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HmgR, a key enzyme in the mevalonate pathway for isoprenoid biosynthesis, is essential for growth of *Listeria monocytogenes* EGDe

Sinead Heuston,[1,2] Máire Begley,[1] Martin S. Davey,[3] Matthias Eberl,[3] Pat G. Casey,[1] Colin Hill[1,2] and Cormac G. M. Gahan[1,2,4]

Correspondence
Colin Hill
c.hill@ucc.ie

[1]Department of Microbiology, University College Cork, Cork, Ireland

[2]Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland

[3]Department of Infection, Immunity and Biochemistry, School of Medicine, Cardiff University, Cardiff, UK

[4]School of Pharmacy, University College Cork, Cork, Ireland

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Isoprenoids may be synthesized via one of two pathways, the classical mevalonate pathway or the alternative 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. While the majority of bacteria utilize a single pathway for isoprenoid biosynthesis, *Listeria monocytogenes* is unusual in possessing the complete set of genes for both pathways. Here, we utilized new molecular tools to create precise gene deletions in selected genes encoding enzymes of both pathways, *gcpE*, *lytB* (encoding proteins in the MEP pathway) and *hmgR* (encoding a protein in the mevalonate pathway). We demonstrate that the *hmgR* gene can only be deleted when the growth medium is supplemented with exogenous mevalonate. Furthermore, full growth of the mutant in the absence of mevalonate was only possible when the intact *hmgR* gene was supplied *in trans* using an IPTG-inducible expression system. Murine competitive index assays performed via the oral and intraperitoneal routes of infection revealed that the mevalonate *hmgR* mutant could not be recovered from livers and spleens 3 days post-infection. We propose that HmgR in *L. monocytogenes* EGDe is involved in essential metabolic functions and that an intact MEP pathway is not capable of sustaining growth.

INTRODUCTION

Isoprenoids are a diverse class of naturally occurring organic compounds, with more than 30 000 identified to date (Sacchettini & Poulter, 1997). All isoprenoids are assembled from the universal 5-carbon building units isopentyl diphosphate (IPP) or dimethylallyl diphosphate (DMAPP). Isoprenoid biosynthesis is essential for the survival of both eukaryotic and prokaryotic cells, as these compounds play a role in a diverse range of processes (Boucher & Doolittle, 2000). Isoprenoids are generally synthesized via one of two pathways: the classical mevalonate pathway utilized by eukaryotic cells and some prokaryotes, or the alternative 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway utilized by the majority of prokaryotes (Fig. 1) (Campos *et al.*, 2001; Goldstein & Brown, 1990; Hecht *et al.*, 2001; McAteer *et al.*, 2001). Several studies have been performed in recent years to determine the role of individual enzymes involved in isoprenoid biosynthetic pathways (for in-depth reviews, see Boucher & Doolittle, 2000; Rodríguez-Concepción & Boronat, 2002; Hunter, 2007). As the majority of pathogenic bacteria use the MEP pathway for isoprenoid biosynthesis (Brown *et al.*, 2010; Buetow *et al.*, 2007; Campos *et al.*, 2001), MEP pathway enzymes have received significant attention as potential drug targets (Hunter, 2007).

Listeria monocytogenes and certain *Streptomyces* species are unusual in that they possess the entire set of genes for both the MEP and mevalonate biosynthetic pathways (Fig. 1) (Begley *et al.*, 2008; Boucher & Doolittle, 2000; Begley *et al.*, 2004). *L. monocytogenes* is an intracellular Gram-positive pathogen and the causative agent of listeriosis, the potentially fatal foodborne disease which can manifest as meningitis and septicaemia in immunocompromised individuals or result in spontaneous abortion in pregnant women (Vázquez-Boland *et al.*, 2001). *Listeria innocua* is a closely related, non-pathogenic species which has lost the final two genes of the MEP pathway, *gcpE* and *lytB*, possibly during adaptation to a non-pathogenic life cycle (Begley *et al.*, 2008). As *Listeria* species are genetically

Abbreviations: IPP, isopentyl diphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; SOE PCR, splicing by overlap extension PCR.

Supplementary methods and six supplementary figures are available with the online version of this paper.

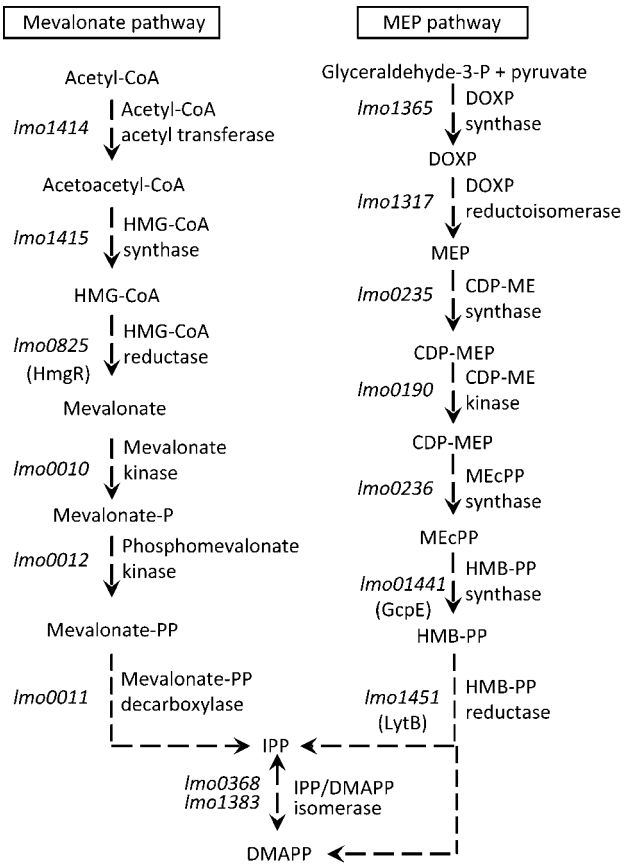

Fig. 1. Genes putatively involved in isoprenoid biosynthesis pathways in *L. monocytogenes* EGDe. The classical mevalonate pathway (left) and the alternative non-mevalonate or MEP pathway (right). Numbers refer to the National Center for Biotechnology Information (NCBI) annotation numbers.

tractable they provide a unique model with which to analyse the roles of the isoprenoid biosynthetic pathways in cellular metabolism and survival in disparate environments.

Previously, we demonstrated that the MEP pathway *gcpE* and *lytB* genes encode enzymes which play a role in the immune stimulation of the human Vγ9Vδ2 subset of T cells (Begley *et al.*, 2004), and also that plasmid insertion mutants in these genes exhibit an impaired virulence potential (Begley *et al.*, 2008). Initial attempts in our laboratory to create single precise deletion mutants in genes encoding enzymes of the mevalonate pathway were unsuccessful. We have recently developed new molecular tools to efficiently create deletion mutants in *L. monocytogenes* and to examine the essentiality of gene systems in this organism (Monk *et al.*, 2008a). The aim of the current study was to utilize these new approaches to create a set of deletion mutants and to use these molecular tools to analyse the role of both the mevalonate and MEP pathways in cellular growth and pathogenesis. This work demonstrates that an intact *hmgR* gene is essential for *L. monocytogenes* growth in the absence of exogenous mevalonate, despite the presence of an intact MEP pathway.

METHODS

Bacterial strains, media, reagents and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strain EC10B was used as the cloning host during the construction of deletion mutants and was grown aerobically at 37 °C in Luria Broth (LB) medium with shaking (Sambrook *et al.*, 1989). *L. monocytogenes* strain EGDe was routinely grown aerobically in brain heart infusion (BHI) broth (Oxoid) at 37 °C with shaking. Broth medium was solidified using 1.5 % technical agar (Merck). For antibiotic selection the following concentrations for *L. monocytogenes* (Lm) and *E. coli* (Ec) were used: erythromycin (Ery) Lm 5 μg ml^{-1}, Ec 250 μg ml^{-1}; chloramphenicol (Cm) Lm 7.5 μg ml^{-1}, Ec 10 μg ml^{-1}; kanamycin (Kan) Lm/Ec 50 μg ml^{-1}; and tetracycline (Tet) Lm/Ec 10 μg ml^{-1}. For growth of the Δ*hmgR* deletion mutant, all BHI media were supplemented with 1 mM mevalonate (M4667 DL-mevalonolactone, Sigma-Aldrich). A 1 M stock solution was prepared by hydrolysing DL-mevalonolactone with 1 M NaOH at 37 °C for 1 h (Eberl *et al.*, 2002). Transformants with pORI280 were selected for by the addition of 100 μg X-Gal ml^{-1} to the media. pIMK4, the IPTG-inducible vector, required the addition of 1 mM IPTG (Sigma-Aldrich) to the media.

Nucleic acid manipulations. Isolation of plasmid DNA was performed using the Qiagen QIAprep Spin Miniprep kit (Qiagen). A Qiagen gel extraction kit was used for extracting DNA from agarose gels (Qiagen). For cloning experiments, KOD Hotstart DNA polymerase (Merck) was used with genomic DNA that was isolated using the GenElute Bacterial Genomic Miniprep kit (Sigma). T4 DNA ligase was supplied by Promega. Restriction enzymes were purchased from Roche and used as directed by the manufacturer's guidelines. Oligonucleotide primers (listed in Table 2) were synthesized by MWG-Biotech.

pORI280 chromosomal mutagenesis. Gene deletions were constructed in the mevalonate pathway *hmgR* gene (using primer sets 0825A, 0825B, 0825C, 0825D) (Table 2) and the MEP pathway *lytB* gene (using primer sets 1451A, 1451B, 1451C, 1451D) (Table 2). These MEP and mevalonate gene deletions were created using the pORI based *repA*$^-$ plasmid system. *L. monocytogenes* EGDe genomic DNA was employed as the template DNA for PCR at a concentration of 10–50 ng per 50 μl reaction. The splicing by overlap extension (SOE) PCR technique was applied (Leenhouts *et al.*, 1998) to generate two 400 bp fragments, one upstream including the ATG start codon giving the AB product, and one downstream generating the CD product beginning at the stop codon. The C primer contained a 20 bp tail complementary to the B primer. These initial PCR products were diluted 1 : 20 in PCR-grade water, and 1 μl of each was used as the template DNA for the second round of PCR using the AD primers to generate an 800 bp product. The SOE PCR product was digested and ligated with similarly digested pORI280. A co-transformation was performed into *L. monocytogenes* with the highly temperature-sensitive plasmid pVE6007 (Maguin *et al.*, 1992). This plasmid supplies the RepA *in trans*. Blue transformants were selected for on BHI agar supplemented with exogenous mevalonate for the Δ*hmgR* deletion and containing Ery at 5 μg ml^{-1} and X-Gal at 100 μg ml^{-1}. Plasmid integration, induced by a single crossover event, was followed by plasmid excision, which was facilitated by continual passaging at 37 °C, as described in detail by Monk *et al.* (2008a). At each passage, the cells were plated onto BHI agar containing X-Gal (with 1 mM mevalonate for Δ*hmgR*) at 37 °C for 24 h. White colonies were selected for and screened by colony PCR (ABgene PCR Extensor

Table 1. Bacterial strains and plasmids used in this study

Kan^r, kanamycin-resistant; Cm^r, chloramphenicol-resistant; Ery^r, erythromycin-resistant; Tet^r, tetracycline-resistant.

Strain or plasmid	Description	Source or reference
***E. coli* strains**		
Top10	Cloning host	Invitrogen
EC101	JM109 derivative, Kan^r, RepA$^+$ integrated in the *glgB* gene	Law *et al.* (1995)
EC10B	DH10B derivative, Kan^r, RepA$^+$ integrated into the chromosome	Monk *et al.* (2008a)
***L. monocytogenes* strains**		
EGDe	Wild-type of serotype 1/2a, genome sequenced	UCC Culture Collection
EGDeΔ*hmgR*	EGDe with the entire *hmgR* gene deleted	This study
EGDeΔ*lytB*	EGDe with the entire *lytB* gene deleted	This study
EGDeΔ*gcpE*	EGDe with the entire *gcpE* gene deleted	This study
EGDeΔ*hmgR*::pIMK4*hmgR*	EGDe with the entire *hmgR* gene deleted and complemented with pIMK4*hmgR* integrated at the tRNA^{Arg} locus	This study
EGDeΔ*gcpE*::pNZ44*gcpE*	Cm^r, EGDeΔ*gcpE* harbouring pNZ44 *gcpE*	This study
EGDe::pIMC3Cm	EGDe transformed with the vector integrated at the tRNA^{Arg} locus on the *L. monocytogenes* EGDe chromosome	Monk *et al.* (2008b)
EGDeΔ*hmgR*::pIMC3Tet	Tet^r, EGDeΔ*hmgR* transformed with pIMC3Tet integrated at the tRNA^{Arg} locus	This study
EGDeΔ*lytB*::pIMC3Kan	Kan^r, EGDeΔ*lytB* transformed with pIMC3Tet integrated at the tRNA^{Arg} locus	This study
EGDeΔ*gcpE*::pIMC3Ery	Ery^r, EGDeΔ*gcpE* transformed with pIMC3Tet integrated at the tRNA^{Arg} locus	This study
Plasmids		
pORI280	Ery^r, RepA gene replacement vector, constitutive *lacZ*, 5.3 kb	Leenhouts *et al.* (1996)
pVE6007	Temperature-sensitive helper plasmid, supplies RepA *in trans*	Maguin *et al.* (1992)
pIMK4	Kan^r, site-specific listerial integrative vector, IPTG-controlled gene expression	Monk *et al.* (2008a)
pIMC3Kan	Kan^r, site-specific listerial integrative vector	Monk *et al.* (2008b)
pIMC3Ery	Ery^r, site-specific listerial integrative vector	Monk *et al.* (2008b)
pIMC3Tet	Tet^r, site-specific listerial integrative vector	Monk *et al.* (2008b)
pIMC3Cm	Cm^r, site-specific listerial integrative vector	Monk *et al.* (2008b)

Ready Mix, Thermo Scientific). Primers external to the SOE PCR product (Out Is–Out IIs) were used to screen for the gene deletions. Deletion mutants were confirmed by sequencing the mutated region (MWG-Biotech).

Using the SOE procedure described above, 0.9 kb of the coding region of *gcpE* (*lmo1441*, total length 1107 bp) was deleted from *L. monocytogenes* EGDe. Products of 0.35 kb flanking the *gcpE* gene were amplified using the primer pairs lmo1441SOEA/lmo1441SOEB and lmo1441SOEC/lmo1441SOED. Briefly, the resulting PCR products were mixed in a 1 : 1 ratio to be reamplified to create the AD product (primer pair lmo1441SOEA/lmo1441SOED). The 0.7 kb AD product was digested with *Pst*I and *Xba*I (see Table 2 for restriction site locations) and ligated into similarly digested pKSV7. The resulting plasmid (designated pKSV7-lmo1441) was electroporated into *L. monocytogenes* EGDe at 30 °C. Chromosomal integration of the plasmid was achieved by a temperature shift up to 42 °C in BHI broth supplemented with chloramphenicol. Plasmid excision and curing were performed on isolated single colonies through continuous passaging at 30 °C in BHI broth without antibiotic. The deletion events were confirmed by PCR analysis using the primers that bind outside the region amplified (Out I–Out II).

Transcriptional analysis. Total RNA was isolated using a combination of the Macaloid and Roche methods, as described by (Raya *et al.*, 1998). *L. monocytogenes* strains were grown overnight at 37 °C with shaking in BHI broth, and overnight cultures were inoculated into fresh BHI (2 % inoculum). Total RNA was extracted from exponential-phase (OD_{600} 0.25) cultures using the Macaloid method (Sambrook *et al.*, 1989). The samples were then further purified using a Roche High Pure RNA Isolation kit according to the manufacturer's guidelines. Samples were then treated with the Ambion DNA-free kit to remove any remaining DNA. For RT-PCR analysis, cDNA was synthesized from RNA using Expand Reverse Transcriptase and random primer p(dN)6 (Roche) performed in accordance with the manufacturer's guidelines. Control PCRs were performed on the cDNA using the *rrnA* (16sRNA) primers U141 and L142 (Table 2). To determine whether gene read-through occurred, PCRs were carried out using the primers RT-0825For and RT-0825Rev (Table 2). PCRs were carried out for 16, 22, 30 and 35 cycles, to allow for optimal quantification of products.

pIMK4 essential gene deletion. pIMK4, the site-specific integration vector which integrates into the tRNA^{Arg} locus on the *L. monocytogenes* chromosome, was used to complement the Δ*hmgR* mutant (Monk *et al.*, 2008a). *L. monocytogenes* EGDe genomic DNA was used as the template, along with the primer pair 0825pIMKFor and 0825pIMKRev (Table 2) to amplify a promoterless copy of the *hmgR* gene from the annotated start (ATG) to the stop codon (TTG). The *hmgR* PCR product was then digested with *Afl*III (this enzyme introduces a single base pair mismatch at the *Nco*I site) and *Pst*I, and then ligated into *Nco*I- and *Pst*I-digested pIMK4. Ligation reactions were precipitated using Pellet Paint (Novagen) and electroporated into chemically competent Top10 cells (Invitrogen). Transformants were selected for on LB agar containing 50 μg kanamycin ml^{-1} and incubated overnight at 37 °C. Transformants containing pIMK4 and the gene were selected by PCR with primers pIMK4FWD and pIMK4REV (Table 2). The sequence of the clone was confirmed by sequencing at MWG-Biotech. The construct was then transformed into the *hmgR L. monocytogenes* deletion mutant. Transformants were selected for on BHI agar containing kanamycin and mevalonate at concentrations of 50 μg ml^{-1} and 1 mM, respectively, then incubated

Table 2. Oligonucleotides used in this study

Bold type indicates restriction sites.

Primer	Sequence (5′–3′)
0825A	ATAT**CTGCAG**GCACCTGCTACTTTAAAAGATTTTTG
0825B	CATTGCTTTATCACCTCAAATTAAAATTTATAC
0825C	TTTGAGGTGATAAAGCAATGTAAAATAAGTTATTAAATCAGCCTT
0825D	TATA**TCTAGA**AAAATAAGTTATTAAATCAGCCTT
0825 Out Is	AGAAATACGACACACATTCCG
0825 Out IIs	CGGTTGTTCCGATGTTCGCC
lmo1441SOEA	GATTTATTCTT**TCTAGA**TTTGGCC
lmo1441SOEB	GCGAAATATTCTTTCATTCAAAGA
lmo1441SOEC	TCTTTGAATGAAAGAATATTTCGCCCGATTAAAGTAGCCGTGCTT
lmo1441SOED	CTACTC**CTGCAG**CAATACCAA
lmo1441outI	ATTCGATTGAGTCATTAGCTA
lmo1441outII	AAATTGCGCCGCACCTGCG
1441 F	ACCCTA**CTGCAG**ACGGAA
1441 R	CATTGCG**GAATTC**GAGGAA
1451A	ATAT**CTGCAG**AAGCTCACGACTTGGTGCCG
1451B	CATTCGAAAACATCCTCATTTCT
1451C	ATGAGGATGTTTTCGAATGTAAAAACCGAGCAACTCCCT
1451D	TCTA**TCTAGA**TCGGCATGGTTTCATTTATC
1451 Out Is	TTGCACGTTGTTTGTCCGTA
1451 Out IIs	CATATGAAGAACCCGCGATT
lmo0825F	ACTC**GTCGAC**ACCCAAGA
lmo0825R	CGCCAAT**GGATCC**CAAAC
0825pIMKFor	ATAT**ACATGT**ATGCTTTTGATAAATTTTAT
0825pIMKRev	ATAT**CTGCAG**ATTTAATAACTTATTTTATTT
RT-0825For	GAACAGGAAGCTATAAACCG
RT-0825Rev	GCTGCGCAATTTCGCGATAAATTC
0008For	AGCCGACTATTTACGATG
0012Rev	AACCGTCGCCGCTGCGCT
pORI280Fwd	CTCGTTCATTATAACCCTC
pORI280Rev	CGCTTCCTTTCCCCCCAT
pIMK4FWD	GTGAGCGTCCACAATTATAAAGCA
pIMK4REV	TCACTACATGTCAAGAATAAACTG
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
PL95	ACATAATCAGTCCAAAGTAGATGC
PL102	TATCAGACCTAACCCAAACCTTCC
U141	TTGCTCTTCCAATGTTAG
L142	GAGTGCTTAATGCGTTAG

at 30 °C for 48 h. Transformants were confirmed using the integrating primers PL95/PL102 and the primer pair, pIMK4FWD/pIMK4REV (Table 2). Final confirmation was made by sequencing the region (MWG-Biotech). Induction of the plasmid was enabled by adding 1 mM IPTG to the growth media.

pIMC3 tagging of deletion mutants. pIMC3 plasmid preparations were precipitated using Pellet Paint and electroporated into *L. monocytogenes* competent cells which were prepared using the method described by Park & Stewart (1990). The mixture was added to a chilled 1 mm cuvette (Bio-Rad) and pulsed at 400 Ω, 25 μF and 10 kV cm^{-1}. One millilitre of recovery medium (BHI broth and 500 mM sucrose) was added immediately to the cuvette and the mixture was transferred into a 1.5 ml tube and incubated statically at 30 °C for 1.5 h. *L. monocytogenes* EGDe wild-type was transformed with pIMC3, Δ*hmgR* was transformed with pIMC3Tet plasmid, which confers tetracycline resistance, and Δ*gcpE* and Δ*lytB* were transformed with plasmids pIMC3Ery and pIMC3Kan, which confer Ery and Kan resistance, respectively. Transformants were then plated onto BHI agar containing the appropriate antibiotic for each plasmid: 7.5 μg Cm ml^{-1}, 10 μg Tet ml^{-1}, 5 μg Ery ml^{-1} and 50 μg Kan ml^{-1} and incubated at 30 °C for 48 h. Transformants were selected and screened by colony PCR using the primer set PL95 and PL102.

hmgR* mutant growth curves in the presence and absence of mevalonate.** Δ*hmgR* was grown in BHI broth to which 1 mM mevalonate was added at various time points. This was used to analyse the ability of the mevalonate pathway mutant to recover to a full growth capacity. Overnight cultures of the mevalonate *hmgR* deletion mutant and the wild-type EGDe were centrifuged at 4000 ***g for 6 min and washed three times in BHI. The pellets were resuspended in equal volumes of BHI. A 1 : 1000 dilution was made into fresh BHI broth with and without 1 mM mevalonate, and cultures were incubated at 37 °C with shaking. Mevalonate was then

added at time 0, 2, 4, 6, 8 and 10 h. Growth was monitored by measuring the OD_{620} at hourly intervals using a Multiskan plate reader over a 24 h period, and plate counts were performed every 2 h onto BHI agar supplemented with mevalonate.

The minimum levels of mevalonate were determined by growth curve analysis over a range of mevalonate concentrations. Overnight cultures of the *hmgR* deletion mutant were centrifuged at 4000 ***g*** for 6 min and then washed three times in sterile quarter-strength Ringer's solution. The washed pellets were resuspended in an equal volume of BHI and inoculated (2 %) into BHI broth containing various concentrations of mevalonate from no mevalonate to 8 μM, 50 μM, 100 μM, 200 μM, 300 μM and 1 mM. Growth was measured by OD_{620} on a Multiskan plate reader over a 24 h period, and cell viability was monitored by performing plate counts at 24 h.

***In vivo* competitive index assay.** To analyse the role that the deleted genes play in *in vivo* growth/virulence potential, a peroral gavage study and intraperitoneal infection were undertaken. Overnight cultures of pIMC3TetΔ*hmgR*, pIMC3EryΔ*gcpE* and pIMC3KanΔ*lytB* deletion mutants and pIMC3 EGDe wild-type were washed three times with PBS. Cultures were then subsequently mixed at ratios of 1 : 1 : 1 : 1 (pIMC3TetΔ*hmgR*, pIMC3EryΔ*gcpE*, pIMC3KanΔ*lytB*, pIMC3 EGDe, respectively). For the gavage inoculations, 8–12-week-old female BALB/c mice were infected with approximately 5×10^8 cells per 20 μl suspension per strain and a total inoculum containing a 1 : 1 : 1 : 1 ratio of 2×10^9 cells. For the intraperitoneal route of infection, 8–12-week-old female BALB/c mice were inoculated with 200 μl containing a 1 : 1 : 1 : 1 ratio of mutants to wild-type with a final concentration of 4×10^4 c.f.u. per 200 μl suspension. The inoculum was serially diluted and plated onto BHI agar containing 1 mM mevalonate with 10 μg Tet ml^{-1}, 5 μg Ery ml^{-1}, 50 μg Kan ml^{-1} and BHI agar containing Cm at a concentration of 7.5 μg ml^{-1}. On days 1, 2 and 3 post-infection, the excretion of viable *Listeria* was measured from faecal samples. Faecal samples were weighed, homogenized in PBS, and serially diluted and plated onto all five BHI agars containing antibiotics. Three days post-infection the mice were sacrificed and the spleens and livers were aseptically removed. The organs were homogenized in 5 ml PBS, serially diluted and enumerated on the agars described above. The ability of the tagged strains to compete within a mouse was determined using the competitive index (CI) formula described by Dramsi *et al.* (2004), where CI=output (mt/wt)/input (mt/wt). Here, 'wt' represents the number of wild-type bacteria and 'mt' the number of mutant bacteria. All murine experiments complied with relevant legislation and were approved by the animal ethics committee at University College Cork. Statistical analysis (one sample *t* test, GraphPad Prism software) was applied to the raw c.f.u. counts through the calculation of c.f.u. or ratio difference (e.g. strain 1 to strain 2, strain 1 to strain 3 and strain 2 to strain 3) per organ (n=4).

Statistical analysis. Statistical analyses were performed using GraphPad Prism software.

RESULTS

Creation and characterization of *L. monocytogenes* MEP and mevalonate pathway deletion mutants

In this study, we created in-frame deletion mutants in genes encoding enzymes in the MEP and mevalonate pathways in *L. monocytogenes* (see Methods). Initial attempts focused on a method used routinely in our laboratory that utilizes plasmid pKSV7 (Smith & Youngman, 1992) and the SOE procedure described by Horton *et al.* (1990). A *gcpE* deletion mutant was successfully created using this approach but several attempts to delete the *hmgR* and *lytB* genes were unsuccessful.

Our laboratory has recently developed a novel gene deletion strategy for *Listeria* species (Monk *et al.*, 2008a). This system utilizes the $RepA^-$ pORI280 plasmid (Leenhouts *et al.*, 1998) used in conjunction with the temperature-sensitive helper plasmid pVE6007 (Maguin *et al.*, 1992), which supplies *repA in trans* (see Methods). Here, the approach was successfully used to create mevalonate pathway *hmgR* (*lmo0825*) and MEP pathway *lytB* (*lmo1451*) deletion mutants.

In order to create the *hmgR* deletion mutant it was necessary to supplement the growth medium with exogenous mevalonate. When passaging of integrants was performed in BHI broth without mevalonate only wild-type colonies were recovered. Δ*hmgR*, the mevalonate pathway mutant, was unable to grow in BHI medium unless supplemented with exogenous mevalonate (Fig. 2).

Characterization of the mevalonate-dependent Δ*hmgR* deletion mutant

The same mevalonate dependency observed in rich liquid media was also observed on solid agar (Fig. 2a). This mevalonate-dependent phenotype was not observed in the previously created *hmgR* pORI19 insertion mutant, suggesting that the original mutant exhibited some read-through of the gene, producing a functional protein. This was confirmed by RT-PCR (Fig. 2b).

When grown in BHI broth the Δ*hmgR* mutant was capable of growing to an initial OD_{620} of approximately 0.2, after which it is likely that the intracellular stores of mevalonate have been utilized and the growth rate is arrested (Fig. 2c). To determine the minimum levels of mevalonate required to fully restore growth varying concentrations were used as illustrated in Fig. 2(c), and 200 and 300 μM mevalonate was sufficient to restore growth rates to wild-type levels. However, at these concentrations a smaller colony morphology was observed as compared with a 1 mM supplementation.

As the Δ*hmgR* mutant demonstrated an initial growth in BHI broth, we further investigated whether full growth could be restored following delayed mevalonate addition over a range of time points (Fig. 2d). We showed that full restoration of growth was possible even when mevalonate was added after 6 and 10 h. Plate counts were performed, which confirmed that the cells remained viable following cessation of growth. In the absence of added mevalonate, cell numbers increased by 1 log unit up to OD ~0.2, after which numbers remained static. Cell numbers increased by an additional 2 log units when mevalonate was added. A similar cessation of growth of the *hmgR* mutant was also observed in LB broth and *Listeria* defined medium (data not shown).

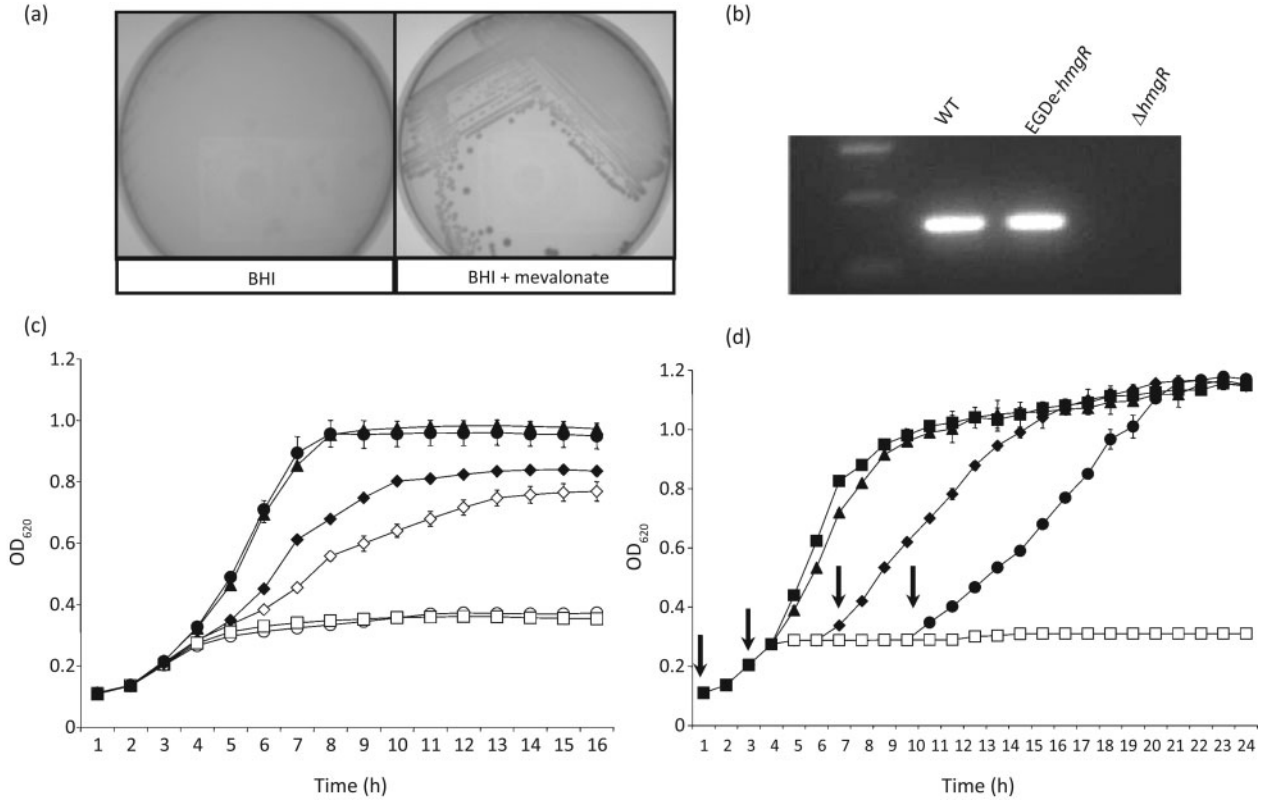

Fig. 2. Growth characteristics of the mevalonate *hmgR* deletion mutant. (a) Comparison of growth on BHI agar and BHI agar supplemented with mevalonate (1 mM). (b) Transcriptional analysis of *hmgR* wild-type (WT), plasmid insertion mutant and deletion mutant by RT-PCR. PCRs were performed using *hmgR* primers (Table 2). (c) Growth analysis of the Δ*hmgR* mutant at various concentrations of mevalonate. Overnight cultures were washed and inoculated at 2 % into fresh medium containing a range of mevalonate concentrations from 8 to 300 μM: BHI alone (□), 8 μM (○), 50 μM (◇), 100 μM (◆), 200 μM (▲), 300 μM (●). Growth was measured as OD_{620} on a Multiskan plate reader. (d) Overnight cultures containing 1 mM mevalonate were washed three times and diluted 1 : 100 into fresh medium with and without mevalonate. Mevalonate was then added at time 0 (■), and after 2 h (▲), 6 h (◆) and 10 h (●) growth; control growth with BHI alone (□) was also measured. Arrows represent the addition of mevalonate at the different time points. Error bars, SD from three independent experiments.

The essentiality of the mevalonate-dependent *hmgR* gene

In order to confirm the essentiality of *hmgR* we employed the pIMK4 plasmid recently developed in our laboratory (Monk *et al.*, 2008a), in which the gene under investigation is placed under the control of an IPTG-inducible promoter to complement the deletion (see Methods). A promoterless copy of *hmgR* was cloned into pIMK4 and then transformed into EGDeΔ*hmgR*. This IPTG-inducible copy of *hmgR* on pIMK4 was integrated into the $tRNA^{Arg}$ locus on the chromosome to create EGDeΔ*hmgR*::pIMK4*hmgR*. Addition of IPTG to Δ*hmgR*::pIMK4*hmgR* restored growth levels close to wild-type (Fig. 3), suggesting that the gene is essential for full wild-type levels of growth in the absence of mevalonate. However, residual growth was observed in the non-induced EGDeΔ*hmgR*::pIMK4*hmgR* (i.e. no IPTG addition). Overall the analysis demonstrates that the gene is essential for growth, as complementation restores growth in the absence of mevalonate.

As a further control, a pIMK4 construct of another *L. monocytogenes* essential gene, *ezrA* (EGDeΔ*ezrA*::pIMK4*ezrA*) (Considine *et al.*, 2011), was included in experiments. The growth of this strain was more tightly controlled by IPTG, confirming that pIMK4 is not leaky for all genes and that our experimental conditions were accurate (data not shown). We suggest that the IPTG promoter may allow sufficient transcription of *hmgR* to permit IPP biosynthesis, whereas the same level of transcription is not adequate in the case of the *ezrA* gene. This is possible, given that EzrA (involved in cell division) is most likely required at higher levels than HmgR. Further work would be necessary to determine the exact levels of HmgR required for activity in *L. monocytogenes*.

Effect of cell wall stresses

As isoprenoids are involved in the synthesis of cell wall and membrane components, we also investigated whether mutants were affected in their responses to various cell envelope

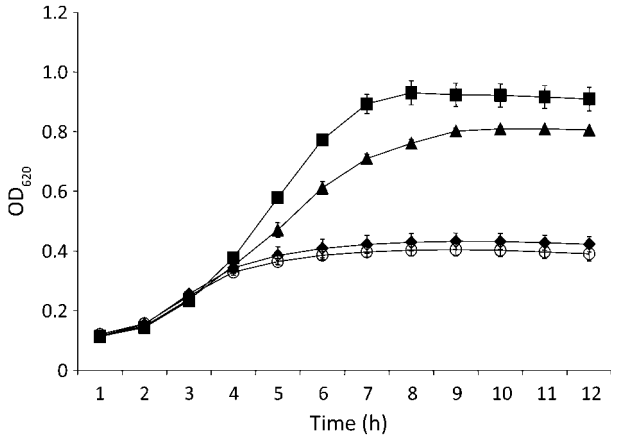

Fig. 3. Growth analysis of the Δ*hmgR* mutant and IPTG-inducible strain. Overnight cultures were washed and resuspended in fresh BHI broth and inoculated at 2% into BHI: Δ*hmgR* (◆), pIMK4::Δ*hmgR* (○), EGDeΔ*hmgR*::pIMK4*hmgR* (▲); or inoculated into BHI/1 mM IPTG (EGDeΔ*hmgR*::pIMK4*hmgR*; ■). Growth was measured as OD_{620} on a Multiskan plate reader. The graph shown is representative of three independent experiments.

stresses. Autolysis assays using Triton X-100 showed that the Δ*hmgR* mutant exhibited the most severe decrease from initial absorbance as compared with the wild-type, Δ*gcpE* also showed a significant decrease when compared with the wild-type (Fig. S1).

Murine experiments

Through the creation of a deletion mutant we have demonstrated that the *hmgR* gene (mevalonate pathway) is essential for cell growth in *L. monocytogenes* in the absence of exogenous mevalonate. In contrast, the MEP pathway deletion mutants Δ*gcpE* and Δ*lytB* showed no difference in growth characteristics compared with the parent strain *L. monocytogenes* EGDe under the conditions tested (BHI broth at 37 °C; data not shown). To allow us to analyse our suite of deletion mutants during murine infection we tagged each of the deletion mutants with a separate genetic marker. Tagging was achieved using the pIMC3 tagging vector constructed by Monk *et al.* (2008b) (see Methods). Δ*hmgR*, Δ*gcpE* and Δ*lytB* were rendered resistant to Tet, Ery and Kan, respectively.

To determine whether the MEP or mevalonate mutants were impaired in their relative *in vivo* growth and survival or virulence potential in a single host, the following murine studies were performed. Oral inoculations were used to analyse the potential role of both pathways in the gastrointestinal stages of infection (Fig. 4a). Intraperitoneal infections were also performed to assess the systemic phase of infection in isolation (Fig. 4b). These competition studies were performed by co-inoculating the mice with a 1:1:1:1 ratio of wild-type to each of the three mutants (see Methods). The number of bacteria in the livers and spleens was determined 3 days post-infection. Both the Δ*hmgR* (mevalonate pathway) and Δ*gcpE* (MEP pathway) mutants showed a significant difference when compared with the wild-type, as determined by Student's *t* test (Fig. 4a, b). Δ*hmgR* was not recovered from the livers and spleens, indicating that the levels of mevalonate present *in vivo* are not sufficient to sustain growth. Gastrointestinal persistence was determined by monitoring the faecal carriage at specified time points, but no difference was observed between mutants and wild-type (data not shown).

Previous findings by Begley *et al.* (2008) indicated that a *gcpE* plasmid disruption mutant is impaired in virulence potential compared with the wild-type. In our competition studies we also observed a significant virulence defect. This

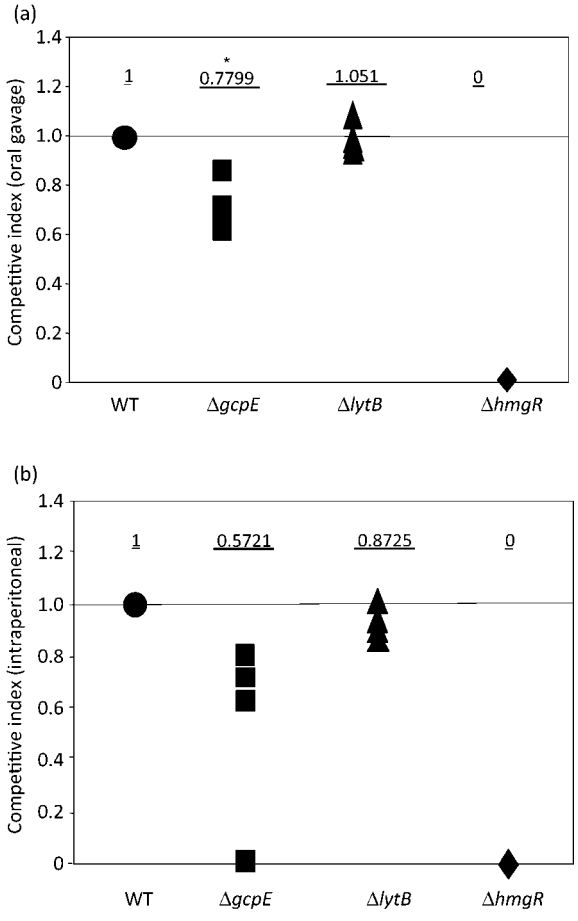

Fig. 4. Effect of Δ*hmgR*, Δ*gcpE* and Δ*lytB* on *in vivo* survival. Mice were co-inoculated via oral gavage (a) in a 1:1:1:1 ratio of each strain containing a total initial inoculum of 2×10^9 per 200 μl and via intraperitoneal infection (b) in a 1:1:1:1 ratio of each strain containing a total initial inoculum of 4×10^4 per 200 μl. Competitive indices were determined for the spleen 3 days post-infection. A competitive index value below 1 indicates that the mutant strain was outcompeted by the wild-type strain. Underlined numbers state the mean for each dataset. Asterisk, $P<0.05$.

virulence defect was verified in repeat experiments using a complemented Δ*gcpE* mutant (Fig. S2).

Deletion mutants confirm a role for the MEP pathway in Vγ9Vδ2 T cell stimulation

We have previously shown a role for an intermediate in the MEP pathway (HMB-PP) as a stimulant of Vγ9Vδ2 T cells (Begley *et al.*, 2004). We used the clean deletion mutants created as part of the current study to re-examine this phenomenon. Mutants were analysed for their ability to stimulate a Vγ9Vδ2 T cell response (Fig. S3). Results were similar to those obtained previously with *gcpE* and *lytB* pORI19 insertion mutants (Begley *et al.*, 2004) in that the Δ*gcpE* mutant failed to stimulate Vγ9Vδ2 T cells, whilst the Δ*lytB* mutant activated this T cell subset to an extent similar to pure HMB-PP. The Δ*hmgR* mutant was not examined in this assay as it failed to survive under the assay conditions. Overall these data suggest that the *L. monocytogenes* MEP pathway is also functional, at least to the extent that it produces the penultimate compound HMB-PP, and that HMB-PP is subsequently metabolized (Begley *et al.*, 2004).

DISCUSSION

Analysis of the genome of *L. monocytogenes* serotype 1/2a strain EGDe (Glaser *et al.*, 2001) revealed that *L. monocytogenes* possesses a complete set of genes for both pathways of isoprenoid biosynthesis (Begley *et al.*, 2004). Whilst it is predicted that both pathways are active, we have not previously been able to generate deletion mutants in both isoprenoid pathways. The aim of the current study was to utilize new molecular tools (Monk *et al.*, 2008a) to construct stable deletion mutants in both pathways of isoprenoid biosynthesis. This approach revealed that the *hmgR* gene, encoding an enzyme in the mevalonate pathway, is essential in *L. monocytogenes*, despite the presence of an intact MEP pathway in this strain.

We utilized new molecular tools (Monk *et al.*, 2008a) to successfully create a suite of *L. monocytogenes* mutants in both the MEP pathway (Δ*gcpE* and Δ*lytB*) and the mevalonate pathway (Δ*hmgR*). In order to create the Δ*hmgR* clean deletion mutant it was necessary to supplement the growth medium with exogenous mevalonate. Wilding *et al.* (2000a, b) also found that genetic disruption of mevalonate pathway genes in *Streptococcus pneumoniae* and *Staphylococcus aureus* was only possible in the presence of exogenous mevalonate. However, both *Strep. pneumoniae* and *Staph. aureus* lack the alternative MEP pathway, which we had previously believed to be functionally active in supporting isoprenoid biosynthesis and growth of *L. monocytogenes* (Begley *et al.*, 2004, 2008). Through the creation of Δ*hmgR* we can now postulate that the MEP pathway alone cannot produce sufficient quantities of isoprenoids for growth under standard laboratory conditions (BHI at 37 °C). As a growth defect was not observed for the MEP pathway mutants (data not shown), we suggest that the mevalonate pathway is the primary pathway required by *L. monocytogenes* for isoprenoid biosynthesis under the specified conditions tested. In the absence of exogenous mevalonate, mutant cells do not grow within the medium, but they remain viable as demonstrated by plate counts recovered on BHI agar containing mevalonate. In addition, exogenous mevalonate can 'rescue' the growth defect, even if it is not added for 10 h following subculturing into mevalonate-free media.

In our study *hmgR* was clearly essential for growth of *L. monocytogenes*, as the Δ*hmgR* mutant was only capable of growth either in the presence of mevalonate or when the *hmgR* gene was provided *in trans*. We employed the plasmid pIMK4 to provide the *hmgR* gene under the influence of an IPTG-inducible promoter (Monk *et al.*, 2008a). In our laboratory we have previously used this system to create mutants in the essential genes *secA* (Monk *et al.*, 2008a) and *ezrA* (Considine *et al.*, 2011). Both of those constructs required induction of the essential gene with IPTG to allow growth. However, our *hmgR* construct was able to grow in BHI broth without added IPTG, albeit to a lower extent than when IPTG was present. Our results are similar to those observed by Dubail *et al.* (2006), who reported the first essential gene deletion in *L. monocytogenes* EGDe but found that there was sufficient gene expression (of *lmo2537*) from the IPTG promoter to support growth in the absence of the inducer. This demonstrates an essential requirement for *hmgR* in *L. monocytogenes* EGDe for *in vitro* growth, and suggests that low expression of the gene is sufficient to support growth.

The creation of deletions in genes encoding enzymes of the two isoprenoid biosynthetic pathways provided a set of mutants with which to analyse the contribution of the specific loci to virulence potential. We appreciate that the Δ*hmgR* mutant is likely to be highly sensitive to complex environments that are low in mevalonate. Indeed, murine infection studies with the *hmgR* deletion mutant revealed that it could not be recovered from the livers and spleens at 3 days post-infection, following either the oral or the intraperitoneal route of infection. The results obtained from this analysis indicate that the *in vivo* levels of mevalonate are not sufficient to sustain growth. Although the plasma mevalonate concentration in mice is not known, the concentration in rats ranges from 0.02 to 0.08 μM and in humans from 0.08 to 0.50 μM (Popják *et al.*, 1979). We now know that these levels are well below the required concentrations to support growth of the Δ*hmgR* mutant.

In a previous study we analysed plasmid insertion mutants in isoprenoid pathway genes and demonstrated a role for the MEP pathway in virulence (Begley *et al.*, 2008). Here, we confirm that Δ*gcpE* is significantly impaired in virulence potential, though we did not detect a virulence defect in our Δ*lytB* mutant. A recent independent study by Schauer *et al.* (2010) illustrated that another gene of the MEP pathway (*lmo0190*) is essential for virulence in *L. monocytogenes*. In

addition, a *gcpE* transposon mutant of *Mycobacterium tuberculosis* was shown to exhibit decreased colonization ability in the liver and intestine of mice (Shin *et al.*, 2006). Further studies are needed to pinpoint the exact role that GcpE plays in the pathogenesis of *L. monocytogenes*. Toledo-Arana *et al.* (2009) recently used tiling arrays to investigate the transcription profile of *L. monocytogenes* EGDe under *in vivo* and *in vitro* conditions. Our analysis of the supplementary data provided with that study determined that in human blood, the *gcpE* gene was upregulated compared with the reference condition (BHI, 37 °C), although the majority of other MEP and mevalonate genes were downregulated.

Previous studies by our group characterized the immune stimulatory capacity of plasmid insertion mutants of *gcpE* and *lytB* (Begley *et al.*, 2004). Here we confirm our previous findings using the deletion mutants and demonstrate that a mutation in *lytB* leads to an increase in the stimulation of Vγ9Vδ2, most likely due to an increased accumulation of HMB-PP (Eberl *et al.*, 2002). Mutation of *gcpE* resulted in a mutant that failed to stimulate Vγ9Vδ2 T cell activation. This result fits with our suggested model that the MEP pathway has an immune stimulatory function and is active in *L. monocytogenes*. This Vγ9Vδ2 T cell stimulatory capacity has been observed in other pathogens, such as *Pseudomonas*, *Salmonella*, *Plasmodium* and *Mycobacterium*, all of which possess the MEP pathway (Behr *et al.*, 1996; Eberl *et al.*, 2003; Hara *et al.*, 1992; Kabelitz *et al.*, 1991; Davey *et al.*, 2011).

In conclusion, through the generation of deletion mutants in representative genes of the mevalonate (*hmgR*) and MEP (*lytB* and *gcpE*) pathways of isoprenoid biosynthesis in *L. monocytogenes*, we have demonstrated that HmgR is essential for growth of the organism. Significantly, the MEP pathway, which was previously assumed to provide an alternative mechanism for isoprenoid biosynthesis, is insufficient to support growth of the organism in the absence of HmgR. Further work will be necessary to examine the energetics of both pathways, and to elucidate their precise role in isoprenoid biosynthesis in this important human pathogen. The current study highlights the possibility of targeting the classical mevalonate pathway *hmgR* gene by novel antibiotics, a possible valid target not only for pathogenic *Listeria* but also for *Staphylococcus* and *Streptococcus* infections.

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