Isoprenoid biosynthesis in bacterial pathogens

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Isoprenoid biosynthesis is essential for cell survival. Over 35 000 isoprenoid molecules have been identified to date in the three domains of life (bacteria, archaea and eukaryotes), and these molecules are involved in a wide variety of vital biological functions. Isoprenoids may be synthesized via one of two independent nonhomologous pathways, the classical mevalonate pathway or the alternative 2C-methyl-D-erythritol 4-phosphate (MEP) pathway. Given that isoprenoids are indispensable, enzymes involved in their production have been investigated as potential drug targets. It has also been observed that the MEP pathway intermediate 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMB-PP) can activate human Vγ9/Vδ2 T cells. Herein we review isoprenoid biosynthesis in bacterial pathogens. The role of isoprenoid biosynthesis pathways in host–pathogen interactions (virulence potential and immune stimulation) is examined. Finally, the design of antimicrobial drugs that target isoprenoid biosynthesis pathways is discussed.

Introduction

Isoprenoids are a large, diverse class of naturally occurring organic chemicals which are essential for cell survival. Over 35 000 isoprenoid molecules have been identified to date in the three domains of life (bacteria, archaea and eukaryotes), and they are involved in a wide variety of vital biological functions. In bacteria, ubiquinone and menaquinone are involved in electron transport, while bactoprenols act as carbohydrate carriers in the biosynthesis of peptidoglycan. Eukaryotic sterols such as cholesterol act as membrane stabilizers and are precursors of bile acids. Archaeal membrane lipids are composed of isoprenoid side chains with ether links that join these side chains to the glycerol backbone. In plants, the main isoprenoids are those of the protective pigments such as carotenoids, which make up part of the photosynthetic machinery. As well as structural components isoprenoids also play a role in hormone-based signalling, protein degradation and in the regulation of transcription.

All isoprenoids are derived from the universal five-carbon precursor isopentyl diphosphate (IPP) or its isomer dimethylallyl diphosphate (DMAPP), which may be synthesized via one of two independent pathways, the classical mevalonate pathway or the alternative 2C-methyl-D-erythritol 4-phosphate (MEP) pathway. The MEP pathway intermediate 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMB-PP) has been shown to activate human Vγ9/Vδ2 T cells. Expansion of this subset of T cells is observed after infection with a broad range of microbial pathogens. Herein we review isoprenoid biosynthesis in bacterial pathogens. The role of the pathways in host–pathogen interactions is examined and the design of drugs that target isoprenoid biosynthesis is discussed.

Isoprenoid biosynthesis pathways

In animals, IPP biosynthesis proceeds exclusively via the mevalonate pathway. The two isoprenoid biosynthesis pathways are mutually exclusive in most bacterial species; Listeria monocytogenes and certain Streptomyces species are exceptions in that they have two complete pathways (Boucher & Doolitttle, 2000). Archaea utilize the mevalonate pathway (Matsumi et al., 2011).

The evolutionary origin of the two pathways remains controversial. A recent study by Lombard & Moreira (2011) examined the origin and evolution of the enzymes of the mevalonate pathway using phylogenomic analyses. Despite the identification of several horizontal gene transfer events, analyses support the hypothesis that the mevalonate pathway was ancestral in archaea, eukaryotes and bacteria. The authors conclude that the mevalonate pathway is likely an ancestral metabolic route in all three domains of life, and was probably present in the last common ancestor of all organisms.

The mevalonate pathway involves six enzymes which convert acetyl-CoA and acetoacetyl-CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is reduced to mevalonate followed by phosphorylation to phosphomevalonate. A subsequent phosphorylation results in the production of diphosphomevalonate, which is converted...
into the C5 building blocks IPP or DMAPP. The MEP pathway requires eight enzymic steps, which proceed via the condensation of pyruvate and glyceraldehyde 3-phosphate to produce 1-deoxy-D-xylulose 5-phosphate (DOXP). DOXP is converted to MEP through the action of the enzyme DOXP reductoisomerase (DXR) (IspC). 4-Diphosphocytidyl-2C-methyl-D-erythritol (CDP-ME) is produced from the reaction of MEP with CTP, catalysed by YghP (IspD). The fourth reaction, catalysed by YchB (IspE), produces 4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-ME2P). The final reactions are catalysed by YghB (IspF), GcpE (IspG) and LytB (IspH) to produce IPP (Fig. 1). The isomerase enzyme which catalyses the interconversion of IPP and DMAPP can be considered as common to both the MEP and the mevalonate pathways.

The reader is referred elsewhere for in-depth reviews on the elucidation and evolution of these pathways (Boucher & Doolittle, 2000; Hunter, 2007; Lange et al., 2000; Matsumi et al., 2011; Rohdich et al., 2001).

**Isoprenoid biosynthesis in pathogens**

**Gram-negative bacteria**

*Escherichia coli.* *E. coli* utilizes the MEP pathway for the synthesis of IPP. Several groups have been involved in deciphering the role and function of the individual MEP enzymes, and this has led to significant insights into the function of isoprenoid biosynthesis in *E. coli* and several other bacteria (Altincicek et al., 2001; Eberl et al., 2002; Herz et al., 2000; Rohdich et al., 1999, 2003a, b).

Mutational studies have proven the essentiality of yghP, ychB, yghB, lytB and gcpE for growth (Altincicek et al., 2001; Campos et al., 2001a; Campbell & Brown, 2002; Freiberg et al., 2001; McAteer et al., 2001). It has been shown that *E. coli* possesses a gene which encodes a type I IPP isomerase (*ipi*), which maintains the supply of DMAPP from IPP; however, this gene is not essential in *E. coli* (Hunter, 2007). Genetic evidence for the branching of the MEP pathway independently of IPI was obtained by Rodriguez-Concepción et al. (2000), who showed that the
MEP pathway branched from the *lytB* gene. The study by Puan *et al.* (2005) proposed that the flavodoxin I gene (*fldA*) (a gene known to be essential in *E. coli*) has a role in the MEP pathway by providing the reducing system required for the enzymic activation of GcpE and LytB. Whilst much of the genetic characterization has been performed using laboratory strains of *E. coli*, it is clear that pathogenic *E. coli* strains (including enterohaemorrhagic, enterogaegregative, enterotoxigenic, enteroinvasive and enteropathogenic strains) also possess the MEP pathway and lack genes encoding enzymes of the mevalonate pathway (Table 1).

**Brucella abortus.** Analysis of the sequenced genome of *B. abortus* identified homologues of seven of eight genes of the MEP pathway. A homologue of the *dxr* gene, encoding DXR, was not found. Sangari *et al.* (2010) identified a new family of enzymes designated DXR-like (DRL) enzymes. The DRL enzyme catalyses the same biochemical reaction

### Table 1. Distribution of the MEP and mevalonate pathways amongst representative strains of Gram-positive and Gram-negative pathogens

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>MEP</th>
<th>Mevalonate</th>
<th>Reference(s)</th>
</tr>
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<tbody>
<tr>
<td><strong>Gram-positive pathogens</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> str. Sterne</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> subsp. subtilis 168</td>
<td>+†</td>
<td>-</td>
<td>Laupitz <em>et al.</em> (2004); Takagi <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> 630</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Clostridium botulinum</em> B1 str. Okra</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Clostridium perfringens</em> E str. JGS1987</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>-</td>
<td>+</td>
<td>Boucher &amp; Doolittle (2000)</td>
</tr>
<tr>
<td><em>L. monocyctogenes</em> EGDe</td>
<td>+</td>
<td>+</td>
<td>Begley <em>et al.</em> (2004)*</td>
</tr>
<tr>
<td><em>L. innocua</em> Clip11262</td>
<td>-§</td>
<td>+</td>
<td>Begley <em>et al.</em> (2004)*</td>
</tr>
<tr>
<td><em>Listeria seeligeri</em></td>
<td>-§</td>
<td>+</td>
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<tr>
<td><em>Nocardia terpenica</em></td>
<td>+</td>
<td>-</td>
<td>Shigemori <em>et al.</em> (1999)</td>
</tr>
<tr>
<td><em>Staph. aureus</em></td>
<td>-</td>
<td>+</td>
<td>Hammond &amp; White (1970)</td>
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<tr>
<td><em>Strep. pneumoniae</em></td>
<td>-</td>
<td>+</td>
<td>Wilding <em>et al.</em> (2000b)</td>
</tr>
<tr>
<td><em>Strep. pyogenes</em></td>
<td>-</td>
<td>+</td>
<td>Wilding <em>et al.</em> (2000b)</td>
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<tr>
<td><strong>Gram-negative pathogens</strong></td>
<td></td>
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<tr>
<td><em>B. abortus</em></td>
<td>+†</td>
<td>-§</td>
<td>Sangari <em>et al.</em> (2010)*</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em></td>
<td>-</td>
<td>+</td>
<td>Boucher &amp; Doolittle (2000)</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> 434/Bu</td>
<td>+</td>
<td>-</td>
<td>Rohdich <em>et al.</em> (2001)</td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
<td>+</td>
<td>-</td>
<td>Eberl <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>S. enterica</em> serovar Typhimurium</td>
<td>+</td>
<td>-§</td>
<td>Cornish <em>et al.</em> (2006)*</td>
</tr>
<tr>
<td><em>E. coli</em> O157 : H7</td>
<td>+</td>
<td>-§</td>
<td>*</td>
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<tr>
<td><em>E. coli</em> O127 : H6</td>
<td>+</td>
<td>-</td>
<td>*</td>
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<tr>
<td><em>F. tularensis</em></td>
<td>+</td>
<td>-</td>
<td>Eberl <em>et al.</em> (2003)</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>+</td>
<td>-</td>
<td>Putra <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>+</td>
<td>-</td>
<td>Gophna <em>et al.</em> (2006)*</td>
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<tr>
<td><em>K. pneumoniae</em> subsp. pneumoniae* MGH 78578</td>
<td>+</td>
<td>-</td>
<td>*</td>
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<tr>
<td><em>Bordetella pertussis</em> Tahoma I</td>
<td>+</td>
<td>-</td>
<td>*</td>
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<tr>
<td><em>H. pylori</em></td>
<td>+</td>
<td>-</td>
<td>Pérez-Gil <em>et al.</em> (2010)</td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em> Sd197</td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em> FA</td>
<td>+</td>
<td>-</td>
<td>Boucher &amp; Doolittle (2000)</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>+</td>
<td>-</td>
<td>*</td>
</tr>
<tr>
<td><em>C. jejuni</em> subsp. jejuni* 81116</td>
<td>+</td>
<td>-</td>
<td>Gabrielsen <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em></td>
<td>+</td>
<td>-</td>
<td>*</td>
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</tbody>
</table>

*Sequences were analysed by performing BLASTP searches (using the web-based BLAST program) on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/).
†Possess a DRL enzyme instead of DXR.
§Possess some of the pathway genes; missing the final two MEP genes.
§§A type II IPP isomerase has been characterized.
¶HMGR enzyme is present.
to produce MEP from DOXP, but has no overall homology to the DXR enzyme. A genomic library of B. abortus was constructed, screened and used to complement a DXR-deficient E. coli mutant (again using the recombinant strain constructed to utilize mevalonate). Cells capable of mevalonate-independent growth were screened and further characterized to confirm the existence of a DRL enzyme in B. abortus.

Sangari et al. (2010) also delineated the phylogenetic distribution of DRL and DXR. Upon examining the distribution of complete MEP pathway enzymes with respect to DRL sequences, three classes of bacteria were determined: class A (B. abortus) possess DRL instead of DXR sequences in their genomes, class B (L. monocytogenes, Pseudomonas aeruginosa) possess sequences similar to both DRL and DXR, and finally class C (archaea, Coxiella burnetii) consists of bacteria with the DRL enzyme but lacking MEP pathway enzymes.

**Salmonella.** The intracellular pathogen Salmonella enterica serovar Typhimurium has the complete set of genes for the MEP pathway of isoprenoid biosynthesis. Experiments by Cornish et al. (2006) demonstrated that mutations in the MEP pathway genes yghP, ychB, yghB, gcpE and lytB were lethal.

**Campylobacter jejuni.** This Gram-negative foodborne pathogen synthesizes IPP exclusively through the MEP pathway. Gabrielsen et al. (2004) used an expression system to characterize the YghP and YchB enzymes (referred to as IspD and IspF, respectively). Analysis of the purified proteins demonstrated the ability of YghB to convert MEP to CDP-MEP, with YchB catalysing the conversion of CDP-MEP to 2C-methyl-d-erythritol 2,4-cyclopyrophosphate (MecPP).

**Vibrio cholerae.** Analysis of the sequenced genomes suggests that synthesis of IPP in this human intestinal pathogen is via the MEP pathway; however, the gene for the mevalonate pathway enzyme HMG-CoA reductase (HMGR) is present in all four sequenced Vibrio species (V. cholerae, Vibrio vulnificus, Vibrio parahaemolyticus and Vibrio fischeri). Gophna et al. (2006) examined the evolutionary history of hmgR within these species, and their data suggest that hmgR was acquired by an ancestral Vibrio in a single lateral gene transfer event. The function of HMGR in Vibrio is as yet unknown, but evidence strongly suggests that it is not involved in isoprenoid biosynthesis, as the remaining genes of the mevalonate pathway are not present. Furthermore, hmgR is located on the minor chromosome, which is typically not associated with metabolic functions, and transcription has been shown to be extremely low under various conditions tested (Gophna et al., 2006). Those authors note that hmgR in Vibrio is under strong purifying selection, which may hinder elucidation of the physiological role of HMGR.

**Helicobacter pylori.** Genome analysis has revealed that H. pylori utilizes the MEP pathway for IPP biosynthesis. Few studies have functionally characterized the genes of the MEP pathway in H. pylori, although recently Pérez-Gil et al. (2010) expressed and characterized ispDF (corresponding to yghPB) from this pathogen in an E. coli strain. The authors demonstrated that this gene product is functional in E. coli and may provide a target for the design of novel drug moieties active against H. pylori.

**Gram-positive bacteria**

**L. monocytogenes.** Analysis of the L. monocytogenes genome revealed that this pathogen possesses the full complement of genes for both the MEP and the mevalonate pathways (Begley et al., 2004). Initial analyses of isoprenoid biosynthesis in L. monocytogenes were performed with plasmid insertion mutants, two MEP pathway mutants [gcpE (lmo1441) and lytB (lmo1451)] and two mevalonate pathway mutants [hmgR (lmo0825) and mvk (lmo0010)]. However, recent experiments in our laboratory utilizing precise deletion mutants suggest that HMGR may be essential for normal growth under laboratory conditions, despite the presence of an intact MEP pathway (S. Heuston, unpublished data). We previously created a double pathway mutant containing deletions in the gcpE (lmo1441) and hmgR (lmo0825) genes which cannot grow in the absence of exogenously provided mevalonate, confirming the requirement of at least one intact pathway for growth (Begley et al., 2008). In silico analyses revealed that the closely related but non-pathogenic species Listeria innocua does not possess the final two genes of the MEP pathway, which encode the enzymes HMB-PP synthase (GcpE) and HMB-PP reductase (LytB). Close analysis of the relevant genomic regions indicates that L. innocua has lost these genes, possibly during adaptation to a non-pathogenic life cycle. This may suggest that the L. monocytogenes gcpE and lytB genes are important for intracellular survival during infection of the host but dispensable for a non-parasitic life cycle (Begley et al., 2008).

Sangari et al. (2010) have reported that, in addition to a functional Dxr, L. monocytogenes also possesses a drl gene. They suggest that the presence of two enzymes with an ability to catalyse the same reaction (DOXP into MEP) could be advantageous in situations where isoprenoid biosynthesis is absolutely required.

To date, HMGR is the only enzyme of the listerial isoprenoid biosynthesis pathways that has been purified. Theivag et al. (2006) cloned hmgR in E. coli and demonstrated that the purified enzyme was a class II HMGR exhibiting dual enzyme specificity, i.e. an ability to utilize both NAD(H) and NADP(H) in catalysis.

**Staphylococcus aureus.** Early studies to decipher the route of IPP biosynthesis in Staph. aureus suggested that it utilized the mevalonate pathway, as predicted by the
organism’s ability to incorporate radiolabelled mevalonate into isoprenoids (Hammond & White, 1970). Bioinformatic analysis of the completed genomes now confirms the presence of the mevalonate pathway genes. Wilding et al. (2000a) verified the essential requirement of the mevalonate pathway gene mvaA, which encodes the well-characterized and rate-limiting enzyme HMG-CoA reductase (Bochar et al., 1999). Initial attempts to delete this gene were unsuccessful, and the mevalonate pathway mutant could only be recovered when the medium was supplemented with exogenous mevalonate (Wilding et al., 2000a). Voynova et al. (2004) expressed the Staph. aureus Mvk protein in E. coli and purified and characterized it. Parallel experiments with heterologous eukaryotic mevalonate kinases indicated that the Staph. aureus Mvk is much less sensitive to feedback inhibition, although there are similarities in structural features that are important for catalytic function (Voynova et al., 2004). A study by Balibar et al. (2009) investigated the cellular responses in Staph. aureus to perturbations in the mevalonate pathway. Three strains of Staph. aureus were constructed in which mevalonate pathway genes (mvaS, mvaA and mvaK1/D/K2) were placed under the control of an inducible promoter. DNA microarray analysis was then used to investigate the associated transcriptional changes. It was observed that decreased expression of the mevalonate pathway leads to downregulation of primary metabolism genes, and upregulation of virulence factors and cell wall biosynthetic genes. The authors suggest that this dramatic shift in the transcriptome may be indicative of an altered metabolic state, where growth is halted and cells are primed for defense.

Bacillus subtilis, a non-pathogenic Gram-positive model. Much of the functional analysis of the MEP pathway in Gram-positive bacteria has been carried out in Bacillus subtilis, and this work is likely to inform future analyses of Gram-positive pathogens. Bacillus subtilis possesses the full complement of MEP pathway genes. Campbell & Brown (2002) have extensively characterized the yacN gene, which is homologous to the E. coli yghB (ispF) gene. Creation of a yacN deletion mutant could only be achieved with conditional complementation under the control of an inducible promoter. This gene was shown to be important, as growth was severely retarded in the absence of inducer. A deletion in this gene resulted in phenotypic changes, with cells exhibiting altered cell morphology. Bacillus subtilis was the organism of choice in several studies to elucidate the structure and function of the type II IPP isomerase which converts IPP to DMAPP (Laupitz et al., 2004). Takagi et al. (2004) identified the ypgA gene product as a type II isomerase, which was found to be a non-essential gene in Bacillus subtilis. Steinbacher et al. (2003) were the first to report the crystal structure of type II isomerase from Bacillus subtilis, paving the way for further studies to characterize this as a novel antibiotic target.

Other pathogens

Mycobacterium tuberculosis. Studies by Putra et al. (1998) revealed that M. tuberculosis does not incorporate radiolabelled mevalonate, suggesting the MEP pathway as the primary source of IPP. This pathogen has been the focus of numerous studies in recent years, and several of the pathway genes have been characterized and shown to play an essential role during in vitro growth, including dxs (Bailey et al., 2002), ddx (ispC) (Brown & Parish, 2008), yghP (ispD) (Eoh et al., 2009; Shi et al., 2007), ychB (ispE) (Eoh et al., 2009) and yghB (ispF) (Buetow et al., 2007). An extensive study by Brown et al. (2010) further demonstrated the essentiality of the dxs and gcpE (ispG) genes. They also demonstrated the presence of two dxs genes designated dxs1 and dxs2; dxs1 was shown to be essential for in vitro growth. The authors hypothesize that HMB-PP synthase is the rate-limiting step of the MEP pathway in M. tuberculosis, as determined by measuring HMB-PP production to analyse the flux through the pathway. From this study the evidence suggested HMB-PP synthase as the bottleneck of the MEP pathway, with DOXP synthase controlling the flux through the pathway at the transcriptional level (Brown et al., 2010).

Other pathogens. Analysis of the entire genome sequences of Yersinia enterocolitica, Clostridium perfringens, Clostridium botulinum, Klebsiella pneumoniae and Clostridium difficile suggests that all of these pathogens utilize the MEP pathway, with Enterococcus faecalis, Legionella pneumophila and Borrelia burgdorferi shown to possess genes of the mevalonate pathway for isoprenoid biosynthesis (Table 1).

Distribution of pathways

The distribution of the MEP and mevalonate pathways of isoprenoid biosynthesis is highly complex, with a clear bias towards the MEP pathway in bacteria and the classical mevalonate pathway in eukaryotes and archaea (Lombard & Moreira, 2011). Indeed, the MEP pathway is present in pathogenic organisms as diverse as Listeria, Salmonella, Mycobacterium, Brucella and Francisella, as highlighted in Table 1, with isoprenoids synthesized by the mevalonate pathway in Borrelia, Staphylococcus, Streptococcus, Lactobacillus casei and Lactobacillus plantarum (Jomaa et al., 1999a). Interestingly, a metagenomic approach by Gill et al. (2006) revealed that MEP pathway genes are abundant in the indigenous microflora of the human distal gut microbiome, perhaps reflecting the abundance of the MEP pathway in bacteria in general. Furthermore, genome sequence analyses have revealed that the common intestinal bacteria Bacteroides, Bifidobacterium, Fusobacterium, E. coli and Clostridium all possess genes of the MEP pathway. It is currently unclear how or why the MEP pathway has evolved in many bacterial species in preference to the ancestral mevalonate pathway. Firstly, there is strong evidence to suggest a role for the MEP pathway during the intracellular survival of bacterial pathogens (Begley et al., 2008; Lai et al., 2001; Shin et al., 2006; Eskra et al., 2001). Also, recent studies
by Ershov (2007) analysed the energy efficiency of the MEP pathway in comparison with the mevalonate pathway. A distinct difference in the ratio of IPP to DMAPP was observed, with the MEP pathway synthesizing a 5:1 ratio of IPP to DMAPP and the mevalonate pathway generating a 3:7 ratio of IPP to DMAPP, highlighting the reduced energy consumption of the MEP pathway. Anaerobic bacteria such as bifidobacteria seem to preferentially utilize this pathway, and it may be that the energetics of this pathway are favoured under the anaerobic conditions in the gut.

**Role of isoprenoid pathways in host–pathogen interactions**

**Virulence potential**

Experiments using the mouse model of infection demonstrated that *L. monocytogenes gcpE* and *lytB* mutants were impaired in virulence potential following intraperitoneal infection (Begley et al., 2004, 2008). A double pathway mutant (*AgcpEΔhmgR*) was not recovered 3 days post-infection, indicating that the intracellular levels of mevalonate were not sufficient to sustain growth of a strain deficient in both IPP biosynthesis pathways (Begley et al., 2008). An independent study by Schauer et al. (2010) reported a requirement for the MEP pathway *ychB* (*lmoO190*) during *in vivo* survival of *L. monocytogenes*. When BALB/c mice were inoculated intravenously, a *ychB* mutant demonstrated impaired virulence potential in the liver and spleens compared with the wild-type. The findings of both of these studies suggest a requirement for the *L. monocytogenes* MEP pathway during host infection.

The MEP pathway has also been implicated in the virulence of other pathogens. A *gcpE* transposon mutant of *M. tuberculosis* exhibited decreased colonization ability in the liver and intestine of BALB/c mice following intraperitoneal infection (Shin et al., 2006). A study by Lai et al. (2001) demonstrated that the *K. pneumoniae dxr* gene was expressed following intraperitoneal infection of BALB/c mice. Expression studies using a GFP reporter system revealed that the *B. abortus dxs* gene was activated during survival within RAW macrophages. The authors of that study proposed that the expression of this gene leads to the accumulation of antiooxidants (produced via the MEP pathway), which allows for initial survival in the oxidative environment within the phagosome of the macrophage (Eskra et al., 2001).

It has been shown that host cholesterol is important for cell entry and intracellular proliferation of *Salmonella*. Garner et al. (2002) demonstrated an accumulation of host cholesterol into the *Salmonella*-containing vacuoles (SCVs) within murine macrophages and epithelial cell lines. Further studies by Catron et al. (2004) investigated the effect of inhibiting the host mevalonate pathway. This involved the targeted inhibition of the host HMG-CoA reductase (HMGMR), the rate-limiting step of the mevalonate pathway. Statins (cholesterol lowering drugs) were used to inhibit the host mevalonate pathway. Inhibition of the host mevalonate pathway resulted in reduced intracellular growth by the bacteria in a mouse model and infected macrophage cell line. These results demonstrate the requirement of *Salmonella typhimurium* for the host mevalonate pathway during intracellular growth.

Evidence suggests that the MEP pathway may be involved in combating oxidative stress. Interestingly Ostrovsky et al. (1998) found that 2-C-methyl-d-erythritol-2,4-cyclopyrophosphate (MEC), an intermediate of the MEP pathway, accumulated in mycobacteria and corynebacteria in response to oxidative stress conditions. Carotenoids have been shown to elicit a protective effect against reactive oxygen species (Ostrovsky et al., 1998). A study of *Chlamydia trachomatis* demonstrated the requirement for 2-C-methyl-d-erythritol-2,4-cyclopyrophosphate (MEC), a molecule which instigates intracellular development through disruption of histone-like protein Hc-1–DNA interactions (Grieshaber et al., 2004). *M. tuberculosis* MEP pathway mutants (*ΔlytB* and *Δdxs*) were impaired in their ability to survive within the phagosome, and therefore this pathway has been linked with the intracellular stress response in this bacterium (Pethe et al., 2004). These studies demonstrate that isoprenoids or derivatives of isoprenoids allow adaptation to the host environment through enhanced stress resistance or other mechanisms. It is worth noting that the DOXP of the MEP pathway not only is involved in IPP biosynthesis but also is an intermediate for thiamine (vitamin B1) and pyridoxil (vitamin B6) biosynthesis, both of which are essential for human health (Hill et al., 1996).

**Immune stimulation**

In humans and primates, V/9/Δ2 T cells constitute 0.5–5% of peripheral blood T cells (Caccamo et al., 2006). The expansion of this subset of T cells has been well documented in the blood plasma of patients suffering from a wide range of microbial infections, including listeriosis, salmonellosis, malaria and tuberculosis (Morita et al., 2007). Early studies implicated IPP biosynthesis in the reactivity of V/9/Δ2 T cells, but only bacteria utilizing the MEP pathway could elicit this immune response (Jomaa et al., 1999a). The stimulatory capacity of several MEP pathway mutants in *E. coli* was analysed and a significant difference was observed between a *ΔgcpE* mutant which exhibited a reduced capacity to elicit an immune response in contrast to the highly immunostimulatory *ΔlytB* mutant. Analysis of *ΔlytB* mutant extracts identified HMB-PP as the most potent stimulator of V/9/Δ2 T cells, with a 10 000-fold increase in the stimulatory capacity of this compound compared with IPP (Altinicek et al., 2001; Eberl et al., 2002; Hecht et al., 2001; Hintz et al., 2001; Reichenberg et al., 2003). These findings made it clear that the inability of the pathogenic bacteria *Staphylococcus*, *Streptococcus* and *Borrelia* to stimulate V/9/Δ2 T cells was due to the lack of an MEP pathway and the absence of HMB-PP (Jomaa et al., 1999a). The ability of isoprenoid biosynthetic pathways to produce non-peptidic compounds...
and stimulate Vγ9/Vδ2 T cells has been reviewed elsewhere (Eberl et al., 2003; Bonneville & Scotet, 2006).

*L. monocytogenes* is an unusual pathogen in possessing two complete pathways for IPP biosynthesis. γδ T cell bioactivity from bacterial extracts was calibrated against a standard of synthetic HMB-PP and the resultant bioactivity was expressed as equivalents of HMB-PP. A ΔgcpE mutant did not produce HMB-PP, resulting in a decreased stimulatory capacity below wild-type levels, but a ΔlytB mutant (HMB-PP+) exhibited increased capacity to stimulate an immune response as compared with the wild-type (Begley et al., 2004). Similarly, a study by Brown et al. (2010) also demonstrated the increased accumulation of HMB-PP in an *M. tuberculosis* strain overexpressing gcpE. Begley et al. (2008) investigated the possibility of restoring the MEP pathway in *L. innocua*. The missing final two genes of the MEP pathway were restored by introducing gcpE on its own and gcpE together with the lytB gene. The presence of gcpE alone conferred the capacity to produce HMB-PP and thereby stimulate Vγ9/Vδ2 T cells, a phenotype that does not exist for the wild-type *L. innocua* strain (Begley et al., 2008). The precise mechanism underpinning the stimulation of Vγ9/Vδ2 T cells by HMB-PP is unclear. The elucidation of this process is complicated by the fact that this particular subset of T cells is only present in humans and higher primates.

Several hypotheses on the role of Vγ9/Vδ2 T cells in the immune response have been proposed, including killing of infected target cells (Bonneville & Scotet, 2006), cytokine production, maturation of dendritic cells, and an ability to act like antigen-presenting cells (Bonneville et al., 2010; Eberl et al., 2009). Initial studies by Bonneville & Scotet (2006) attempted to determine the mechanisms by which HMB-PP stimulates Vγ9/Vδ2 T cells. This study demonstrated the cytotoxic effects of Vγ9/Vδ2 cells in the killing of *Mycobacterium bovis* BCG-infected dendritic cells but did not determine the mode of HMB-PP presentation to the Vγ9/Vδ2 T cells. Eberl and colleagues then confirmed the functioning of monocytes as ‘feeder’ cells in the stimulation of Vγ9/Vδ2 T cells with HMB-PP during bacterial infections (Eberl et al., 2009). A recent study by Davey et al. (2011) delved further into the relationship between Vγ9/Vδ2 T cells and HMB-PP within the host. They demonstrated that HMB-PP is in fact released from infected neutrophils into the microenvironment. This study was performed using the non-pathogenic *L. innocua*, which cannot produce HMB-PP, a strain with the ability to produce HMB-PP conferred by the presence of the gcpE gene, and a strain of *Mycobacterium smegmatis* engineered to overproduce HMB-PP. They demonstrated that the capacity of Vγ9/Vδ2 T cells to respond to infected neutrophils is dependent on cell to cell contact with monocytes coupled with the ability of the invading bacteria to produce HMB-PP, demonstrating that only HMB-PP-producing bacteria could activate the surrounding Vγ9/Vδ2 T cell population (Fig. 2). The cell to cell contact between monocytes and Vγ9/Vδ2 T cells proved crucial in eliciting an optimum response to the phagocytosed bacteria from the infected neutrophil (Davey et al., 2011). However, the exact mechanism by which HMB-PP is recognized by the Vγ9/Vδ2 T cell still remains to be determined. Interestingly, recent evidence has shown that aminobisphosphonate and alkylamine (isoprenoid biosynthesis) inhibitors can indirectly stimulate Vγ9/Vδ2 T cells. This highlights that there are multiple ways to stimulate Vγ9/Vδ2 T cells, and emphasizes the requirement to characterize both direct and indirect pathways for Vγ9/Vδ2 T cell stimulation (Wang et al., 2011a).

**Isoprenoid biosynthesis pathways as drug targets**

**Mevalonate pathway drug targets**

It is significant that human/mammalian isoprenoid biosynthesis is the target of several FDA-approved drugs,
including those used in the treatment of cancer (taxol), bone disease (bisphosphonates) and hypercholesterolaemia (statins) (Buhaescu & Izzedine, 2007). The potential of the mevalonate pathway as a drug target has been reviewed in detail by Buhaescu & Izzedine (2007). In humans, cholesterol biosynthesis proceeds via the mevalonate pathway, which is responsible for the production of the IPP precursor molecule. Some of the best-characterized and most effective inhibitors of IPP biosynthesis are the cholesterol-lowering statins. Statins disrupt a reaction in the mevalonate pathway by binding to and inhibiting HMGCR, the rate-limiting enzyme for cholesterol biosynthesis in mammals. Nitrogenous bisphosphonates (NBPs) such as pamidronate and zoledronate, which are predominantly used to treat bone disease, also target the mevalonate pathway by inhibiting farnesyl pyrophosphate synthase. The IPP isomeres have been considered as potential targets; however, as humans possess the type I isomerase, specific inhibitors of the type II enzyme could function to target pathogenic bacteria such as multidrug-resistant Staphylococcus (Kuzuyama et al., 2000).

The MEP pathway as a drug target

Enzymes of the MEP pathway have been classified as metabolic chokepoints, meaning that they can consume or generate a specific product and their function cannot be compensated for by any other enzyme (Hasan et al., 2006). This, together with the fact that IPP synthesis in humans proceeds via the mevalonate pathway (so there are no human MEP enzyme homologues), has resulted in the MEP pathway as a drug target (reviewed by Ershov, 2007; Obiol-Pardo et al., 2011; Rohdich et al., 2005; Testa & Brown, 2003).

The identification and characterization of E. coli DXR catalytic residues was described by Kuzuyama et al. (2000), and further characterization studies have determined the crystal structure of E. coli DXR when complexed with cofactors (NADPH) and fosmidomycin (Steinbacher et al., 2003). Solving the crystal structures of these enzymes was a crucial step to pave the way for drug design. In 1999, Jomaa and colleagues provided evidence that the MEP pathway is used by the malaria parasite Plasmodium falciparum, and that fosmidomycin and its analogue FR-900098 had the ability to inhibit DXR from Plasmodium falciparum in a dose-dependent manner. Both inhibitors cured mice infected with the rodent malaria parasite Plasmodium vinckei, demonstrating their use as anti-malarial compounds (Jomaa et al., 1999b). Clinical trials proved that fosmidomycin was effective in the treatment of patients suffering from uncomplicated Plasmodium falciparum malaria (Lell et al., 2003). The anti-malarial activity of FR-900098 was approximately twofold greater than that of fosmidomycin. Fosmidomycin treatment led to a fast reduction in fever and clearance of the parasite, but was precluded as a monotherapy due to inefficient elimination of the parasite. A combination of fosmidomycin with clindamycin could result in more synergistic effects (Singh et al., 2007). Kurz et al. (2006) reported the synthesis of chain-substituted pivaloyloxymethyl ester derivatives of fosmidomycin and FR900098 with comparable in vitro antimalarial activity. Kunz et al. (2005) synthesized two phosphonohydroxamic acids which showed inhibitory activity towards DXR as well as antibacterial activity against E. coli in vitro. Indeed, Behrendt et al. (2011) very recently reported the antiplasmodial activity of IspC/Dxr inhibitors.

YchP, YcbB and YghB have all been shown to be essential in E. coli, Bacillus subtilis, Haemophilus influenzae and M. tuberculosis (Bueto et al., 2007; Campbell & Brown, 2002; Campos et al., 2001a, b; Freiberg et al., 2001). Several characterization and screening studies have analysed these MEP pathway enzymes as potential drug targets. Illarionova et al. (2006) described the enzyme-assisted preparation of isoprenoid biosynthesis intermediates and high-throughput methods for screening of IspC/Dxr, IspD/YghP and IspE/YcbB against large compound libraries. 13C NMR assays were subsequently employed to verify hits via direct detection of the primary reaction product. The IspD/YghP enzyme of M. tuberculosis was cloned and expressed in an E. coli system, and the purified recombinant protein was subjected to biochemical and enzymic characterization in order to gain insight into the structure of this protein for therapeutic drug design (Shi et al., 2007). A recent publication by Tsang et al. (2011) details the kinetic characterization of the IspD enzyme of Francisella tularensis, and their results validate the targeting of this enzyme for novel therapeutics against F. tularensis. Detailed biochemical characterization and crystal structure models (to analyse substrate-binding sites) for YcbB were determined using an efficient expression system with YcbB from the thermophilic bacterium Aquifex aeolicus. Sequence–structure comparisons revealed that the active site and ligand interactions are highly conserved for YcbB and that A. aeolicus YcbB represents a suitable model for the human pathogen M. tuberculosis (Sgraja et al., 2008). A. aeolicus IspE has proved invaluable for providing structural information in the structure-based design approach for the generation of IspE inhibitors for organisms such as Plasmodium falciparum and M. tuberculosis (Crane et al., 2008; Hirsch et al., 2008). Co-crystal structure analysis (Crane et al., 2008; Hirsch et al., 2008) and structure–activity relationships (SARs) (Hirsch et al., 2008) of A. aeolicus IspE have validated the binding mode of several new inhibitors.

Bueto et al. (2007) determined the crystal structure of M. smegmatis YghB, which has a high degree of similarity to YgbB of M. tuberculosis. This study allowed the identification of active sites and binding pockets, and provides a template for structure-based inhibitor design (Bueto et al., 2007).

Ramsden et al. (2009) utilized the structure of E. coli IspF bound to CDP to identify features considered important
for ligand binding and affinity. The rigid nucleotide–Zn2+ binding pocket of the active site was noted. Subsequently, a computational docking approach was employed to screen, in silico, a database of approximately one million commercially available compounds against the nucleotide–Zn2+ binding pocket. Selected hits from the virtual screen were examined experimentally with X-ray crystallography and a surface plasmon resonance assay, and two weakly binding ligands were identified (Ramsden et al., 2009). These initial results prompted additional experiments using a more focused set of compounds to probe the IspF cytidine and Zn2+ binding sites, and yielded a ligand with low micromolar binding affinity. An additional study by Jawaid et al. (2009) examined the IspF enzyme of F. tularensis. The enzyme was cloned and expressed in E. coli, the recombinant protein was then purified and enzymically characterized, and subsequent analysis showed that IspF is an excellent target for antibiotic development.

The 3D structures for YghP, YchB and YghB are available for M. tuberculosis, Plasmodium Falciparum and C. jejuni, respectively (Miallau et al., 2003; Richard et al., 2001; Steinbacher et al., 2003). The crystal structures for these enzymes in E. coli have also been solved; however, despite these developments, no successful inhibitors for these three enzymes have been identified to date (Miallau et al., 2003; Richard et al., 2001; Steinbacher et al., 2003).

The last two enzymes of the MEP pathway, IspG/GcpE and IspH/LytB, are 4Fe–4S proteins, which are involved in essential reduction steps in isoprenoid biosynthesis. The crystal structure for IspH is known for both E. coli (Gräwert et al., 2010) and A. aeolicus (Rekittke et al., 2008). Pyridine-based inhibitors of IspH have undergone spectroscopic and chemical investigation to elucidate their inhibitor functions (Wang et al., 2011b). Indeed, Wang et al. (2010) screened a series of small molecules using EPR spectroscopy and put forward the proposal that diphosphonates inhibit IspH through interaction with the active site, thereby preventing HMB-PP from binding to the 4Fe–4S cluster.

There are two classes of IPP isomerases: humans and animals possess the type I isomerase, and the majority of pathogenic bacteria possess the type II enzyme. So far, no specific type II inhibitors have been identified, but this enzyme has been pinpointed as an attractive target in infections by Gram-positive cocci, especially multidrug-resistant Staph. aureus (Rohdich et al., 2005). Both farnesyl diphosphate synthase (FPPS) and undecaprenyl diphosphate synthase (UPPS) enzymes are essential for bacterial cell growth. FPPS catalyses the condensation of DMAPP with IPP to form geranyl diphosphate (GPP), and UPPS elongates FPP. UPPS is of particular interest, as like the MEP pathway enzymes it is also absent in humans. Interestingly, Durrant et al. (2011) described a novel screening approach to identify non-bisphosphonate FPP synthase inhibitors which also showed inhibitory properties against UPP synthase enzymes. These compounds were shown to be effective at micromolar concentrations (Durrant et al., 2011), and furthermore they appear to be more amenable to clinical use than their bisphosphonate counterparts, as they are less vulnerable to rapid removal from the circulatory system (Jahnke et al., 2010).

It is estimated that approximately 70% of the human gut microbiome is unculturable under standard laboratory conditions, but with recent and rapid advances in culture-independent sequencing techniques the genetic makeup of previously uncharacterized gut commensals has now been determined. Metagenomic analysis suggests that commensals utilize the MEP pathway (Gill et al., 2006; S. Heuston, unpublished data). Although the MEP pathway represents an attractive and valid target for pathogenic bacteria, careful consideration is required, as drugs targeting this pathway will also likely affect the human gut commensal population, possibly resulting in an undesirable effect on the human superorganism.

Conclusions and future directions

The MEP pathway is the principal means of IPP biosynthesis in the majority of human pathogens. The penultimate intermediate of the MEP pathway (HMB-PP) is known to be the most potent activator of human V9/V82 T cells. Several elegant studies have demonstrated the ability of MEP-utilizing bacteria to stimulate V9/V82 T cells. The in vitro and in vivo experimental analysis discussed in this review indicates a link between the MEP pathway and the intracellular survival of pathogenic bacteria.

IPP biosynthesis in humans proceeds via the mevalonate pathway, and so the development of novel drugs that target the MEP pathway would result in a new class of broad-spectrum antibiotics. However, as discussed in this review, extensive metagenomic analysis of the human gut microbiome has demonstrated an over-representation of the MEP pathway in gut bacteria. As a result, drugs targeting the MEP pathway would consequently target gut bacteria and possibly result in detrimental effects on the human host. The targeting of MEP pathway enzymes may need to be re-evaluated from the perspective of the host, and due consideration should be given to dual therapies which would involve MEP-targeted antibiotics used in conjunction with probiotic therapies (to restore the host microbiome).

One unanswered question is why gut bacteria favour the MEP pathway. The MEP pathway could be favoured due to its energy requirements. Ershov (2007) discussed the energy efficiency of the MEP pathway in comparison with the mevalonate pathway. A distinct difference in the ratio of IPP to DMAPP was noted, with the MEP pathway synthesizing a 5 : 1 ratio of IPP to DMAPP and the mevalonate pathway generating a 3 : 7 ratio. These factors could have important implications in the evolutionary selection of the MEP pathway. Anaerobic bacteria such as bifidobacteria seem to preferentially utilize this pathway (Ershov, 2007).
Evidence suggests a role for the MEP pathway during the intracellular survival of bacterial pathogens. Interestingly, several extracellular pathogens appear to utilize the mevalonate pathway rather than the MEP pathway \( (Staph. aureus, Streptococcus pneumoniae and Streptococcus pyogenes)\). Studies in both \( Staph. aureus \) and \( Strep. pyogenes \) have demonstrated their inability to activate \( V_7/N\delta2 \) T cells \( (Jomaa et al., 1999a) \). Furthermore, an adaptation to a non-pathogenic extracellular life cycle in \( L. innocua \) has clearly led to the loss of two genes of the MEP pathway, with \( L. innocua \) possessing only the mevalonate pathway \( (Begley et al., 2008) \). MEP-utilizing bacteria can elicit an immune response through the production of the non-peptidic penultimate compound of the MEP pathway, HMB-PP, but it is not clear whether this immuno-stimulatory capacity is advantageous to the bacterium residing in the host. As the MEP pathway is over-represented in the human gut metagenome, this immune stimulation is not limited to gut pathogens but extends to autochthonous gut microbes. This suggests that the stimulatory capacity of the bacteria may in fact induce a state of tolerance within the hosts and enable the bacteria to evade the immune system. This particular subset of T cells can function as a bridge linking innate and adaptive immune responses. However, the exact reason why bacteria would produce HMB-PP to stimulate an immune response still remains unclear.

Future studies may be best focused on determining a role for the MEP pathway during the gastrointestinal phase of the bacterial life cycle. The ability of the MEP pathway to stimulate an immune response from the perspective of the host warrants further characterization, but these studies cannot be performed in murine models, as mice do not possess the \( V_7/N\delta2 \) subset of T cells. Future studies would need to be performed in primate models such as Rhesus Macaques \( (Macaca mulatta) \) and Night Monkeys \( (Aotus nancymaae) \). Recent studies have contributed greatly to elucidating the methods by which HMB-PP is released from infected cells, but the exact mechanisms by which HMB-PP is recognized by \( V_7/N\delta2 \) T cells still remain unclear.

To our knowledge there are very few studies which document the levels of IPP production in bacterial mutant strains, with most experimental data correlating HMB-PP concentration with the bioactivity of \( V_7/N\delta2 \). Effective methods to measure the production of pathway intermediates as well as end products would greatly increase our understanding of the functioning of these pathways. A fundamental understanding of the flux or regulation of these pathways during different stages in the bacterial life cycle will also be essential. Studies to determine whether some of the dominant intracellular gut pathogens have the ability to utilize the host mevalonate pathway during intracellular survival would be very useful to help determine the roles of these pathways \( in vivo \).

In conclusion, numerous elegant studies, largely biochemical and immunological, have led to significant advances in our understanding of isoprenoid biosynthesis pathways. Many questions remain unanswered regarding the use of these pathways by human pathogens and host commensals, and it is highly likely that isoprenoids have as-yet-unidentified and possibly unexpected roles in bacterial physiology.

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