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Lethal Mutations in the Isoprenoid Pathway of *Salmonella enterica*

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Essential isoprenoid compounds are synthesized using the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway in many gram-negative bacteria, some gram-positive bacteria, some apicomplexan parasites, and plant chloroplasts. The alternative mevalonate pathway is found in archaea and eukaryotes, including cytosolic biosynthesis in plants. The existence of orthogonal essential pathways in eukaryotes and bacteria makes the MEP pathway an attractive target for the development of antimicrobial agents. A system is described for identifying mutations in the MEP pathway of *Salmonella enterica* serovar Typhimurium. Using this system, point mutations induced by diethyl sulfate were found in the all genes of the essential MEP pathway and also in genes involved in uptake of methylerythritol. Curiously, none of the MEP pathway genes could be identified in the same parent strain by transposon mutagenesis, despite extensive searches. The results complement the biochemical and bioinformatic approaches to the elucidation of the genes involved in the MEP pathway and also identify key residues for activity in the enzymes of the pathway.

It was recently discovered that bacteria synthesize isoprenoids by a pathway that differs from that found in eukaryotes. In bacteria, pyruvate and glyceraldehyde 3-phosphate are converted, through the 2-*C*-methyl-D-erythritol phosphate (MEP) pathway, to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). This has focused recent research on the elucidation of the biosynthetic steps and genes encoding the catalytic enzymes in bacteria. To date, all synthetic steps of the MEP pathway and the genes encoding the biosynthetic enzymes have been identified. Isoprenoid biosynthesis begins with the condensation of pyruvate and glyceraldehyde 3-phosphate by 1-deoxy-D-xylulose 5-phosphate (DXP) synthase, encoded by *dxs* (26). Next, in the first committed step of the pathway, DXP reductoisomerase, encoded by *dxr*, converts DXP to MEP (16, 32). In the following three steps, a cytidyl monophosphate moiety is attached to the phosphate of MEP; the resulting diphosphodiester is phosphorylated at the C-2 hydroxyl in the methylerythritol moiety and then cyclized to form an eight-membered cyclic diphosphate diester, and these steps are catalyzed by the proteins encoded by *ispD* (25), *ispE* (18), and *ispF* (11), respectively. The enzyme encoded by *ispG*, 1-hydroxy-2-methyl-2-(*E*)-butenol 4-diphosphate (HDMAPP) synthase, catalyzes the ring opening and reduction of 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate to yield HDMAPP (10). Finally, HDMAPP is converted to a mixture of IPP and DMAPP by HDMAPP reductase encoded by *ispH* (1).

Mutants blocked in the MEP pathway of *Salmonella* spp. or *Escherichia coli* are expected to be lethal, since these organisms are unable to utilize exogenously supplied IPP, DMAPP, or their corresponding alcohols. To allow viability of such mutants, genes of the alternative mevalonate pathway were introduced into bacteria, either on plasmids or in the chromosome (2, 5, 9, 15, 24). Strains containing the genes of the mevalonate pathway can synthesize isoprenoids using the eukaryotic pathway when mevalonate is provided. This allows identification of mutants blocked in the alternative bacterial pathway by their requirement for mevalonate. An approach similar to that described here was used recently in an *E. coli* study (28) and revealed point mutations for all MEP pathway genes except *ispH*.

The system described here for *Salmonella* bacteria employs a strain with genes of the MVA pathway of yeast inserted in the bacterial gene *dxs*, which encodes deoxyxylulose phosphate synthase in the bacterial MEP pathway. This strain uses the MEP pathway when supplied with methylerythritol and the MVA pathway when supplied with MVA. Extensive attempts to isolate insertion mutations in the MEP pathway of *Salmonella* bacteria by this means were unsuccessful. However, following mutagenesis with diethyl sulfate (DES), mutants were recovered for all steps in the MEP pathway downstream of *dxr* that were identified by alternative biochemical or bioinformatic methods. Mutants with mutations in the *srl* operon, which has been implicated in the uptake and phosphorylation of exogenously supplied ME, were also recovered.

The failure of the genetic approach using transposons was surprising, since constructed *Salmonella* mutations for each of the genes showed the expected phenotype when introduced into the parental strain—an absolute requirement for mevalonate (33). The evidence described here demonstrates the efficacy of the genetic system and raises the question of why transposon mutagenesis failed. This genetic system promises to be a useful adjunct to other methods in further analysis of the bacterial MEP pathway, which remains an attractive target for the design of antibiotic, antimalarial, and herbicidal compounds, as demonstrated by the use of fosmidomycin, a MEP pathway inhibitor used in treatment of bacterial infections and malaria (13).

Structural information about the active sites of the MEP pathway proteins can be an invaluable tool for the rational design of inhibitors. While the crystal structures have been reported for the proteins encoded by *dxr* (21, 39), *ispD* (13, 23), *ispE* (20, 36), and *ispF* (13, 23, 31), little structural data are

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TABLE 1. *Salmonella enterica* serovar Typhimurium LT2 strains used in this study

Strain	Description	Reference and/or source
RMC26	LT2 *dxs*(swap)::MVA_{operon}	This work, 28
CR4	RMC26 *ispD*(swap)::CAT	This work, 28
CR33	RMC26 *ispE*(swap)::CAT	This work
CR34	RMC26 *ispG*(swap)::CAT	This work
CR35	RMC26 *ispH*(swap)::CAT	This work
CR5	RMC26 *srlE*(swap)::CAT	This work
DM269	*thiI*::Tn*10*d-tet	32

TABLE 2. Oligonucleotide sequences used in this study

Primer	Sequence (5′–3′)
sAMPygbPB	GCACAGCCACTGCTCGGTAA
asAMPygbPB	AGCCGCGTCAACGATGAT
sAMPychB	GGTAAAAACTGGAAAGTGG
asAMPychB	AAACCCGAATGCGTTAGA
sAMPgcpE	TTCGCCAGGCAGATAATC
asAMPgcpE	CGCGTCTGACCCTTAATG
sAMPlytB	CGGGCATACCGTTCACTTTGA
asAMPlytB	CAACGTAGCGTCATCAGGCA
sAMPsrlAEB	GAAGCGGCACAAGAGAAT
asAMPsrlAEB	CGACATTCGCGGCTTTAT
sSEQygbPB1	CGCAGCAAAATGTGCTCGCCTT
sSEQygbPB2	ACGCCACATCGCCATCGGAA
sSEQygbPB3	GTTCGCCGCGTTTCATGGTG
sSEQychB1	TATGACAGCAAAACGCAGCC
sSEQychB2	TTGGCAATGCGGGCTTTCC
aSEQgcpE	TGCCACGTTTATTCTCTTTC
asSEQgcpE	GCAGTAACAGACGGGTAA
sSEQlytB1	TGATATTGAAGTGCTGGAAA
sSEQlytB2	GGATGTACCTGGTGGAGTCG
sSEQsrlAEB1	TGATTACAATGAACAGGAAA
sSEQsrlAEB2	GCTGGTAGGTCTGGTGACGA
sSEQsrlAEB3	CCAGCCTGCCAAAACGACAT
sSEQsrlAEB4	TCGGCGTACCTTCTGTGCTG

available for the other MEP pathway proteins. In addition, the oxygen sensitivity of the iron sulfur proteins encoded by *ispG* and *ispH* (30, 38) presents special problems when work is performed with the purified enzymes. In such cases, alternative methods, such as random mutagenesis, are useful for identifying residues in the enzymes essential for function and a method for testing the in vivo efficacy of potential inhibitors.

MATERIALS AND METHODS

Genetic media and methods. Chloramphenicol (Cam), kanamycin (Kan), tetracycline (Tet), L-arabinose (L-ara), mevalonolactone, and DES were purchased from Sigma. Klentaq-LA polymerase was purchased from Clontech. Luria-Bertani (LB) full medium was used with or without supplementation for all growth conditions (27). E minimal medium was prepared without carbon as described by Vogel and Bonner (35). Cam was used at a final concentration of 20 μg/ml, Kan at 40 μg/ml, and Tet at 30 μg/ml unless otherwise noted. Methylerythritol was synthesized using the method of Duvold (8) and supplemented at a final concentration of 50 μg/ml. L-Arabinose was used at a final concentration of 0.02%. Mevalonic acid was prepared by hydrolysis of 1 volume of 1 M mevalonolactone with 1.02 volumes of 1 M KOH, followed by incubation at 37°C for 30 min, and used at a final concentration of 5 mM.

Transductions were mediated by the high-frequency P22 mutant HT*105/1 int-201* as previously described (29). Phage P22 lysates were prepared as previously described (7). All DNA sequencing was performed at the Health Sciences Center Sequencing Facility, Eccles Institute of Human Genetics, University of Utah.

Cloning and sequencing methods. Genomic DNA was isolated using Easy DNA kits (Invitrogen). PCR was performed using a Perkin-Elmer GeneAmp PCR system 2400 DNA thermal cycler as directed by the polymerase vendor. PCR nucleotide mix was purchased from Roche Molecular Biochemicals. All oligonucleotides were synthesized by the Protein/DNA Core Facility of the Utah Regional Cancer Center. Agarose gel purifications and PCR purifications of DNA were performed using QIAquick gel extraction kits and PCR purification kits (QIAGEN), respectively. Sequence searches were performed on the National Center for Biotechnology Information (NCBI) and Washington University School of Medicine Genome Sequencing Center BLAST servers. Gene sequences were downloaded from NCBI. Sequence alignments were performed using Vector NTI Align-X software. Strains used in this work are listed in Table 1, and oligonucleotides are described in Table 2.

Identifying and classifying mutations. Mutants were generated by exposing stationary-phase cultures of RMC26 to a saturated solution of DES in E medium. A saturated DES solution was prepared by placing two drops of DES in 5 ml of E medium. The solution was mixed and incubated at 37° for 10 min. To the saturated DES solution, 0.1 ml of RMC26 stationary-phase culture was added. After the cells were incubated at 37° for 30 min, 0.1 ml aliquots were used to inoculate 5 ml overnight cultures of LB–MVA–L-ara–Kan medium. The following day, full-cell suspensions were diluted 10^6-fold, and 0.1 ml of diluted cells was plated to LB–MVA–L-ara–Kan plates. A separate overnight culture was used for each plate in an effort to reduce the isolation of sibling mutants. After 2 days growth at 37°C, colonies were replica printed to LB–MVA–L-ara–Kan and LB-ME-Kan media. Mutants were isolated that demonstrated viability in the presence of MVA–L-ara but not in the presence of ME. Mutants were verified by patching to media supplemented with MVA–L-ara and printing to both MVA–L-ara media and ME–L-ara media.

Identification of isolated mutants. Strains CR5, CR33, CR34, and CR35 (see Table 1) were constructed from parental strain RMC26 to contain a chloramphenicol acetyltransferase (CAT) cassette in place of individual MEP pathway genes or *srlE* by using linear recombination as previously described (33). Isolated mutants were used as recipients in P22-mediated transductional crosses with donor strains having known MEP genes or genes within the *srl* operon replaced by a CAT cassette. The following strains, all with the MVA operon inserted in the chromosomal copy of *dxs*, were used as donors in the P22 crosses: CR4 for the *ispD* replacement, CR33 for the *ispE* replacement, CR34 for the *ispG* replacement, CR35 for the *ispH* replacement, and CR5 for the *srlE* replacement. RMC26 was also used as a donor in a control cross. DES-generated random mutants were used as recipients in the transductional crosses and were plated to LB-ME-Kan media. The number of transductants from each cross was counted. Any recipient point mutant that could not generate recombinants able to use 2-*C*-methyl-D-erythritol (ME) when crossed with a particular donor mutant was likely to have a mutation in the same gene that was lacking in the donor strain.

Once identified by cross, the mutant allele was PCR amplified from the genomic template by use of Klen*Taq* LA polymerase mix, followed by sequencing of the amplificate to identify the position of mutation. In cases where the results of complementation by strains with disruptions in MEP pathway genes were ambiguous, the implicated genes were amplified and sequenced. PCRs were performed in duplicate, followed by sequencing in duplicate. Primers used to amplify the target genes were as follows: for *ispD* and *ispF*, primers sAMPygbPB and asAMPygbPB; for *ispE*, primers sAMPychB and asAMPychB; for *ispG*, primers sAMPgcpE and asAMPgcpE; for *ispH*, primers sAMPlytB and asAMPlytB; and for *srlA*, *srlB*, and *srlE*, primers sAMPsrlAEB and asAMPsrlAEB. The following sequencing primers were used for each target gene: primers sSEQygbPB1, sSEQygbPB2, and sSEQygbPB3 were used to sequence *ispD* and *ispF* amplificates; primers sSEQychB1 and sSEQychB2 were used for *ispE* amplificates; primers sSEQgcpE and asSEQgcpE were used for *ispG*; sSEQlytB1 and sSEQlytB2 were used for *ispH*; and sSEQsrlAEB1–sSEQsrlAEB4 were used for *srlA*, *srlE*, and *srlB*.

A subset of mutant strains that failed to grow on ME as an isoprenoid precursor did not appear to have mutations in any known MEP pathway gene, as determined by the recombination test described above. To test their dependence on the MVA operon, these mutants were used as recipients in transductional crosses with donor DM269 (37), which harbors a Tn*10*d-Tet insertion in the chromosomal copy of *thiI*, a thiamine biosynthesis gene in close proximity to *dxs*. As a control, RMC26, CR4, and CR5 were also used as recipients in the same cross. Recombinants were selected on LB–MVA–L-ara–Tet media. After 2 days growth at 37°C, transductants were replica printed to LB–MVA–L-ara–Kan media and scored for the presence of the MVA operon as indicated by Kan resistance.

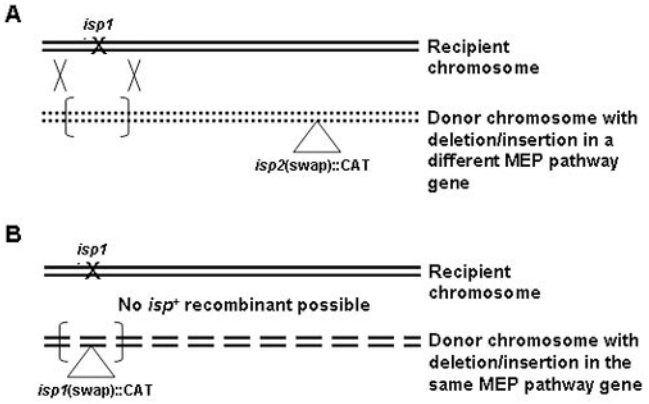

FIG. 1. Identification of DES-generated mutations by complementation. A) Bracketed region suggests the presence of a 44-kb recombination fragment. A donor chromosome with a mutation in a MEP pathway gene, different (*isp2*) than the unidentified mutants (*isp1*), produces a recombination fragment able to repair the unidentified mutation. B) A donor chromosome with a mutation in the same gene as the recipient chromosome does not produce a recombination fragment able to repair the lethal mutation.

TABLE 3. Mutants of RMC26 unable to utilize ME for the biosynthesis of isoprenoids

Mutant	Amino acid substitution[c]							
	ispD	*ispE*	*ispF*[a]	*ispG*	*ispH*	*srlA*[b]	*srlE*	None
PM1								unk
PM3				R237C				
PM5				R133H				
PM6							G78A	
PM7							G295A	
PM8								unk
PM9							G281A	
PM10			D38N					
PM11	E196K							
PM12								unk
PM13								unk
PM14						W11Stp		
PM15						G20Q	W315Stp	
PM16			A33V					
PM17	G130E							
PM18	Q100Stp							
PM19				G309R				
PM21					C96Y			
PM23	G92Q							
PM24		G239R						
PM25				G206R				
PM26								unk
PM28								unk

[a] Complementation deficiency mapped with deletion insertion in *ispD*.
[b] Complementation deficiency mapped with deletion insertion in *srlE*.
[c] Mutations noted are amino acid substitutions. Numbering is consistent with the enzymes from *S. typhimurium*. unk, not identified; Stp, stop codons.

RESULTS

Structure of the parental stain RMC26. Strain RMC26 has a synthetic operon encoding the *Saccharomyces cerevisiae* genes ERG12, ERG8, and ERG19 (for the conversion of MVA to IPP) inserted into the chromosomal copy of *dxs* and expressed from an arabinose-inducible promoter (1). This strain has an absolute growth requirement for an early compound of either the yeast MVA pathway or for the bacterial MEP pathway. When either 1-deoxy-D-xylulose or ME is supplied, the bacterial pathway is used. When MVA and the inducer L-ara are provided, the yeast pathway is used. Mutants with disruptions in the MEP pathway were identified by their viability on media supplemented with MVA–L-ara but not on medium with ME.

Isolating mutants in strain RMC26. Random mutants were generated in *Salmonella* serovar Typhimurium strain RMC26 by exposure to DES, a DNA-alkylating agent that results in primarily G:C to A:T transitions (12). Approximately 20,000 colonies were screened for mutations that allow growth in the presence of MVA–L-ara but not in the presence of ME. A total of 28 mutants were isolated that grew less well on media containing ME than on media supplemented with MVA–L-ara. Of the total, 23 exhibited a complete lack of growth on ME and were further characterized. The remaining five mutants showed only slightly impaired growth on ME, suggestive of partial loss of functions, and were not pursued.

Characterization of mutations. To identify the mutated genes, each of the mutants was used as recipient in a transduction cross with individual donor strains bearing CAT replacements in one of the known MEP pathway genes downstream of *dxr* and *srlE* (Fig. 1). Selection was made for ability to grow on ME. A failure to yield recombinants indicated that the recipient point mutation affected the gene that was deleted in the donor. The implicated region of the mutant chromosome was amplified from genomic DNA using Klen*Taq* LA polymerase mix followed by sequencing of the amplificate. Each mutant allele was PCR amplified and sequenced in duplicate to reduce the chance of reporting a mutation introduced by PCR or an error in sequencing. This possibility was further reduced by using Klen*Taq* LA polymerase mix (3), which contains a small amount of editing 3′ exonuclease which provides proofreading and reduces the probability of introducing mutations by PCR.

All of the sequenced mutations were G:C-to-A:T transitions, as expected by the use of the ethylating agent DES (12). Of the 23 mutants characterized, four independent mutations were found in *ispD*, one in *ispE*, two in *ispF*, four in *ispG*, one in *ispH*, and six in the *srl* operon (Table 3). The remaining six mutants were recombinationally repaired in crosses with any of the donor strains lacking one of the known genes in the MEP pathway, suggesting that their failure to grow on ME was not due to lack of a known MEP enzyme.

If these strains carried some novel block in the MEP pathway, they would be expected to require their MVA operon to supply isoprenoids. To test this, the MVA operon was removed from each mutant by a cross with donor strain DM269 (37), which carries a Tn*10* insertion near the *dxs* gene in which the MVA operon was inserted. In all six cases, more than 90% of Tet^r transductants lost the MVA operon (and became *dsx*$^+$), as determined by their Kan sensitivity. This is the same loss frequency observed in crosses between donor DM269 and recipient RMC26, with no additional blocks in the MEP pathway, and recipient CR5, which has the insertion of a CAT cassette in the chromosomal copy of *srlE*. These *dsx*$^+$ transductants grew normally with without added MVA or MEP, demonstrating that their de novo MEP pathway is unaffected by any of the six unlinked mutations. When this cross was done using a recipient with a block in MEP (e.g., CR4 which has a CAT insertion in the chromosomal copy of *ispD*), none of the transductants were Kan sensitive, suggesting the MVA operon is

essential when the endogenous MEP pathway is blocked. We conclude that these six mutants have an intact MEP pathway and failed to grow on ME in the original background because they interfere with assimilation of ME (outside the normal MEP pathway).

DISCUSSION

A system is described for isolation of mutants with blocks in the MEP pathway of *S. typhimurium*. Using this screen, approximately 20,000 DES-mutagenized colonies were tested, and 23 mutants were isolated that exhibited a strong growth defect on media containing ME compared to the results seen with media containing MVA–L-ara. The gene affected by each of the 23 mutations was identified by failure to recombine with deletions of one of the known genes in the MEP pathway, and their base changes were determined by DNA sequencing. The resulting mutations included blocks in each of the genes of the MEP pathway downstream of *dxr*. In the study of *E. coli* reported by Sauret-Gueto et al. (28), a screen of ~27,000 strains yielded no mutations in *ispH*, when the expected frequency of hits was ~2.7. Those authors suggested that the region of the *E. coli* chromosome might be poorly accessible to the mutagen. This now appears unlikely, and the absence of an *ispH* mutant more likely reflects the small number of mutations isolated. In addition, we isolated several strains bearing mutations in the sorbitol operon, in agreement with previous work suggesting its involvement in the import and phosphorylation of exogenous ME (33).

The results reported here for a chemical mutagen contrast with extensive earlier mutant screens looking for insertional inactivation of MEP pathway genes. These hunts employed a MutJ(Cm) element, a derivative of phage Mu carrying chloramphenicol resistance (6). No insertion mutations were found in any gene of the MEP pathway. The only genes detected by insertions were in the sorbitol phosphotransferase system (PTS) (33). Sufficient insertion mutations (150,000) were screened to assure complete coverage—on the order of 30 hits per gene. At this point we have no explanation for this discrepancy but suggest several possibilities below.

The puzzle posed by failure to identify insertion mutations might reflect any of the following possibilities. (i) The *isp* genes could be poor targets for the insertion element used. This seems unlikely, since this transposon has been used extensively in other systems and shows very little target specificity. While rare genes might be poor targets, it seems unlikely that all of the scattered genes for one particular pathway would be poor targets. (ii) The chloramphenicol resistance might be unexpressed in strains using the MVA instead of the MEP pathway, or chloramphenicol might interfere with functioning of the MVA pathway. This seems unlikely, since successfully constructed MEP mutants carried a Cam^r gene very similar to that cloned into the various MEP genes and all these mutants showed simultaneous resistance to chloramphenicol and ability to use MVA. (iii) Intermediates in the MEP or MVA pathways might affect transposition. We have shown that transposition occurs in cells of the parent strain grown on mevalonate both with and without added methylerythritol. Thus, no intermediate in the MVA pathway interferes with transposition and no intermediate in the MEP pathway is required for transposition. Any methylerythritol phosphate accumulated in the parent (*dxs*) strain does not inhibit transposition. The idea that a particular internal MEP intermediate inhibits transposition does not explain the results, since the mutational block would not be imposed until after transposition had occurred and methylerythritol was provided. (iv) The explanation we favor is that polar insertions in the MEP genes are nonviable. While it seems unlikely that essential genes are located downstream of each MEP gene, this possibility fits our data quite well, based on the evidence summarized below.

The insertion element used is strongly polar, and such polarity has been shown to be sufficient to eliminate expression of downstream genes in other systems. The constructed MEP pathway mutants received a Cam^r cassette that is known to have outward-directed promoters at both sides and would thus provide for expression of downstream genes. The idea of lethal polar effects is consistent with the rarity of nonsense mutations among the recovered MEP point mutations; such mutations would be expected to show polarity and may also be counterselected. The general idea predicts that all isoprenoid biosynthetic genes are in multigene operons with essential distal genes. This prediction has not yet been tested, but such mixed-function operons are known (17, 19, 34).

Of the genes downstream of *dxr* in the MEP pathway, crystal structures are reported for the proteins encoded by *ispD* (14, 22), *ispE* (20, 36), and *ispF* (13, 23, 31). Four mutants, defective in *ispD*, were isolated. PM18 had a nonsense mutation (Q100). The remaining three had missense mutations in PM11 (E196K), PM17 (G130E), and PM23 (G92Q). The crystal structure of *E. coli* 4-diphosphocytidyl-2-*C*-methyl-D-erythritol (CDP-ME) synthase, encoded by *ispD*, suggests that the protein is a homodimer (22). Each subunit is comprised of two structurally distinct domains, a larger core domain, comprised of residues 1 to 136 and 160 to 236, and a smaller lobe domain, comprised of residues 137 to 159. The core domain is globular in structure and includes a distinctive parallel β-sheet motif. The lobe domains of the two subunits resemble "curved arms" which interlock to mediate dimer formation and organize parts of the catalytic site (22). The truncated IspD protein resulting from the nonsense mutation in PM18 lacks both the small lobe domain and a portion of the large globular domain, making dimer formation and catalysis unlikely. The three missense mutations do not appear in amino acids thought to be involved directly in substrate binding or catalysis as predicted by the crystal structure. In addition, none of the three mutated residues are strictly conserved, as determined on the basis of sequence alignments of the proteins from 35 organisms. However, in all three cases a change in polarity of the residue occurs that may translate into altered protein structure. Negatively charged Glu196 is changed to positively charged lysine, while nonpolar Gly92 and Gly130 are mutated to larger polar glutamine and negatively charged glutamate, respectively. Aside from polarity, the protein structure may be compromised by steric interactions in which small buried glycine residues are replaced by the much larger glutamine and glutamate residues.

A single missense mutation was isolated in *ispE* (G239R). This same mutation was isolated by Sauret-Güeto and coworkers (28) in ethyl methanesulfonate-generated *E. coli* mutants containing a MVA operon. They identified a strictly conserved

motif characterized by the consensus sequence G-[S,T]-G that is critical for enzyme function. The crystal structure of the IspE protein from *E. coli* implicates Gly239 as an important residue in substrate binding. The Gly239 amide is thought to aid in positioning Asn12 through hydrogen bonding interactions, which allow the residue to in turn hydrogen bond with the O4 hydroxyl group in CDP-ME (20). The substitution of the much larger and charged Arg residue may disrupt the secondary structure of the protein backbone sufficiently to inhibit formation of the stabilizing hydrogen bond between the amide and Asn12, which in turn likely disrupts substrate binding.

Two missense mutations were isolated in the protein encoded by *ispF*. The crystal structures of *E. coli* 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate synthase show that the protein is a bell-shaped homotrimer (13, 23, 31). Each monomer is comprised of a large and a small β-sheet and four α-helices. The catalytic sites are located at the interfaces of each of the three subunits and are characterized by three distinct binding pockets: a central pocket binds the sugar and diphosphate of the cytidyl moiety; a second pocket binds the nucleoside; and a third pocket binds the 2-phosphate and carbon chain of the 2*C*-methyl-D-erythritol moiety (31). Both mutations, A33V and D38N, appear to be very near residues involved in substrate binding by the third pocket. The hydrophilic side chains of Ser35, Ser73, and Asp63 are thought to be important for binding and positioning the 2*C*-methyl-D-erythritol moiety (31). Additionally, His34 is thought to stabilize a flexible loop which caps the active site (31). The pentapeptide sequence starting at His34, including fully conserved residues Ser35 and Asp38, is highly conserved and is positioned along the side of the active site, suggesting a role in substrate binding (13). Though Asp38 is not directly implicated in substrate binding or catalysis by the current crystal structures, its involvement in enzyme activity is not surprising given the close proximity to the binding site and fully conserved nature of the residue. Similarly, the close proximity of Ala33 may suggest that it is important for correct positioning of the conserved pentapeptide within the active site.

Though crystal structure data are not available for the remaining enzymes of the MEP pathway, some structural information can be inferred based on function and homology to enzymes with better characterized motifs. The amino acid sequence of the IspG protein has strong homology to enzymes possessing a [4Fe-4S] cluster (30), and a conserved ferrodoxin motif spanning residues 300 to 319 in *E. coli* has been identified (38). However, alignments have failed to identify additional conserved motifs that would give an insight into the mechanism of catalysis (2, 4). IspG possesses three fully conserved cysteines, residues 269, 272, and 305 in the *S. typhimurium* enzyme, which have been suggested to participate in iron binding (10). The current screen identified four missense mutations in *ispG*: R237C (PM3), R133H (PM5), G309R (PM19), and G206R (PM25). Of these, only G309R is not in a fully conserved residue. However, Gly309 is highly conserved, located within the ferrodoxin motif and near a conserved Cys305. The other three residues, Arg133, Arg237, and Gly206, are fully conserved among bacteria, plants, and the malaria parasite *Plasmodium falciparum*. The polar-charged nature of Arg133 and Arg237 allows the possibility of involvement in substrate binding or catalysis, while the small size and nonpolar aliphatic nature of Gly206 would suggest a structural role. Nonetheless, identification of these residues by the current screen confirms that they are essential for enzyme function.

A single missense mutation in *ispH* was isolated. Like IspG, IspH is a [4Fe-4S] protein and possesses three fully conserved cysteine residues at positions 12, 96, and 197 in *Salmonella* serovar Typhimurium. It has been suggested that these cysteines are involved in iron binding (2). Therefore, it is not surprising that the missense mutation isolated in *ispH* (C96Y) results in a loss of function phenotype, which may be attributed to the inability of the protein to form the [4Fe-4S] cluster.

Five DES-generated mutants, showing MVA auxotrophy, were isolated in the sorbitol operon, which was previously implicated in the import and phosphorylation of exogenous ME (33). While these mutations are not directly relevant to the function of the MEP pathway, isolation of these mutations supports the current model in which exogenous ME is imported and phosphorylated by the sorbitol phosphoenolpyruvate: phosphotransferase system. While use of an insertion element to generate gene disruptions in previous screens for MVA auxotrophic mutants resulted in the isolation of only *srlE*, the current screen also identified a random mutation in *srlA*. Both *srlA* and *srlB* had been previously implicated in import and phosphorylation by directed disruption (28). A single mutant, PM15, carried two independent mutations, a missense mutation in *srlA* (G20Q) and a nonsense mutation in *srlE* (W315), as determined by sequencing. It is unclear at this time which mutation results in the MVA-dependent phenotype. One additional nonsense mutation was isolated in *srlA* at W11. Thus, inactivation of the SrlA protein is not surprising given the early translation termination. Three additional point mutations were isolated in *srlE*, G78A, G281A, and G295A. The substitution of glycine to alanine is conservative. However, two of these glycines, G78 and G295, are fully conserved among sorbitol PTSs, which may explain the loss of function for the similar substitution. It is unclear why G281A causes a loss-of-function phenotype. Although glycine 281 is highly conserved, alanine substitutes for glycine at that position in *S. mutans*.

Six mutants isolated in the screen failed to use ME but were not completely characterized, because they do not affect function of the MEP pathway. All six were used as recipients in a transductional cross with donor DM269, which has a Tn*10* insertion in the *thiI* gene closely linked to the *dxs* insertion (containing MVA pathway genes) in the parent strain. Many Tet^r recombinants lost the *dxs* insertion and the MVA genes and regained a functional *dxs* gene. These transductants still carried the new mutation but were clearly able to use the bacterial MEP pathway. All of the six mutants thus failed to grow on ME in the parent *dxs* background due to some defect in uptake or phosphorylation of methylerythritol. They have no impairment in the MEP pathway. The behavior of these Dxs^+ transductants contrasts with that for the same recombinants obtained using recipient strain CR4, which bears a dysfunctional copy of the MEP pathway gene *ispD.* We suggest (but have not directly tested) that these six mutations affect the sorbitol PTS, which requires proteins in addition to SrlA, SrlE, and SrlB. The six mutations could thus block uptake of ME without blocking the MEP pathway or affecting the *srl* operon. Essential genes outside of the known MEP pathway were not

identified in this screen or that described by Sauret-Güeto (28). This agrees with biochemical and bioinformatic studies indicating that all genes required for biosynthesis of IPP and DMAPP by MEP pathway have been identified. Both IspG (GcpE) and IspH (LytB) enzymes require regeneration systems such as flavodoxin, flavodoxin reductase, and NADPH for activity in vitro (30). Regeneration systems specific to the [4Fe-4S] proteins, IspG and IspH, have been hypothesized to exist in vivo, but this has not been conclusively established. Genes encoding enzymes for the specific reduction of either IspG or IspH are unlikely to exist. Similarly, screens performed with *E. coli* failed to isolate strains with mutations in additional genes (28).

Six of the mutants recovered that failed to grow on ME were shown to possess an intact MEP pathway. We suggest that these mutants are likely to interfere with some early step in uptake and metabolism of ME or to make strains sensitive to growth inhibition by ME.

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