis using the AlkPhos Direct system (GE Healthcare) according to the manufacturer's instructions.

Gene switching. Gene switching was carried out as previously described (5, 29). Strains carrying integrated plasmids were electroporated with an integrating plasmid bearing an alternative selection marker, and transformants were isolated by selecting for the incoming plasmid. Switching was confirmed by patch testing for the loss of the appropriate resistance.

Construction of overexpression strains. *dxs1*, *dxs2*, *dxr* (expressed from the first possible start codon), dxr_t (truncated *dxr*, expressed from the last possible start codon), and *gcpE* were amplified using gene specific primers with *Pfu* DNA polymerase and cloned into pSMT3 under the control of the strong, constitutive *hsp60* promoter from *Mycobacterium bovis* (19) or into pJFR19, under the control of the inducible acetamidase gene from *M. smegmatis* (10). Constructs were introduced into *M. tuberculosis* via electroporation (15) and selected on plates containing hygromycin. Three independent transformants were selected for each plasmid. Overexpression was confirmed by reverse transcription-PCR (RT-PCR).

Construction of *dxr* expression vectors. *dxr* was amplified and cloned into pBAD202 (Invitrogen). Insert integrity was confirmed by sequencing. Growth was measured in transformants grown in half-strength LB with various concentrations of arabinose. The truncated *dxr* gene (dxr_t) was amplified and cloned into

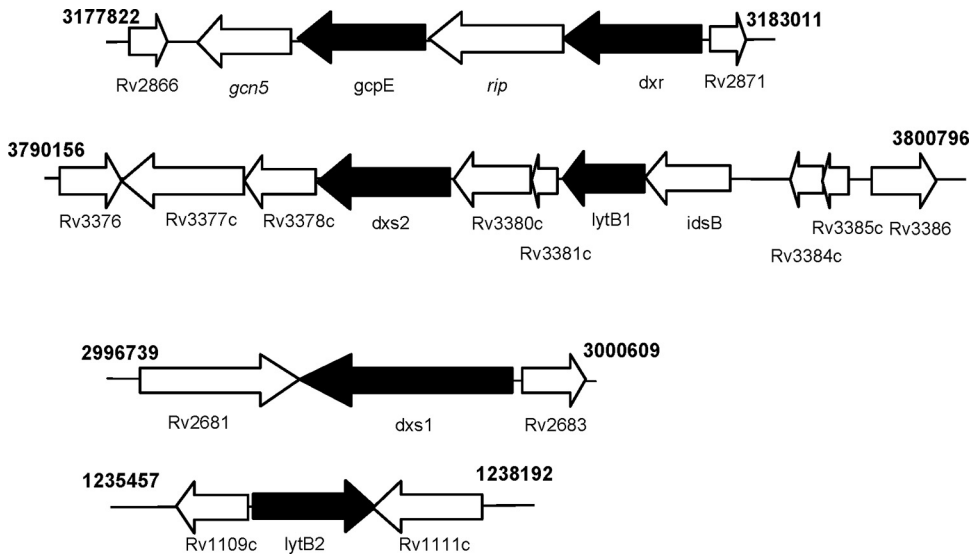


FIG. 2. Genomic organization of the DOXP genes. The chromosomal arrangement is indicated. DOXP pathway genes are in black. The genetic coordinates for the H37Rv strain of *M. tuberculosis* are given.

pBAD202 or pSMT3. Plasmids were introduced into *M. smegmatis* and *M. tuberculosis* via electroporation and selected on hygromycin. Three independent transformants were analyzed for each vector.

RT-PCR. RNA was prepared from *M. tuberculosis* standing cultures as previously described and DNase treated (34). cDNA was synthesized from 1 μg of total RNA using SuperScript II RT (Invitrogen) and random primers according to the manufacturer's instructions. An RT-minus control for each sample was also included and processed in tandem. Cycling conditions were 30 cycles of 94°C for 30″, 56°C for 30″, and 72°C for 1′ using GoTaq PCR Mastermix (Promega). The amplified products were run on a 1% agarose gel and visualized with ethidium bromide.

Promoter analyses. The upstream regions of *dxr* and *gcpE* were amplified and cloned into the integrating promoter reporter vector pSM128 (12). Plasmids were electroporated into *M. tuberculosis* and transformants selected on streptomycin. Three transformants for each were used to inoculate standing cultures. Cell extracts and β-galactosidase assays were performed as described previously (9).

Semiquantitative RT-PCR analysis. We used limiting dilution RT-PCR to determine mRNA levels (31). PCR was carried out using undiluted cDNA and serial fourfold dilutions (1:4, 1:16, 1:64, 1:256, and 1:1,026) using RT primers for *dxs1*, *dxs2*, *dxr*, *gcpE*, and *sigA*. The cycling conditions were 30 cycles of 94°C for 30″, 56°C for 30″, and 72°C for 1′ using GoTaq PCR Mastermix (Promega). The amplified products were run on a 1% agarose gel and visualized with ethidium bromide.

Macrophage infection assays. Macrophage infection assays were carried out using human monocytic leukemia cell line (THP-1) (ATCC–TIB-202) as described previously (23). Determination of the initial inoculum was assessed by plating serial dilutions and the number of intracellular bacteria was monitored over 7 days.

HMB-PP assays using stimulation of γδ cells. Strains were cultured aerobically in 100 ml of medium in roller bottles for 7 days. Cells were harvested by centrifugation at 4,000 × *g*, washed twice with 10 ml of 10 mM Tris (pH 7.5), resuspended in 1 ml of 10 mM Tris (pH 7.5), and transferred to Lysing Matrix B tubes (MP Biomedicals). Cells were disrupted using a FastPrep (MP Biomedicals) at speed 6.5 for 40 s and passed through a 0.2-μm-pore-size filter. Protein concentrations were measured by using a BCA protein concentration kit (Pierce) and adjusted to 0.5 mg/ml. Low-molecular-weight filtrates for stimulation assays were prepared by passing the cell-free effluents through 0.1-μm-pore-size Durapore polyvinylidene difluoride Ultrafree-MC and 3,000 MWCO regenerated cellulose Ultracel-YM3 filters (Millipore), as previously described (13). PBMC were cultured in RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 50 μg of penicillin-streptomycin/ml, 50 μM β-mercaptoethanol, and 10% fetal calf serum (Invitrogen). A total of 200,000 PBMC per well were stimulated by addition of serial dilutions of synthetic 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP) (32) or low-molecular-weight filtrates, in the presence of 20 to 100 U of recombinant interleukin-2 (Proleukin; Chiron)/ml. γδ T-cell activation was determined by measuring the upregulation of CD25 and CD69 on the cell surface and the expansion of Vγ9/V δ 2 T cells over 6 days, and the bioactivity of the bacterial preparation was expressed as micrograms of HMB-PP per gram of protein, as described previously (13).

RESULTS

***dxs1* and *gcpE* are essential for *in vitro* growth of *M. tuberculosis*.** Several genes encoding enzymes of the DOXP pathway are essential for the viability of *M. tuberculosis* in culture (6, 7, 14). However, we had not previously investigated *dxs1* or *gcpE*. We attempted to construct in-frame, unmarked deletions for each gene in a wild-type background and in a merodiploid background (in which a functional copy of the gene was provided on an integrating plasmid). For both genes we were unable to isolate chromosomal deletions strains in the wild-type background ($n = 40$ for each gene). We confirmed that both genes were essential by obtaining chromosomal deletions of *dxs1* and *gcpE* when the second copy of the gene was introduced (20 of 40 and 23 of 40 strains tested were deletion alleles for *dxs1* and *gcpE*, respectively). The expected genotypes of selected strains were confirmed by Southern analysis (data not shown).

The essentiality of *dxr* has been demonstrated in our laboratory, but the strain generated during this process had a deletion in which the upstream region of *dxr*, as well as *dxr* itself and the start codon of the downstream gene *rip*, was deleted (6). Since we wanted to have a strain in which *dxr* alone was deleted for further analysis, we constructed a second deletion delivery vector (in which *dxr* was deleted and the promoter region and adjacent genes remained intact). Using this deletion vector, we confirmed our previous results demonstrating the essentiality of *dxr* in that we were unable to obtain a chromosomal deletion in the wild-type background (40 strains tested) but were able to do so in the merodiploid (19 of 40

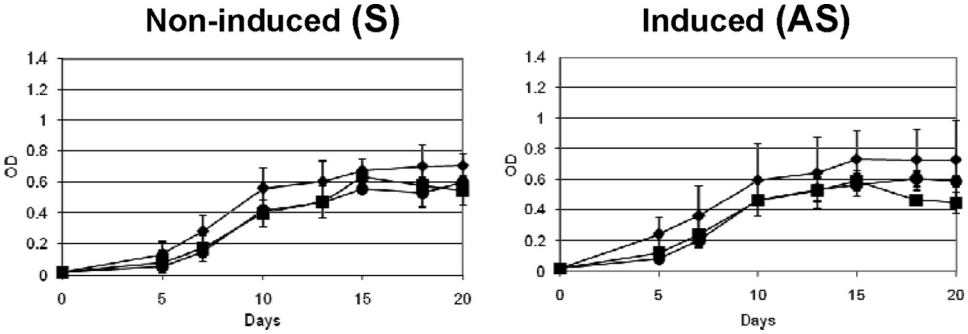


FIG. 3. Overexpression of Dxs1 or Dxs2 is inhibitory to growth in mycobacteria. Transformants overexpressing Dxs1 or Dxs2 were grown in liquid medium under noninduced (S = succinate) or induced (AS = acetamide, succinate) conditions. Symbols: ◆, control vector pJFR19; ■, *dxs1*-overexpressing vector; ●, *dxs2*-overexpressing vector. The results are expressed as the means and standard deviations of three independent transformants.

strains tested). The expected genotypes of selected strains were confirmed by Southern analysis (data not shown).

We further confirmed essentiality of all three genes using gene switching to demonstrate that the complementing vector could not be removed when the chromosomal gene copy had been deleted (5, 29). We attempted to replace the complementing vector with an empty vector (pUC-Hyg-Int) (24) carrying a different resistance marker (*hyg*). We did not obtain any transformants in which the resident complemented vector was replaced by pUC-Hyg-Int for any of the genes. Controls in which the empty vector was switched into the merodiploid strains (where the chromosomal gene copy was still intact and an integrated copy was present) gave a typical switching efficiency of $10^7/\mu g$. These data confirm that viability is dependent on the presence of the complementation vector for all three genes. Therefore, we were able to conclude that in addition to *dxr*, *dxs1*, and *gcpE* are essential for the *in vitro* growth of *M. tuberculosis*.

We also attempted to make deletion mutants of *dxs2*. We used a number of alternative deletion delivery constructs but were unable to obtain single crossovers without rearrangements in the chromosome. This is likely to be caused by the presence of an insertion element in the area; since our deletion constructs also contained the insertion element, it could easily lead to problems with rearrangements and recombination between repeat elements.

Overexpression of Dxs1 or Dxs2, but not GcpE, is inhibitory to growth in *M. tuberculosis*. Since we had confirmed that Dxr, Dxs1, and GcpE are all essential for the viability of *M. tuberculosis* in culture, we were unable to construct deletion mutants for phenotypic studies. Instead, we decided to look at the effect of overexpression of each. We first attempted to overexpress Dxs1 and Dxs2 independently; plasmids carrying either *dxs1* or *dxs2* under the control of the strong, constitutive *hsp60* promoter were constructed and transformed into *M. smegmatis* and *M. tuberculosis*. No transformants were obtained on several occasions, suggesting that high level expression was toxic to the cells. We therefore constructed alternative expression vectors in pJFR19 under the control of the inducible acetamidase gene from *M. smegmatis* (10). The acetamidase promoter has a basal level of activity in the "noninduced" state and is further upregulated by the addition of acetamide; thus, overexpression is normally achieved in the absence of induction but can be boosted by the addition of the inducer acetamide.

We looked at the effect of overexpression of *dxs1* and *dxs2* using the acetamidase promoter expressing at the basal level and after further induction by acetamide in *M. tuberculosis* (Fig. 3). Growth was slightly retarded regardless of the addition of inducer, which could be explained since expression from the acetamidase promoter is leaky (10, 33). Overexpression had a slightly detrimental effect on the growth of *M. tuberculosis* (Fig. 3), suggesting that high level expression of either of the two Dxs enzymes is toxic. This was particularly interesting given that recombinant Dxs2 does not have DOXP synthase activity (2) and suggests that it may have an alternative, as-yet-unidentified function. This effect was specific to Dxs1/Dxs2, since overexpression of GcpE did not result in retarded growth (data not shown).

We also looked at the effect of Dxs overexpression on other phenotypes that could be affected by changes in cell wall synthesis, but no effect on antibiotic resistance (rifampin, isoniazid, novobiocin, ethambutol, and ampicillin), gross colony morphology, or replication in macrophages was seen (data not shown).

We used the same strategy to overexpress *gcpE* from the *hsp60* promoter. No problems were seen with toxicity and plasmids were easily transformed into *M. smegmatis* and *M. tuberculosis.* No effect on growth in liquid culture, antibiotic resistance or gross colony morphology was found, nor was there any defect in or replication in macrophages (data not shown).

Overexpression of Dxr in *Escherichia coli* and mycobacteria. We also looked at the effect of overexpression of Dxr. Our initial attempts to clone *dxr* into routine cloning vectors (pGEM T-Easy, pSMT3, and pJRF19) failed. We either obtained no transformants, or the few transformants found had mutations in the coding sequence. This suggested that the clone was toxic to *E. coli* and could not be tolerated. In order to overcome potential toxicity we cloned *dxr* under the control of the tightly repressed arabinose promoter using the pBAD system in which the protein is expressed as a thioredoxin fusion protein. Plasmids were easily obtained without mutations in the *dxr* coding sequence. We assessed growth in *E. coli* with increasing concentrations of arabinose to induce expression of

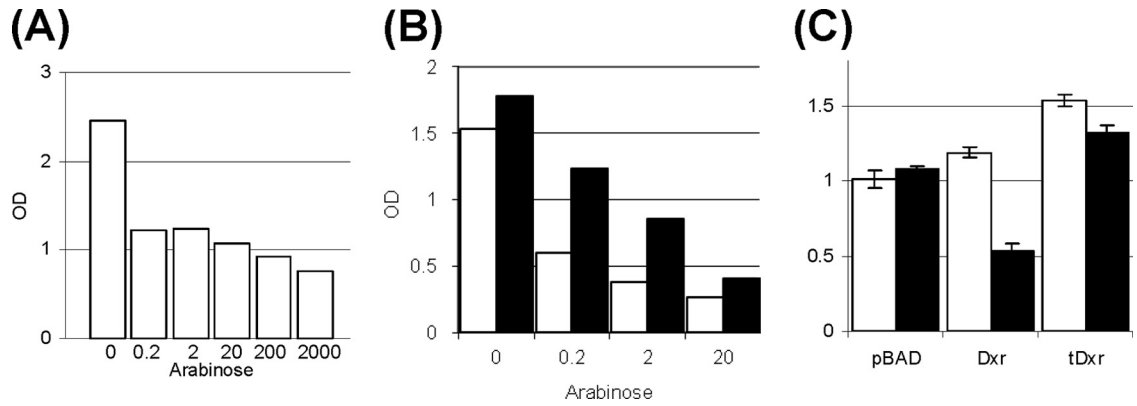


FIG. 4. Induction of Dxr expression results in growth inhibition in *E. coli*, which is partially relieved by pyruvate addition. The growth of transformants expressing Dxr from the arabinose-inducible pBAD plasmid was measured. (A) Inhibition of growth in response to increased Dxr expression. Growth was measured in half-strength LB medium. Various concentrations of arabinose (μg/ml) were used to induce expression from the pBAD promoter, and growth was measured after 4 h. (B) Relief of toxicity by pyruvate. Strains were grown in the presence of various concentrations of arabinose (indicated in μg/ml). □, No sodium pyruvate; ■, plus 0.5% sodium pyruvate. Growth was measured after 20 h. (C) Truncated Dxr is not toxic. Strains carrying the full-length ("Dxr") or truncated ("tDxr") constructs were grown in the presence of no arabinose (□) or 1% arabinose (■) to induce expression. Growth was measured after 20 h. The results are expressed as the means of three independent transformants.

dxr; this clearly had a toxic effect and inhibited growth (Fig. 4). We hypothesized that toxicity could be due to depletion of pyruvate from the cells, since this is the substrate of the pathway. Addition of 0.5% sodium pyruvate to the medium partially relieved toxicity, allowing increased cell growth in the presence of elevated Dxr expression, but it did not completely reverse the phenotype (Fig. 4). Thus, the toxicity was not wholly dependent on Dxr enzyme activity.

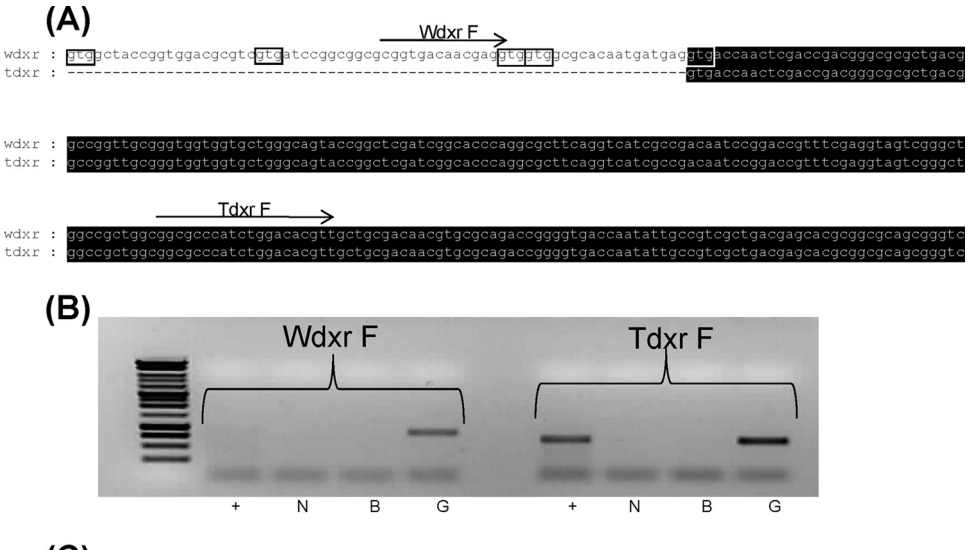
The translational start site for Dxr has not been identified by experimental means and could be any one of five initiation codons (Fig. 5). In our attempts to clone *dxr*, we initially amplified a region containing all of these potential start codons, to ensure production of a full-length protein. In addition, previous work with enzymatically active Dxr was achieved using a shorter version (11). We decided to attempt to clone a smaller fragment of DNA starting at the sequence from the putative start codon furthest downstream. We cloned this short fragment into the arabinose-controlled system (pBAD) and assessed toxicity in *E. coli*. Overexpression of Dxr from this construct was not toxic for *E. coli* (Fig. 5). Since this version was tolerated, we cloned the region into the pSMT3 overexpression vector and easily isolated a recombinant plasmid with no mutations in *dxr.* The resultant plasmid transformed *M. smegmatis* and *M. tuberculosis* at a high efficiency. Growth curves for *M. smegmatis* and *M. tuberculosis* demonstrated no change in growth rate after Dxr overexpression; no effect on antibiotic resistance, gross colony morphology or replication in macrophages was seen (data not shown). Thus, the original toxicity noted appears to derive from inclusion of the upstream region.

Determination of the translational start site of Dxr. Our results showed that the inclusion of the upstream region of Dxr construct resulted in toxicity. Since expression of the clone in pBAD results in a fusion protein by virtue of the cloning method, the upstream region would be translated into protein, and this itself could be responsible for a toxic effect. In order to determine whether this had any bearing on the normal expression of Dxr and whether the native protein might contain a toxic N terminus, we wanted to identify which of the five potential translational start sites were utilized in *M. tuberculosis*. In order to identify which start codon is used in the native host, we used two approaches.

First, we determined the 5′ limit of transcription for the *dxr* gene transcript in *M. tuberculosis.* RNA was isolated from *M. tuberculosis* and subjected to RT-PCR using primers designed to amplify different regions at the 5′ end of *dxr*. An amplification product was obtained with one set of primers only, demonstrating that the shorter transcript is the only one present and that the region upstream is not transcribed (Fig. 5).

Second, we looked at the functionality of the translational start codons in *M. tuberculosis*. We made use of the strain we had constructed in which the chromosomal copy of *dxr* is deleted and the only functional copy is present on the integrating vector (which could be replaced). A number of complementing vectors were constructed, in which each putative start codon was mutated to alanine (Fig. 5). Vectors were tested for functional complementation of the chromosomal deletion by gene switching, i.e., we replaced the sole functional copy with the mutant allele. Functional complementation was obtained with five of the six mutant constructs; the only construct that failed to complement was that with the furthest downstream V-to-A mutation. We also constructed a mutation in which a stop codon was introduced immediately upstream; this vector was able to functionally complement the deletion. These data confirm that the distal site is the functional translational start site in *M. tuberculosis.*

Dxr is expressed as an operon with GcpE. Dxr appears to be expressed as an operon with three other genes (Fig. 2) since the gaps between the genes are small, although the ORFs do not overlap. In order to define whether the genes are cotranscribed as an operon, we conducted RT-PCR to look at coexpression of each pair of genes. Primers were designed to amplify the junctions between *dxr*/*rip*, *rip*/*gcpE*, and *gcpE*/*gcn5* and used on cDNA from *M. tuberculosis*; in each case a specific



(C)

Plasmid	Upstream sequence	Functional complementation
pOPH	VATGGRVVIRRGDNEVVAHNDEV	Yes
pOP1	**A**ATGGRVVIRRGDNEVVAHNDEV	Yes
pOP2	VATGGR**AA**IRRGDNEVVAHNDEV	Yes
pOP3	VATGGRVVIRRGDNE**AA**AHNDEV	Yes
pOP4	VATGGRVVIRRGDNEVVAHNDE**A**	No
pOP6	VATGGRVVIRRGDNEVVAHN*EV	Yes

FIG. 5. Determination of the correct translational start site for Dxr. (A) The upstream region of Dxr is shown, indicating the region included in the full-length, toxic clone (wDxr) and the shorter clone (tDxr). Potential translational start sites are boxed. The upstream primers used for RT-PCR are indicated. (B) RT-PCR analysis of the *dxr* transcript. RNA was isolated from wild-type *M. tuberculosis*, cDNA was synthesized and amplified with the indicated primers plus a common downstream primer: +, plus RT; N, no RT; B, blank (no template); and G, genomic DNA. (C) Functional analysis of the Dxr start codon. Mutations were made in the upstream region as indicated in boldface underline and tested for functionality using gene switching. The potential translated sequence is shown, pOPH encodes the wild-type sequence.

product was detected, indicating that the four genes are co-transcribed and form a bona fide operon (Fig. 6).

We also identified the promoter region of the operon. We amplified the region upstream of *dxr* and tested it for promoter activity using a LacZ reporter. Significant promoter activity (204 ± 20 Miller units) was detected in the upstream region confirming that a promoter is present (Fig. 6). Since we were particularly interested to know whether *dxr* and *gcpE* were coexpressed, we also tested the region upstream of *gcpE* for promoter, but none was detected (2.2 ± 0.4 Miller units; control plasmid 2.8 ± 0.2 Miller units), indicating that there is not an alternative functional promoter (Fig. 6).

Although our data demonstrated that there was no promoter activity from the region upstream of *gcpE*, and that *gcpE* would be coexpressed with *dxr*, we considered that there was the possibility that a promoter could exist which was only functional under specific conditions. Therefore, we used a functional assay for the expression of *gcpE* in cells. We used the *M. tuberculosis* strain in which the *dxr* promoter (and *dxr*) was deleted and where functional complementation was provided by the complete *dxr* operon (6). In this strain the remainder of the *dxr* operon was intact, including *gcpE* and its upstream region. We constructed a complementing vector, in which *gcpE* was deleted from the operon and tested it for functional complementation by gene switching. No viable colonies were isolated, indicating that *gcpE* was not being expressed from the chromosomal location. In control switching experiments in which the same vector was used, but the strain background was wild type, a transformation efficiency of 10^8 per μg of plasmid DNA was achieved. Thus, we confirmed that in the normal chromosomal location *gcpE* is expressed from the promoter upstream of *dxr* and that this represents a functional operon.

Overexpression of DOXP genes leads to the accumulation of HMB-PP. Many synthetic pathways are subject to posttranslational control with the first enzymatic reaction being a regulatory step controlling flux through the pathway by, for example, feedback inhibition of enzyme activity by the end product.

In order to assess the effect on flux through the synthetic pathway resulting from overexpression of enzymes from either the beginning or the end of the pathway, we measured accumulation of one of the end products of the pathway; HMB-PP is the product of the GcpE-catalyzed reaction and can be measured using a biological assay (stimulation of γδ T cells) (13).

We used this assay to measure changes in HMB-PP levels in the overexpressing strains (Fig. 7). As expected, Dxs2 overexpression did not affect HMB-PP levels, since it is not a functional enzyme. In contrast, overexpression of either Dxs1 or

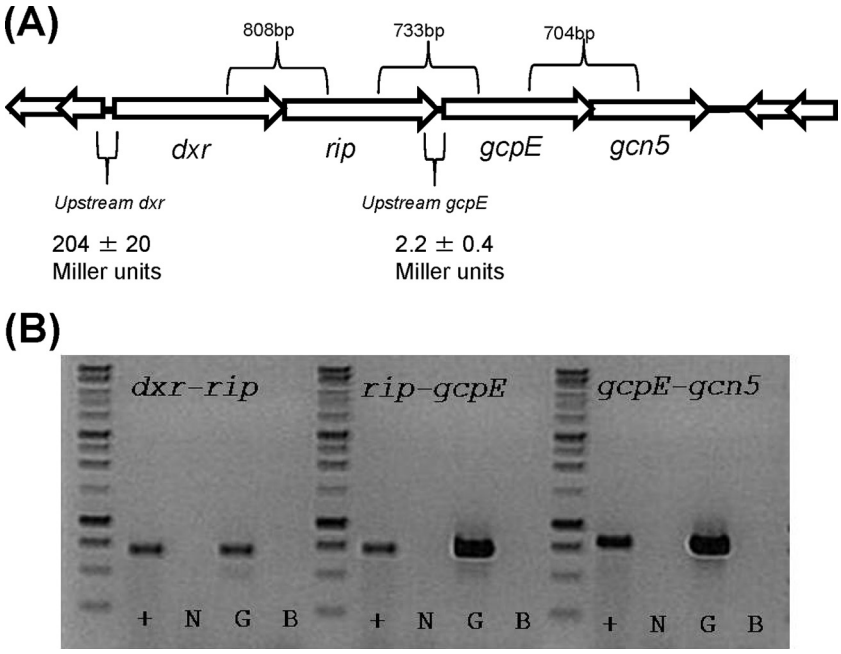


FIG. 6. Dxr is part of a multicistronic operon containing *gcpE*. (A) The *dxr* operon is shown. The upstream regions (~0.2 kb) of *dxr* and *gcpE* tested for promoter activity are indicated. The results are the mean β-galactosidase activities (± the standard deviations) for three independent transformants assayed in duplicate in Miller units. The activity from the control plasmid pSM128 was 2.8 ± 0.2 Miller units. (B) Cotranscription of the *dxr* operon was confirmed by RT-PCR using primers to amplify the junctions between each gene pair indicate in panel A. Primers were designed to amplify across the gene junctions; the potential amplification products are marked on the operon. +, Plus RT; N, no RT; B, blank (no template); G, genomic DNA.

GcpE led to a twofold increase in HMB-PP levels over the wild type. Overexpression of Dxr had no effect. These results were unexpected, in that we found overexpression of the first or last enzyme, but not the intermediate enzyme led to increased end product accumulation. Normally, one enzyme will be the rate-limiting step in the metabolic pathway, and this choke point would determine flux through the pathway. For example, if Dxs were the limiting step, then overexpression of GcpE should make no difference to HMB-PP accumulation; similarly, if GcpE were the limiting step, then overexpression of Dxs would have no effect. This led us to hypothesize that regulatory control over and above enzymatic activity was occurring, possibly at the level of transcription.

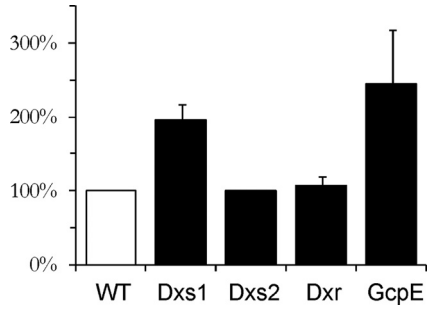
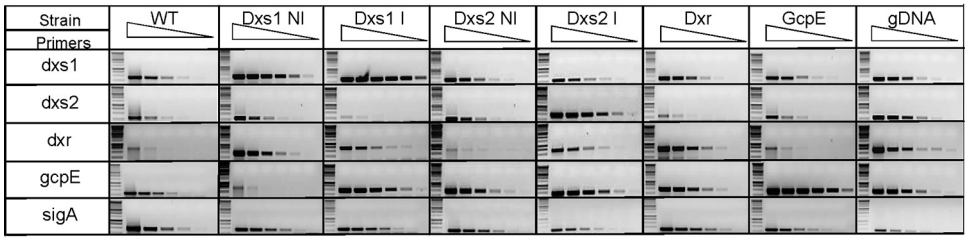


FIG. 7. Overexpression of Dxs1 and GcpE leads to increased levels of HMB-PP. *M. tuberculosis* strains overexpressing Dxs1, Dxs2, Dxr, or GcpE were analyzed for HMB-PP content using an assay for stimulation of γδ T cells. The results are the mean ± the standard deviation of extracts from three individual transformants. The results are expressed relative to levels in the wild-type strain. Dxs1 and Dxs2 were expressed from the acetamidase promoter and assayed under inducing conditions. tDxr (dxr_t) and GcpE were expressed from the constitutive hsp60 promoter. WT, wild-type *M. tuberculosis*.

Overexpression of Dxs leads to upregulation of the Dxr operon. In order to address this possibility that overexpression of one enzyme could lead to transcriptional changes, we looked at the level of expression of Dxs1, Dxr and GcpE in the overexpressing strains. For each strain, we confirmed that the expected genes were being overexpressed from the plasmid constructs; levels of overexpression were approximately 16-fold under induced conditions (Fig. 8). The data also confirmed that all of the genes were expressed in the wild-type strain.

Overexpression of Dxr or GcpE showed no increase in the expression levels of the other genes tested. In contrast, overexpression of Dxs1 and Dxs2 led to increased levels of *dxr* transcripts (Fig. 8); for Dxs1 it also resulted in increased levels of *gcpE* transcripts. These data explain how Dxs1 overexpression leads to increased production of HMB-PP, since it also results in the upregulation of GcpE, the last step of the pathway. *gcpE* was not increased in the Dxs2-overexpressing strain, but the amplification product showed some degree of smearing, suggesting that the mRNA was being degraded from the 3′ end; since GcpE is not upregulated in this strain it would account for the lack of increase in HMB-PP in this strain. In addition, Dxs2 was downregulated in *dxs1*- and *dxr*-overexpressing strains. From these data we would therefore predict that GcpE is the rate-limiting step, but that Dxs1 and Dxs2 are involved in transcriptional control and thus control flux at the start point.

(A)



(B)

Gene	Strain: WT	Dxs1 NI	Dxs1 I	Dxs2 NI	Dxs2 I	Dxr	GcpE
dxs1	1	4	16	1	1	1	1
dxs2	1	1	0.06	4	16	0.25	1
dxr	1	16	16	1	16	16	1
gcpE	1	0.25	4	1	4	1	16
sigA	1	1	1	1	1	1	1

FIG. 8. Quantitation of expression levels of DOXP pathway genes in overexpressor strains. Limiting dilution RT-PCR was carried out with gene specific primers for *dxs1*, *dxs2*, dxr_t, and *gcpE* on cDNA made from the overexpressing strains. Serial 4-fold dilutions (1:1, 1:4, 1:16, 1:64, 1:256, and 1:1,024) of cDNA were used as a template for PCR with gene-specific primers. (A) Gel pictures obtained after PCR, showing product intensity from increasing diluted cDNA; (B) relative expression levels for each gene. RNA input was standardized using *sigA* expression; values were then normalized to the WT value to give an indication of the relative fold difference. WT, wild-type H37Rv; Dxs1 (NI), strain expressing *dxs1* from acetamidase promoter in noninducing conditions (basal level); Dxs1 (I), strain expressing *dxs1* from acetamidase promoter in inducing conditions. Dxs2 (NI), strain expressing *dxs2* from acetamidase promoter in noninducing conditions (basal level); Dxs2 (I), strain expressing *dxs2* from acetamidase promoter in inducing conditions. Dxr, strain constitutively overexpressing dxr_t from the hsp60 promoter; GcpE, strain constitutively overexpressing *gcpE* from the hsp60 promoter; gDNA, genomic DNA control.

DISCUSSION

The DOXP pathway of isoprenoid biosynthesis is an attractive target in the search for novel therapeutics. Successful targeting of this pathway in other organisms has already been achieved, since the antibiotic fosmidomycin inhibits Dxr. The majority of the genes of the pathway are essential, and therefore this is a crucial metabolic pathway in *M. tuberculosis* worthy of further study.

GcpE is essential in many other species, including *E. coli* (1), which rely on the DOXP pathway as the sole biosynthetic pathway for isoprenoids, and we have demonstrated that this also holds true for *M. tuberculosis*. Surprisingly, inactivation of *gcpE* by transposon mutagenesis has been achieved in *M. avium* subsp. *paratuberculosis* (35), raising the possibility that there are other pathways for isoprenoid synthesis in this species but not *M. tuberculosis*.

M. tuberculosis is unusual in that it possesses two homologs of the DOXP synthase. Dxs1 is a fully functional enzyme, but recombinant Dxs2 has no synthase activity due to an N-terminal truncation resulting in the loss of a critical histidine residue (2). Our data demonstrate that as expected Dxs1 is the key enzyme in the synthetic pathway, being essential for *in vitro* growth, and shows that its loss cannot be complemented for by Dxs2.

Overexpression of both *dxs1* and *dxs2* had a slightly detrimental effect on growth of *M. tuberculosis*. High-level expression of recombinant Dxs is reported to result in reduced growth in *E. coli*; this phenotype was attributed to a drain of metabolites (21). Similarly, in *M. tuberculosis*, Dxs1 overexpression could deplete the substrates pyruvate and glyceraldehyde-3-phosphate (G3P), crucial cellular components required for the production of acetyl coenzyme A, glycolysis, and other metabolic pathways. However, this cannot account for growth inhibition by Dxs2, given that *dxs2* does not encode a functional enzyme. However, the differences were small, so it remains to be determined whether this results from increased enzyme activity or from a general stress resulting from protein overexpression.

Increases in flux through the pathway, as measured by production of HMB-PP were seen after overexpression of GcpE or Dxs1. We hypothesize that GcpE is the limiting step of the pathway, and therefore overexpression will lead to increased flux. In contrast increased flux after Dxs1 overexpression results from a concomitant increase in GcpE expression. This is the first demonstration of a link between activity of an enzyme in the DOXP pathway and transcriptional regulation of the pathway. Dxs has been previously implicated as the rate-limiting step for the synthesis of IPP in *E. coli* strains engineered to overproduce lycopene (16, 20). In contrast, we found that GcpE is the bottleneck in *M. tuberculosis* and that Dxs appears to regulate flux through the pathway by mediating transcriptional control of GcpE, once again, indicating how mycobacterial systems differ from those of other bacteria (3). Interestingly, Dxs2 also caused transcriptional changes, despite having no enzyme activity; this suggests that regulation is not due to accumulation of the product of Dxs, but another mechanism (2). Future work to identify the regulatory proteins and mechanisms could cast light on the control of isoprenoid synthesis. It is possible that the level of expression of the enzymes is linked to avoid the accumulation of toxic intermediates. For example, in *E. coli* expressing the mevalonate pathway, toxicity is seen due to the production of the intermediate 3-hydroxy-3-methylglutaryl-coenzyme A (22).

The regulatory control mechanisms of the DOXP pathway have not been elucidated in any system, although in *E. coli*, expression of all of the pathway genes from *dxs* to *gcpE* on an

artificial operon results in increased levels of HMB-PP (17). We have shown that overexpression of *dxs1* results in increased HMB-PP, mediated by increased transcription of the *dxr* operon, including *gcpE*. Overexpression of Dxr or GcpE did not result in upregulation of the *dxr* operon, indicating that only Dxs affects the transcriptional regulatory control. Other mycobacteria (*M. smegmatis*, *M. bovis*, *Mycobacterium marinum*, and *Mycobacterium leprae*) also have a similar genetic arrangement with an apparent operon including *dxr* and *gcpE*. However, this arrangement does not seem to be present in other bacterial species.

The DOXP biosynthetic pathway of *M. tuberculosis* is specific and essential and represents an attractive potential target for the design of new antimycobacterial agents. Several DOXP genes are located in operons, representing controlled transcription, and *dxr* and *gcpE* are cotranscribed from a single promoter element upstream of *dxr*. Tight regulation of Dxs appears to be required to prevent depletion of essential components from the cell, as demonstrated by a reduced growth phenotype due to Dxs overexpression. Flux through the pathway appears to be regulated by Dxs at the transcriptional level, since unusually it appears that it directly affects transcription of other DOXP genes. Future work to identify the regulator would shed further light on regulation of this key pathway.

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