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Analysis of the Isoprenoid Biosynthesis Pathways in *Listeria monocytogenes* Reveals a Role for the Alternative 2-C-Methyl-D-Erythritol 4-Phosphate Pathway in Murine Infection[▽]

Máire Begley,[1,2,3]† Peter A. Bron,[1]† Sinead Heuston,[1,2] Pat G. Casey,[1,2] Nadine Englert,[4] Jochen Wiesner,[4] Hassan Jomaa,[4] Cormac G. M. Gahan,[1,2,5]* and Colin Hill[1,2]

Alimentary Pharmabiotic Centre,[1] Department of Microbiology, University College Cork,[2] and Teagasc Dairy Products Research Centre, Moorepark, Fermoy County Cork,[3] Cork, Ireland; Institut für Klinische Chemie und Pathobiochemie, Justus-Liebig-Universität Giessen, Giessen, Germany[4]; and School of Pharmacy, University College Cork, Cork, Ireland[5]

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Most bacteria synthesize isoprenoids through one of two essential pathways which provide the basic building block, isopentyl diphosphate (IPP): either the classical mevalonate pathway or the alternative non-mevalonate 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. However, postgenomic analyses of the *Listeria monocytogenes* genome revealed that this pathogen possesses the genetic capacity to produce the complete set of enzymes involved in both pathways. The nonpathogenic species *Listeria innocua* naturally lacks the last two genes (*gcpE* and *lytB*) of the MEP pathway, and bioinformatic analyses strongly suggest that the genes have been lost through evolution. In the present study we show that heterologous expression of *gcpE* and *lytB* in *L. innocua* can functionally restore the MEP pathway in this organism and confer on it the ability to induce Vγ9Vδ2 T cells. We have previously confirmed that both pathways are functional in *L. monocytogenes* and can provide sufficient IPP for normal growth in laboratory media (M. Begley, C. G. Gahan, A. K. Kollas, M. Hintz, C. Hill, H. Jomaa, and M. Eberl, FEBS Lett. 561:99–104, 2004). Here we describe a targeted mutagenesis strategy to create a double pathway mutant in *L. monocytogenes* which cannot grow in the absence of exogenously provided mevalonate, confirming the requirement for at least one intact pathway for growth. In addition, murine studies revealed that mutants lacking the MEP pathway were impaired in virulence relative to the parent strain during intraperitoneal infection, while mutants lacking the classical mevalonate pathway were not impaired in virulence potential. In vivo bioluminescence imaging also confirmed in vivo expression of the *gcpE* gene (MEP pathway) during murine infection.

To date, more than 30,000 isoprenoid molecules are known and have been shown to be involved in diverse processes. Well-known examples include the sterols of eukaryotes, which act as membrane stabilizers and are precursors of bile acids, carotenoids, which form part of the photosynthetic machinery of plants, and bactoprenols, which act as carbohydrate carriers in the biosynthesis of bacterial peptidoglycan (4).

Despite their diversity of structure and function, all isoprenoids derive from the five-carbon building unit isopentyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP). IPP and DMAPP are synthesized by one of two distinct pathways: either the classical mevalonate pathway or the alternative non-mevalonate 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. These pathways have been the focus of several elegant studies in recent years, and the enzymes involved have been elucidated (4, 17, 27). Biochemical studies have revealed that an intermediate of the MEP pathway, namely, (*E*)-4-hydroxy-3-methyl-*but*-2-enyl pyrophosphate (HMB-PP), is a potent activator of human Vγ9Vδ2 T cells (1, 2, 9, 15, 26). Elevated levels of this subset of T cells in the peripheral blood of patients have been documented in a variety of microbial infections, such as salmonellosis, listeriosis, brucellosis, and tuberculosis (7, 19). Their precise role is unknown, but it has been postulated that they play a role in clearance and protective immunity or may be involved in immune evasion of the pathogen and establishment of chronic disease (10). As humans use only the mevalonate pathway, the MEP pathway is considered a novel target for the development of antibacterial drugs (18, 28).

Analysis of the bacterial genomes sequenced to date reveals that the gram-positive pathogen *Listeria monocytogenes* and certain *Streptomyces* species are unusual in containing genes for both pathways. In all other organisms examined thus far these pathways are mutually exclusive (reviewed in reference 4). *Listeria innocua*, which is closely related to *L. monocytogenes* but is nonpathogenic, possesses the first five of the seven genes of the MEP pathway but lacks genes encoding the final two enzymes (GcpE and LytB). In the current communication we describe our in depth in silico analyses of the *gcpE* and *lytB* genomic regions in both *Listeria* species and the possibility of restoring the MEP pathway in *L. innocua*, and the effect on Vγ9Vδ2 T-cell activation is examined. The essentiality of isoprenoid biosynthesis pathways in *L. monocytogenes* is investigated through the creation of a double pathway mutant. Finally, the contribution of the two pathways to the pathogenesis of

* Corresponding author. Mailing address: Department of Microbiology, University College Cork, Cork, Ireland. Phone: 353-214901363. Fax: 353-214903101. E-mail: c.gahan@ucc.ie.

† M.B. and P.A.B. contributed equally to this work.

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L. monocytogenes is examined using the mouse model of infection.

MATERIALS AND METHODS

Bioinformatic analyses. The following genome sequences were accessed through the National Center for Biotechnology Information (NCBI) Genome database (http:/www.ncbi.nlm.nih.gov/): *L. monocytogenes* EGDe (accession number NC_003210), *L. monocytogenes* 10403S (accession number NZ_AARZ00000000), *L. monocytogenes* strain 4b F2365 (accession number NC_002973), *L. monocytogenes* strain 1/2a F6854 (accession number NZ_AADQ00000000), *L. monocytogenes* strain 4b H7858 (accession number NZ_AADR00000000), *L. monocytogenes* HPB2262 (accession number NZ_AATL00000000), *L. monocytogenes* J2818 (accession number NZ_AARX00000000), *L. monocytogenes* F6900 (accession number NZ_AARU00000000), *L. monocytogenes* FSL N3-165 (accession number NZ_AARQ00000000), *L. monocytogenes* LO28 (accession number NZ_AARY00000000), *L. innocua* CLIP11262 (accession number NC_003212), and *Bacillus subtilis* subsp. *subtilis* strain 168 (accession number NC_000964). Sequences were analyzed using various Web-based programs by using the ExPASy server (http://www.expasy.ch/tools/). The Web-based program δρ-Web was used for compositional bias of dinucleotide frequency analysis (http://deltarho.amc.nl). Genomic dissimilarity (δ*) values were calculated. A high δ* value between an input sequence and the host genome sequence indicates a heterologous origin of the input sequence. Predicted promoter regions were analyzed using BPROM (http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). Alignments were performed with CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw/index.html). Phylogenetic and molecular evolutionary analyses were conducted using MEGA4 (http://www.megasoftware.net/). Phylogenetic trees were constructed using neighbor-joining algorithms, and bootstrap analyses were performed with 1,000 replicates.

Bacterial strains, media, and growth conditions. The strains, plasmids, and primers used in this study are listed in Table 1. *Escherichia coli* strain DH10B (Invitrogen, Paisley, United Kingdom) and *Bacillus subtilis* 168 (20) were grown aerobically at 37°C in Luria-Bertani medium (29). *Listeria* strains were grown aerobically at 37°C in brain heart infusion (BHI) medium (Oxoid, Basingstoke, United Kingdom). When appropriate, antibiotics were added to the medium: for *E. coli*, chloramphenicol (15 μg/ml) and erythromycin (250 μg/ml); for *L. monocytogenes*, chloramphenicol (7.5 μg/ml) and erythromycin (5 μg/ml); for *L. innocua*, chloramphenicol (7.5 μg/ml).

Molecular techniques. Plasmid DNA was isolated from *E. coli* using the QIAprep Spin Miniprep kit according to the manufacturer's instructions (Qiagen, Crawley, United Kingdom). Genomic DNA was isolated from *L. monocytogenes* EGDe and *B. subtilis* 168 using the Genelute bacterial genomic DNA kit (Sigma, Steinheim, Germany) following the recommendations of the manufacturer. Primers were purchased from Sigma Genosys (Haverhill, United Kingdom). PCR products required for cloning were obtained with KOD hot start high-fidelity DNA polymerase (Merck, Nottingham, United Kingdom) using 10 ng genomic or plasmid DNA as template. Standard procedures were applied for DNA manipulations in *E. coli* (29). Restriction endonucleases (Roche Diagnostics, Mannheim, Germany), T4 DNA ligase (Roche), and 2× PCR mix (Promega, Madison, WI) were used following the recommendations of the manufacturers. Transformations of *L. monocytogenes* and *L. innocua* were performed as described previously (25).

Plasmid, strain, and mutant construction. A mutant in which 0.9 kb of the coding region of lmo1441 (*gcpE*) was deleted from *L. monocytogenes* EGDe was created using a construct generated by the splicing by overlap extension (SOE) procedure (16). Primer pairs were designed (lmo1441SOEA-lmo1441SOEB and lmo1441SOEC-lmo1441SOED) to amplify 0.35-kb products flanking the lmo1441 (*gcpE*) gene. The resulting products were mixed in a 1:1 ratio and reamplified using primer lmo1441SOEA and lmo1441SOED. The resulting 0.7-kb product was digested with PstI and XbaI (restriction sites introduced on the lmo1441SOEA and lmo1441SOED primers) and cloned into the temperature-sensitive plasmid pKSV7 (32), previously digested with the same restriction enzymes. The resulting plasmid was designated pKSV7-lmo1441 and was electroporated into *L. monocytogenes* EGDe at 30°C. Chromosomal integration of the plasmid was established by overnight growth at 42°C in BHI broth containing chloramphenicol and spread plating onto BHI agar containing chloramphenicol. Single colonies were selected, and plasmid excision and curing were brought about by continuous passaging at 30°C in BHI broth without chloramphenicol, followed by spread plating onto BHI plates. Chloramphenicol-sensitive colonies were identified and a PCR, using template DNA obtained directly from the colonies by a microwave treatment (3 min, 600 W) and the primer pair lmo1441out and lmo1441outII confirmed the deletion event. One colony was selected for further analysis and was designated EGDeΔ*gcpE*.

To create a double pathway mutant in which both the MEP and the mevalonate pathways are blocked, a similar SOE procedure (16) was employed to replace 1.2 kb of the coding region of lmo0825 (*hmgR*) in EGDeΔ*gcpE* with the erythromycin resistance gene (*ery*). The primer pairs lmo0825A-lmo0825B and lmo0825C-lmo0825D were used to amplify 0.4-kb flanking regions of lmo0825, and the primer pair eryF-eryR was used to amplify a 1.1-kb fragment harboring *ery* from pRV300 (23) template DNA. The three PCR products were mixed and reamplified using the primer pair lmo0825A and lmo0825D. The resulting 1.9-kb fragment was digested with XbaI-HindIII and cloned into similarly digested pKSV7. The resulting plasmid was designated pKSV7-lmo0825*ery* and transformed into EGDeΔ*gcpE*. Plasmid integration, excision, and curing were established as described above. However, erythromycin was added to all media during these procedures, allowing direct selection against plasmid excision, resulting in wild-type chromosomal organization. Moreover, mevalonate was added at all times, providing a route to synthesize the essential molecule IPP in the double pathway mutant. Candidate mutants were tested for their chloramphenicol phenotype, and sensitive colonies were checked directly in a PCR, using the primer pair lmo0825out and lmo0825outII. One colony for which the PCR confirmed the replacement event was designated EGDe-Δ*hmgR*Δ*gcpE*.

The *L. monocytogenes* double pathway mutant was complemented using the listerial site-specific integration vector pPL2a (5, 22). lmo0825 was amplified from *L. monocytogenes* EGDe chromosomal DNA using the primer pair 0825F-0825R. The 1.5-kb amplicon was digested with XbaI-SalI and ligated to similarly digested pPL2a. The resulting plasmid was designated pPL2-lmo0825 and was transformed into EGDe-Δ*hmgR*Δ*gcpE*. Chromosomal integration of the plasmid was confirmed by a colony PCR using the primer pair PL95-PL102 (22).

The luciferase-based reporter system pPL2*lux* (5) was utilized to investigate whether the lmo0010 (*mvk*), lmo0825 (*hmgR*), lmo1441 (*gcpE*), and lmo1451 (*lytB*) genes are expressed during infection. Chromosomal DNA from *L. monocytogenes* EGDe was used as template in PCRs using the primer pairs lmo0010luxF and -R, lmo0825F and -R, lmo1441F and -R, and lmo1451F and -R (Table 1). The resulting PCR products, harboring the 0.5-kb promoter regions immediately upstream of the corresponding genes, were digested with XhoI and cloned into SwaI-SalI-digested pPL2*lux*, as described previously (5). The resulting plasmids (Table 1) harbor exact translational fusions of the promoter regions to the *luxABCDE* operon, allowing analysis of promoter activities by monitoring bioluminescence levels. Subsequently, the pPL2*lux* derivatives were transformed into *L. monocytogenes* EGDe, and candidate integrants were checked for site-specific integration by colony PCR, using the primer pair PL95-PL102. From each transformation, one colony possessing the anticipated genotype was selected for further analysis (Table 1).

The *gcpE* and *lytB* homologues of *B. subtilis* 168 were introduced into *L. innocua* using plasmid pNZ44 (24). Chromosomal DNA of *B. subtilis* 168 and the primer pairs gcpEF-gcpER and lytBF-lytBR were used to amplify 1.2- and 1.1-kb fragments harboring BG11671 (*gcpE* homologue) and BG11662 (*lytB* homologue), respectively. Firstly, pNZ44 was digested with Ecl136II and blunt end ligated with the BG11671 amplicon. The ligation mixtures were digested with Ecl136II prior to transformation to *E. coli* DH10b (Invitrogen) to reduce the number of transformants harboring back-ligated vector DNA. After 40 h, full-grown colonies were used directly as templates in a PCR using the primer pair P44F-gcpER. This PCR generates a product only when an insert is cloned in the orientation that allows transcription of BG11671 through the constitutive, lactococcal P44 promoter. One clone that generated the expected amplicon was designated pNZ44-*gcpE*. This plasmid was digested with KpnI and ligated to the BG11662 PCR product previously digested with the same enzyme. The appropriate orientation in which the BG11662-BG11671 transcription is driven from the P44 promoter was selected for by a PCR strategy using the primer pair P44F-lytBR, yielding pNZ44-*lytB-gcpE*. Both pNZ44-*gcpE* and pNZ44-*lytB-gcpE* were electroporated into *L. innocua* (Table 1).

Growth curves. Growth of the double pathway mutant in the presence and absence of mevalonate was investigated. A stock solution of 1 M mevalonate was prepared by hydrolyzing DL-mevalonolactone (M4667; Sigma Aldrich, Dublin, Ireland) with 1 M NaOH at 37°C for 1 h (9). Mevalonate at 1 mM was added to broth when required. The cells were pelleted by centrifugation (7,000 × *g* for 5 min), washed three times with phosphate-buffered saline (PBS), and diluted 1:100 in fresh BHI medium with or without mevalonate. Subsequently, growth at 37°C was monitored over time in triplicate for all strains, using flat-bottom 96-well plates (Sarstedt, Newton, MA), and measurements of the optical density at 600 nm (OD_{600}) in a Genios microtiter plate reader (Tecan, Salzburg, Austria).

Murine infection studies. Overnight cultures of the *L. monocytogenes* strains were pelleted by centrifugation (7,000 × *g* for 5 min), washed twice with PBS, and used to inoculate 8- to 12-week-old BALB/c mice intraperitoneally with 2 ×

TABLE 1. Strains, plasmids and primers used in this study and their relevant characteristics

Strain, plasmid, or primer	Relevant feature[a]	Source or reference
Strains		
E. coli DH10B	Cloning host	Invitrogen
L. monocytogenes strains		
EGDe	Wild type of serotype 1/2a, for which the genome sequence is available	
EGDe *mvk*	Ery^r, *L. monocytogenes* EGDe harboring a disrupted copy of lmo0010	3
EGDe *hmgR*	Ery^r, *L. monocytogenes* EGDe harboring a disrupted copy of lmo0825	3
EGDe-*gcpE*	Ery^r, *L. monocytogenes* EGDe harboring a disrupted copy of lmo1441	3
EGDe-*lytB*	Ery^r, *L. monocytogenes* EGDe harboring a disrupted copy of lmo1451	3
EGDe Δ*gcpE*	*L. monocytogenes* EGDe harboring a clean deletion of lmo1441 (*gcpE*)	This study
EGDe Δ*hmgR*Δ*gcpE*	Ery^r, EGDe Δ*gcpE* with a chromosomal replacement of lmo0825 (*hmgR*) by *ery*	This study
EGDe Δ*hmgR*Δ*gcpE*::pPL2-lmo0825	Ery^r Cm^r, EGDe Δ*hmgR*Δ*gcpE* containing pPL2-lmo0825 chromosomally integrated at tRNA^{Arg} locus	This study
EGDe::pPL2*lux*-$P_{lmo0010}$	Cm^r, contains pPL2*lux*-$P_{lmo0010}$ integrated at tRNA^{Arg} locus on the *L. monocytogenes* EGDe chromosome	This study
EGDe::pPL2*lux*-$P_{lmo0825}$	Cm^r, contains pPL2*lux*-$P_{lmo0825}$ integrated at tRNA^{Arg} locus on the *L. monocytogenes* EGDe chromosome	This study
EGDe::pPL2*lux*-$P_{lmo1441}$	Cm^r, contains pPL2*lux*-$P_{lmo1441}$ integrated at tRNA^{Arg} locus on the *L. monocytogenes* EGDe chromosome	This study
EGDe::pPL2*lux*-$P_{lmo1451}$	Cm^r, contains pPL2*lux*-$P_{lmo1451}$ integrated at tRNA^{Arg} locus on the *L. monocytogenes* EGDe chromosome	This study
B. subtilis 168		20
L. innocua strains		
APC	Wild type	3
pNZ44	Cm^r, *L. innocua* APC harboring pNZ44	This study
pNZ44-*gcpE*	Cm^r, *L. innocua* APC harboring pNZ44-*gcpE*	This study
pNZ44-*lytB-gcpE*	Cm^r, *L. innocua* APC harboring pNZ44-*lytB-gcpE*	This study
Plasmids		
pKSV7	Cm^r, temperature-sensitive integration vector used routinely to construct *L. monocytogenes* deletion mutants	32
pKSV7-lmo1441	Cm^r, pKSV7 harboring flanking regions of lmo1441	This study
pRV300	Ery^r	23
pKSV7-lmo0825*ery*	Ery^r Cm^r, pKSV7 harboring *ery* derived from pRV300 and flanking regions of lmo0825	This study
pPL2a	Cm^r, listerial integration vector	22
pPL2-lmo0825	Cm^r, pPL2a harboring the promoter and coding region of lmo0825	This study
pNZ44	Cm^r, expression vector for obtaining the lactococcal P44 promoter	24
pNZ44-*gcpE*	Cm^r, pNZ44 harboring *B. subtilis* 168 *gcpE* homologue	This study
pNZ44-*lytB-gcpE*	Cm^r, pNZ44-*gcpE* harboring *B. subtilis* 168 *lytB* homologue	This study
pPL2*lux*-$P_{lmo0010}$	Cm^r, pPL2*lux* derivative containing 0.5-kb promoter region of lmo0010 translationally fused to *luxABCDE*	This study
pPL2*lux*-$P_{lmo0825}$	Cm^r, pPL2*lux* derivative containing 0.5-kb promoter region of lmo0825 translationally fused to *luxABCDE*	This study
pPL2*lux*-$P_{lmo1441}$	Cm^r, pPL2*lux* derivative containing 0.5-kb promoter region of lmo1441 translationally fused to *luxABCDE*	This study
pPL2*lux*-$P_{lmo1451}$	Cm^r, pPL2*lux* derivative containing 0.5-kb promoter region of lmo1451 translationally fused to *luxABCDE*	This study
Primers		
lmo1441 SOEA	5′-GATTTATTCTTTCTAGATTTGGCC-3′	This study
lmo1441 SOEB	5′-GCGAAATATTCTTTCATTCAAAGA-3′	This study
lmo1441 SOEC	5′-TCTTTGAATGAAAGAATATTTCGCCCGATTAAAGTAGCCGTGCTT-3′	This study
lmo1441 SOED	5′-CTACTCCTGCAGCAATACCAA-3′	This study
lmo1441out	5′-ATTCGATTGAGTCATTAGCTA-3′	This study
lmo1441outII	5′-AAATTGCGCCGCACCTGCG-3′	This study
lmo0825SOEA	5′-CTACTTTAAAAGCTTTTTGTTTTT-3′	This study
lmo0825SOEB	5′-CTGATTGGTGTATCATTTCGTTAGCATTCATTGCTTTATCACC-3′	This study
eryF	5′-AACGAAATGATACACCAATCAG −3′	This study
eryR	5′-CTCTTTAGCTCCTTGGAAGC-3′	This study
lmo0825SOEC	5′-GCTTCCAAGGAGCTAAAGAGGCAAACAATTAAACGTAGTCGCT-3′	This study
lmo0825SOED	5′-GTACTTATCTAGAATATCGCG-3′	This study
lmo0825out	5′-CACTCGAGAAGAAGCACT-3′	This study
lmo0825outII	5′-CCGGCACTTACTAATCCG-3′	This study

Continued on following page

TABLE 1—*Continued*

Strain, plasmid, or primer	Relevant feature[a]	Source or reference
0825F	5′-GGGGGTCTAGATGAAACTTTTAGAAATACGACACAC-3′	This study
0825R	5′-AAAAAGTCGACCCGAAAAGAAAAATTCCAAGTCCTCCG-3′	This study
PL95	5′-ACATAATCAGTCCAAAGTAGATGC-3′	22
PL102	5′-TATCAGACCTAACCCAAACCTTCC-3′	22
gcpEF	5′-ATTGTTATATCTAGAAGCGG-3′	This study
gcpER	5′-TTCCCGTCAAGCTTTTTGTG-3′	This study
lytBF	5′-CCGGGGTACCTACTCACTTCAGTATTGGAG-3′	This study
lytBR	5′-CCGGGGTACCGTCACGAGAAAACTTACATC-3′	This study
P44F	5′-TATGAATCGTGATGTGTGAG-3′	This study
IM109	5′-GTAAGTTGGGGATAACTCCG-3′	This study
IM110	5′-GCGCTTTACGCCCAATAAATC-3′	This study
BG11662F	5′-TTTGATCACGAAGATCCATCAAC-3′	This study
BG11671F	5′-AGTGGGACCTTTAACAATAGGC-3′	This study
BG11671R	5′-TTCGGATTTTATCTGCGCCGC-3′	This study
lmo0010luxF	5′-CCATCCAACTCGAGCAGAACGCCGTTTTATTTTAGAG-3′	This study
lmo0010luxR	5′-CAATGCACGCCAATCCTTTCTCC-3′	This study
lmo0825luxF	5′-CCATCCAACTCGAGCCCGGATGAAACTTTTAGAAATACG-3′	This study
lmo0825luxR	5′-CATTGCTTTATCACCTCAAATTAAAATTTATAC-3′	This study
lmo1441luxF	5′-CCATCCAACTCGAGGCAGCTGGTGTTTCGCAATCTG-3′	This study
lmo1441luxR	5′-CAAAGAGACCACTCCTTTAGACTAGC-3′	This study
lmo1451luxF	5′-CCATCCAACTCGAGTTGCACGTTGTTTGTCCGTACC-3′	This study
lmo1451luxR	5′-CATCCTCATTTCTGTATTAGATTC-3′	This study

[a] Ery^r, erythromycin resistant; Cm^r, chloramphenicol resistant. Underlined sequences indicate restriction sites used in subsequent cloning procedures.

10^5 CFU in 200 μl of PBS. Three days postinfection, the mice were sacrificed by cervical dislocation. Livers and spleens were homogenized in PBS, and serial dilutions were plated onto BHI agar, followed by overnight incubation at 37°C. Full-grown colonies were counted, and the results were used to determine the bacterial load in the organs. When bioluminescent strains were used, bioluminescent imaging was conducted on the individual organs using 5-min exposure times at a binning value of 4 on an IVIS 100 system (Xenogen, Alameda, CA). Murine studies were carried out according to institutional guidelines.

Macrophage assays. J774.A1 mouse macrophage cells were routinely grown in antibiotic-free Dulbecco modified Eagle medium (DMEM) plus Glutamax (Gibco) supplemented with 10% fetal calf serum (Sigma) in T75 flasks (Sarstedt) and incubated at 37°C with 5% CO_2. For infection assays, macrophage cells were grown to confluence in 24-well tissue culture plates. One ml of overnight bacterial culture was washed once in PBS and diluted 1/100 in DMEM to a final concentration of 3×10^7 CFU/ml. DMEM was removed from each tissue culture well, and 1 ml of DMEM containing bacteria was added. Plates were centrifuged at 1,500 × *g* for 10 min to increase contact between the bacteria and macrophages and incubated for 1 h in 5% CO_2 at 37°C. Gentamicin (15 μg/well; Sigma) was added for 30 min. Bacterial counts were determined at this point (*T*0) and after 6 h (*T*6) by washing the cells twice with 1 ml PBS followed by lysis using 250 μl of cold sterile distilled water containing 0.1% Triton X-100. A 100-μl aliquot was serially diluted and plated onto BHI agar plates, which were incubated at 37°C overnight. The double pathway mutant was starved of mevalonate prior to experiments by inoculating cultures which were grown overnight in BHI supplemented with mevalonate into fresh BHI without mevalonate and incubated shaking at 37°C until the OD_{600} remained steady (approximately 6 h; see Fig. 4B below). The double mutant was plated onto BHI agar supplemented with 1 mM mevalonate at all times.

RNA isolation and reverse transcription-PCR analysis. RNA was isolated from 10 ml of full-grown cultures of *L. innocua*(pNZ44), *L. innocua*(pNZ44-*gcpE*), and *L. innocua*(pNZ44-*lytB-gcpE*) using the protocol described previously (5). Subsequently, 2 μg of the isolated RNA was subjected to DNase I treatment according to the manufacturer's instructions (Ambion, Huntingdon, United Kingdom). cDNA was synthesized in a 40-μl reaction mixture containing 0.2 nmol of random hexamer primer, 10 mM dithiothreitol, 1× Expand reverse transcriptase buffer, 50 U Expand reverse transcriptase (Roche Diagnostics, Mannheim, Germany), each deoxynucleoside triphosphate at a concentration of 0.25 mM, and 1.5 μg DNase I-treated RNA. Reverse transcription was initiated for 10 min at 30°C, followed by 45 min at 42°C and inactivation of the reverse transcriptase by incubation at 95°C for 2 min. Subsequently, 1 μl of the synthesized cDNA was used in PCR mixtures containing 1× PCR mix (Promega) and 1 pmol of primer pairs (IM109-IM110 for 16S RNA, BG11662F-BG11671R for *gcpE-lytB*, and BG11671F-BG11671R for *gcpE*) (Table 1).

γδ T-cell stimulation assays. γδ T-cell stimulation assays were performed essentially as described previously (3). Low-molecular-weight (LMW) extracts were prepared by sonication of grown cultures and subsequent ultrafiltration over Centriprep 3-kDa filters (Millipore, Eschborn, Germany). The extracts were tested for stimulation of human Vγ9Vδ2 T cells in peripheral blood mononuclear cell preparations by using flow cytometry. The results are expressed as the percentage of $V\gamma9^+$ cells among total $CD3^+$ cells.

RESULTS

Bioinformatic analyses of listerial isoprenoid genes. Genes for both pathways of isoprenoid biosynthesis are present in the *L. monocytogenes* EGDe genome (Fig. 1). All genes are also present in the genomes of the other 10 *L. monocytogenes* strains examined (genomes and accession numbers are listed in Materials and Methods). Analysis of the genome of *L. innocua* revealed that this closely related but nonpathogenic species possesses all mevalonate pathway loci but has the genes encoding only the first five enzymes of the MEP pathway (1-deoxy-D-xylulose 5-phosphate [DOXP]; MEP DOXP synthase, DOXP reductoisomerase, 4-diphosphocytidyl-2-C-methyl-D-erythritol [CDP-ME] synthase, CDP-ME kinase, and 2-C-methyl-D-erythritol 2,4-cyclopyrophosphate [MEcPP] synthase). Genes encoding the final two enzymes, HMB-PP synthase (GcpE) and HMB-PP reductase (LytB), are absent from its genome (Fig. 2A).

Further in silico analysis was carried out in order to determine whether *L. monocytogenes* may have acquired the two genes or, alternatively, whether the nonpathogenic species may have deleted them. The GC content of the genes was found to be similar to the overall chromosomal content (39.76% for lmo1451 and 39.11% for lmo1441, compared to a genome average of 39%). There is no evidence of a transposon inser-

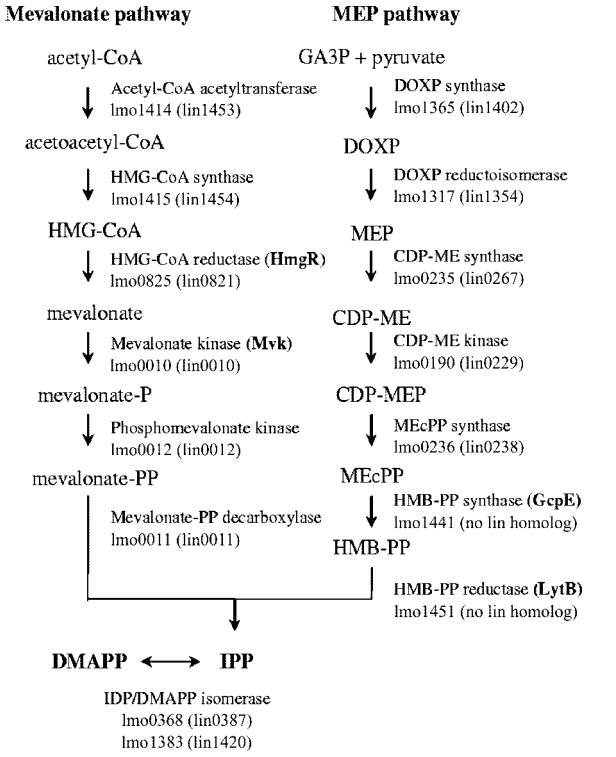

FIG. 1. Homologues of the genes involved in the classical mevalonate pathway and the alternative MEP pathway of isoprenoid biosynthesis that are present in the *L. monocytogenes* EGDe genome. Data used to generate this figure were obtained from the NCBI site (www.ncbi.nlm.nih.gov). *L. innocua* Clip 11262 gene annotation numbers are in parentheses. Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A. This figure is adapted from Begley et al. (3) with permission from the publisher.

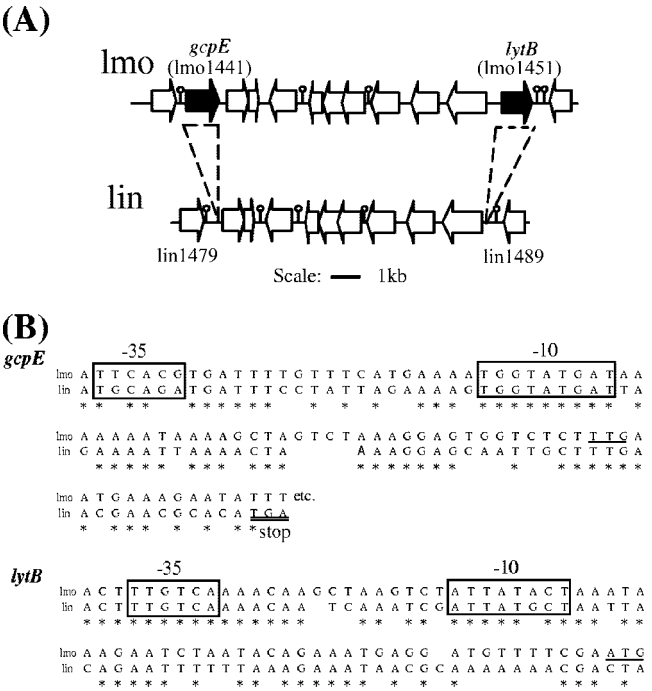

FIG. 2. (A) Genetic organization of *gcpE* and *lytB* genomic regions in *L. monocytogenes* EGDe and *L. innocua* Clip11262. Numbers refer to the NCBI annotation numbers. Arrows indicate orientations of genes. Hairpins depict putative terminators. All genes have been drawn approximately to scale. Those genes shaded in black were examined in the present study. (B) Comparison of *gcpE* and *lytB* region genomic sequences in *L. monocytogenes* EGDe and *L. innocua* Clip11262. Sequences were obtained from the Genome database on the NCBI website and aligned using ClustalW. The asterisks indicate identical nucleotides. Putative promoters were predicted with the program BPROM. The underlined nucleotides (TTG, ATG) represent predicted start codons. The double-underlined nucleotides (TGA) represent a predicted stop codon.

tion site or transposon-related repeat structures in this region of the chromosome. Genomic dissimilarity values (δ* values, calculated by δρ-Web) for both genes were low, suggesting that these genes are not likely to have been acquired by horizontal gene transfer. Furthermore, the computer-based approaches employed by Hain et al. (14) to detect genes of foreign origin in the *L. monocytogenes* genome did not designate either gene as "alien."

As no evidence of gene acquisition by *L. monocytogenes* was found, efforts focused on examining the two gene sequences and surrounding genomic regions for evidence of gene loss by *L. innocua*. Close examination of the nucleotide sequence between lin1479 and lin1480 revealed that *L. innocua* possesses the putative promoter and ribosomal binding site of *gcpE*. Vestigial sequence encoding the first 3 amino acids of GcpE were also identified; however, these codons were followed by a stop codon, and the remainder of the gene was not present (Fig. 2B). Inspection of the *lytB* region of the genome indicates that *L. innocua* still possesses the putative promoter region, as predicted by BPROM; however, none of the coding sequence could be identified (Fig. 2B). Finally, phylogenetic analyses of both *lytB* and *gcpE* across the sequenced *L. monocytogenes* strains suggest that these loci have evolved at the same rate as the native housekeeping gene *gyrB* while the same genes in *Bacillus subtilis* have significantly diverged through evolution (data not shown).

To conclude, our bioinformatics analyses provide very strong evidence that *L. innocua* has lost *gcpE* and *lytB* through evolution yet has retained the remaining five genes of the MEP pathway.

The ability to activate Vγ9Vδ2 T cells can be introduced into *L. innocua*. We have previously shown that *L. innocua* is not capable of activating Vγ9Vδ2 T cells (3). The possibility of restoring the MEP pathway and introducing the ability to activate Vγ9Vδ2 T cells was assessed by introduction of plasmid pNZ44 containing *gcpE* (pNZ44-*gcpE*) or *gcpE* together with *lytB* (pNZ44-*lytB-gcpE*). *L. innocua* was also transformed with an empty vector to serve as a control. To verify the production of the anticipated transcripts, RNA was isolated from all three strains, followed by cDNA synthesis. Control PCRs were performed with the 16S RNA primer pair and confirmed that cDNA concentrations were comparable in all samples. PCRs with *gcpE* primers generated an amplicon only when cDNA derived from the *L. innocua*(pNZ44-*gcpE*) or *L. innocua*-(pNZ44-*lytB-gcpE*) cultures was used as template, whereas the *gcpE*/*lytB* primer pair generated a PCR product only when the cDNA corresponding to *L. innocua*(pNZ44-*lytB-gcpE*) was

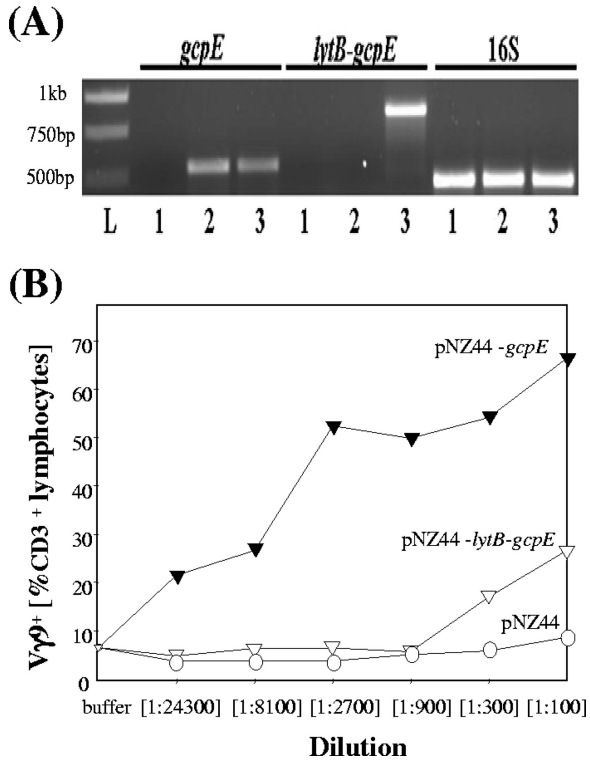

FIG. 3. Introduction of the MEP pathway into *L. innocua*. (A) *gcpE* and/or both *gcpE* and *lytB* were introduced into *L. innocua*, and proper transcription of the genes was confirmed by reverse transcription-PCR (see text for details). Lanes: L, ladder (molecular size standards); 1, *L. innocua*(pNZ44); 2, *L. innocua*(pNZ44-*gcpE*); 3, *L. innocua*(pNZ44-*lytB-gcpE*). (B) LMW extracts of strains were prepared and Vγ9Vδ2 T-cell activation was measured for different dilutions ranging from 100- to 24,300-fold. Buffer represents a background measurement to which no LMW extract was added.

used (Fig. 3A). These results demonstrate that the introduction of the pNZ44-*gcpE* and pNZ44-*lytB-gcpE* plasmids into *L. innocua* results in the functional production of a *gcpE* and *lytB-gcpE* transcript, respectively. Subsequently, the strains were tested in a Vγ9Vδ2 T-cell assay. With LMW extracts derived from the control strain *L. innocua*(pNZ44), no significant Vγ9Vδ2 T-cell activity was detected, corroborating our earlier findings which suggested that *L. innocua* is not capable of activating Vγ9Vδ2 T cells (3). Using LMW extracts isolated from *L. innocua*(pNZ44-*gcpE*), the Vγ9Vδ2 T-cell activity was even detectable at extremely high dilutions (1:2,430,000) (Fig. 3B). This experiment indirectly indicates the synthesis of very large amounts of HMB-PP and suggests the presence of a functional set of enzymes leading to the substrate of HMB-PP synthase, namely, MEcPP. The absence of HMB-PP reductase prevents the conversion of HMB-PP into IPP, resulting in HMB-PP accumulation and, as a consequence, very strong Vγ9Vδ2 T-cell activation. With the LMW extracts derived from *L. innocua*-pNZ44-*lytB-gcpE*, activity was observed which was detectable at the lower dilutions (1:10,000 and 1:30,000) (Fig. 3B). The lower activities achieved may be explained by the fact that this strain now contains a complete MEP pathway and the HMB-PP produced by HMB-PP synthase is likely to be rapidly consumed by HMB-PP reductase into IPP. It can be inferred from these results that the first set of enzymes of the MEP pathway present in *L. innocua* are functional and we can resume the discussion that the intermediates of the MEP pathway are involved in other functions beside the synthesis of isoprenoid precursors. Furthermore, these results provide proof of principle that it is feasible to reintroduce both the MEP pathway and Vγ9Vδ2 T-cell activation into a bacterium lacking a complete MEP pathway.

Creation of an *L. monocytogenes* double mutant lacking both the classical and alternative pathways. We have previously mutated two genes of each pathway in *L. monocytogenes*, namely, lmo0010 (*mvk*), lmo0825 (*hmgR*), lmo1441 (*gcpE*), and lmo1451 (*lytB*) (3). These mutants were not impaired in growth in BHI medium, suggesting that both pathways are functional and that disruption of one pathway can be complemented by the action of the other. In order to analyze the cellular requirement for both pathways in the creation of the essential compound IPP, we created a double mutant in which the two pathways were inactivated in a single strain. The temperature-sensitive plasmid pKSV7 was used to create a deletion in the lmo1441 gene of the MEP pathway (see Materials and Methods). Subsequently, the lmo0825 gene of the mevalonate pathway was deleted in this background, resulting in the double pathway mutant EGDe-Δ*hmgR*Δ*gcpE*. This double pathway mutant could be created only when mevalonate was added to the medium, and the mutant relied on an exogenous supply of mevalonate for growth at all times (Fig. 4A). Restoration of the mevalonate pathway by introduction of lmo0825 (*hmgR*) on a plasmid permitted growth in the absence of mevalonate (Fig. 4B). These results formally prove that *L. monocytogenes* requires at least one pathway of isoprenoid biosynthesis for growth under in vitro laboratory conditions and that further means of isoprenoid biosynthesis do not exist in this organism.

A double pathway mutant is mevalonate dependent. The growth characteristics of the double pathway mutant were examined in more detail in broth with and without added mevalonate. In medium without added mevalonate, the double pathway mutant was capable of moderate growth and could reach an OD_{600} of only approximately 0.2, after which growth was arrested (Fig. 4B). It is possible that intracellular stores of IPP allowed this initial growth, and in support of this hypothesis, medium inoculated with a higher dilution (1:1,000) of overnight culture did not show any measurable growth (data not shown). Although growth of the mutant was arrested, cells remained viable as determined by the ability to form colonies on agar plates. Indeed, the addition of mevalonate after 6 or 10 h after the beginning of the experiment resulted in an immediate regeneration of growth (Fig. 4B). However, it was observed that both the growth rate and final optical density reached were lower than for the mutant culture to which mevalonate had been added at the start of the experiment. Also, mevalonate addition after 10 h resulted in more pronounced growth defects compared to mevalonate addition after 6 h.

However, in the presence of mevalonate, the double pathway mutant displayed virtually identical growth characteristics as the complemented strain to which no mevalonate was added (Fig. 4B). Moreover, growth of these strains under the conditions tested was similar to the wild-type *L. monocytogenes* EGDe strain, demonstrating that either the addition of meva-

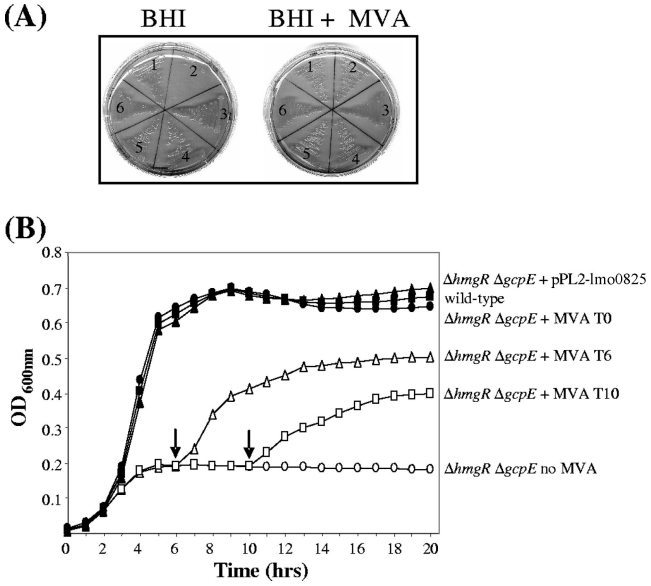

FIG. 4. (A) Growth of wild-type and mutant strains on BHI agar and BHI agar supplemented with 1 mM mevalonate (MVA). Quadrants: 1, wild type; 2, EGDe Δ*hmgR*Δ*gcpE* (double mutant); 3, EGDe *hmgR* mutant; 4, EGDe *mvk* mutant; 5, EGDe *gcpE* mutant; 6, EGDe *lytB* mutant. (B) Growth characteristics of a mevalonate-MEP double pathway mutant. Overnight cultures of wild-type, EGDe Δ*hmgR*Δ*gcpE*, and EGDe Δ*hmgR*Δ*gcpE*::pPL2-lmo0825 were washed and diluted 1:100 into fresh medium without mevalonate, and growth was monitored over time (closed squares, open circles, and closed triangles, respectively). In addition, EGDe Δ*hmgR*Δ*gcpE* overnight cultures were initially inoculated into medium without mevalonate, which was then added after 0, 6, or 10 h of growth (closed circles, open triangles, and open squares, respectively). Arrows indicate the time points of mevalonate addition. The presented experiment is representative of three independent experiments.

lonate or the restoration of the mevalonate pathway results in full complementation of the double pathway mutant.

The MEP pathway plays a role in murine infection. As mice do not have the Vγ9Vδ2 subset of T cells (10), the mouse model of infection cannot be used to examine the direct immunomodulatory role of HMP-BB during infection by *L. monocytogenes*. However, as the nonpathogenic species *L. innocua* lacks a complete MEP pathway and this species cannot grow intracellularly, the mouse model can be employed to investigate whether the MEP pathway contributes to growth of *L. monocytogenes* in this environment. The wild-type *L. monocytogenes* EGDe, and previously described single mutants in both pathways (EGDe-*mvk*, EGDe-*gcpE*, and EGDe-*lytB* mutants) (3) were used to intraperitoneally infect groups of four mice. Three days postinfection the mice were sacrificed and the numbers of bacteria present in the spleens and livers were determined (Fig. 5A). The bacterial numbers observed in livers and spleens derived from the groups of mice infected with the wild-type *L. monocytogenes* EGDe strain and the mevalonate pathway mutant (EGDe-*mvk*) were similar. In contrast, the numbers obtained for both MEP pathway mutants (EGDe-*gcpE* and EGDe-*lytB*) were significantly lower (1 to 2 logs) than the wild type in both the livers and the spleens, suggesting that the MEP pathway contributes to the fitness and survival of *L. monocytogenes* during the infection process. Complementation of the *gcpE* deletion mutant by transformation of the strain with pNZ44-*gcpE* restored virulence back to wild-type levels (data not shown).

The survival of the double pathway mutant during murine infection was also investigated in a similar manner. This mutant was not recovered from the livers and spleens, suggesting that in vivo mevalonate and/or IPP levels are too low to allow growth of a strain that cannot synthesize its own supply (Fig. 5B). Cloning *hmgR* from the classical pathway into the double mutant restored virulence to wild-type levels. This was unexpected, as this strain still lacks a functional MEP pathway. Experiments by Sauret-Güeto et al. (30) in *E. coli* have shown that spontaneous mutations arise at a high frequency to block the otherwise-lethal obstruction of isoprenoid biosynthesis. We suggest that the restoration of virulence of our *L. monocytogenes* double mutant may be due to mutations or alterations in transcription of a gene(s) that arose during the creation of the mutant.

***gcpE* is expressed in the liver during murine infection.** In order to examine expression of genes in vivo, a luciferase-based reporter system was employed. The promoter regions of the genes (lmo0010 [*mvk*], lmo0825 [*hmgR*], lmo1441 [*gcpE*], and lmo1451 [*lytB*]) were cloned into pPL2*lux* and the constructed plasmids were integrated into the chromosome of *L. monocytogenes* EGDe, allowing direct assessment of gene expression levels by monitoring bioluminescence levels (5). All constructs were stable, and in vitro bioluminescence imaging determined that all constructs are functional (Luria-Bertani, pH 7.4) (data not shown). Mice infected with the constructed strains were sacrificed 3 days postinfection, and bioluminescence imaging was conducted on the livers (Fig. 5C). Of the four genes tested, only *gcpE* expression (MEP pathway) was detectable in the livers of mice. Cell numbers recovered from the livers of the mice infected with the different strains were very similar, excluding the possibility that the observed differences in expression levels are caused by differences in infection efficacies.

Mutants are not affected in survival in J774 macrophages. As *L. monocytogenes* virulence defects observed following murine infection via the intraperitoneal route can often be attributed to an inability to survive in macrophages, the capacity of strains to survive in murine J774 macrophages was examined as outlined in Materials and Methods. It was observed that single mutants were unaffected in uptake or in intracellular survival, as the numbers of bacteria recovered were comparable to the wild type over the short time course of this experiment (data not shown). The double mutant that was starved of mevalonate prior to infection (see Materials and Methods for details) was also recovered in similar numbers to the wild type, suggesting that levels of mevalonate and/or IPP in the macrophages were sufficiently high to permit growth.

DISCUSSION

We have previously noted that although *L. monocytogenes* possesses all genes for both pathways of isoprenoid biosynthesis, the closely related nonpathogenic species *L. innocua* lacks the last two genes of the MEP pathway (3). The present study was initiated to further explore this phenomenon and to identify a possible role for the genes in *L. monocytogenes*.

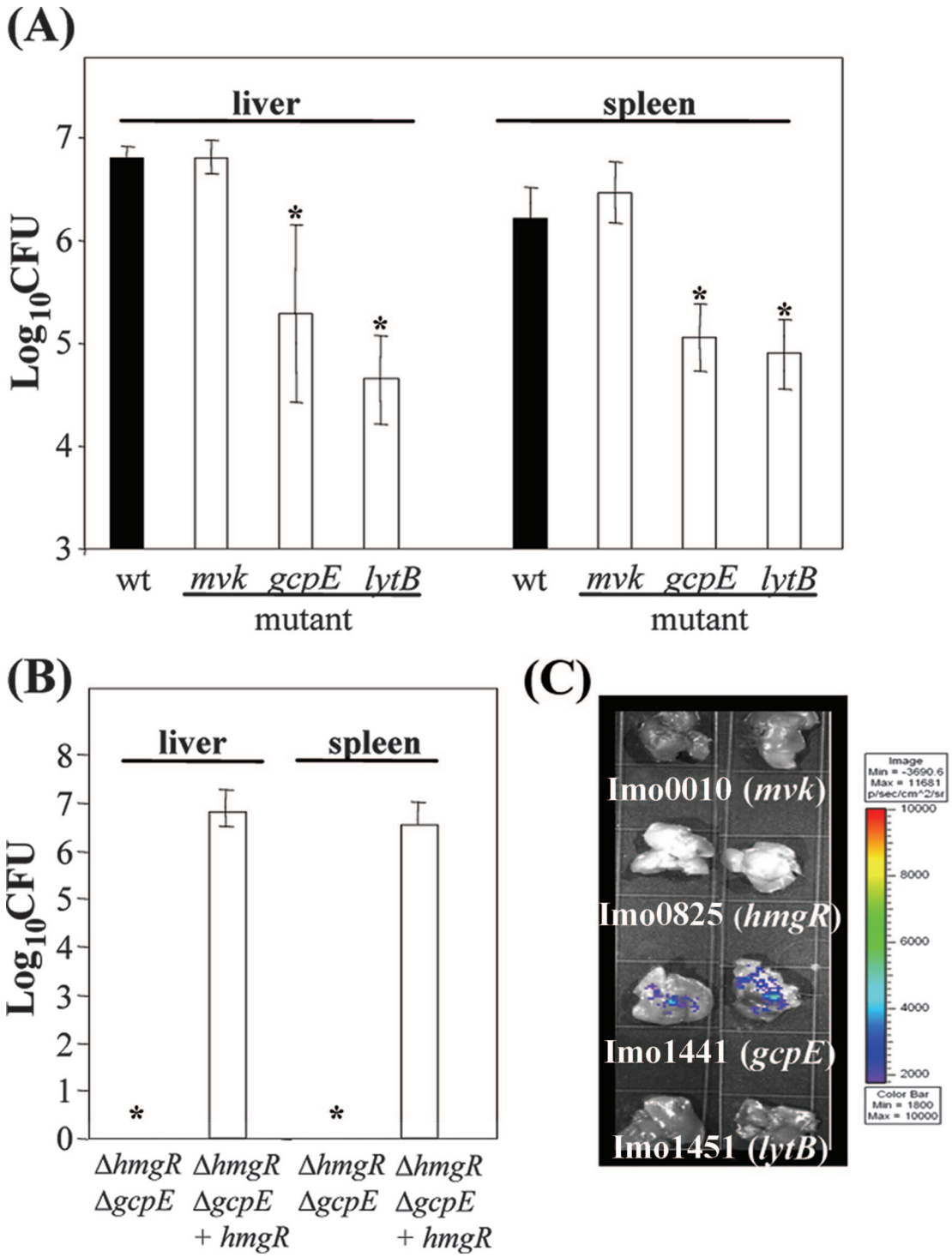

FIG. 5. (A) In vivo survival of MEP and mevalonate pathway mutants. Mice were infected with *L. monocytogenes* EGDe or EGDe *mvk*, EGDe *gcpE*, and EGDe *lytB* mutants. Three days postinfection the mice were sacrificed, and the average numbers of bacteria in the livers and spleens were determined. *, $P < 0.001$ as determined by one-way analysis of variance with post hoc comparison by the Holm Sidak method ($n = 4$ mice per group). All calculations were performed using Sigmastat 3.5 (Synstat Inc., Erkrath, Germany). The presented experiment is representative of two independent experiments. wt, wild type. (B) In vivo survival of a MEP-mevalonate double pathway mutant. Mice were infected with EGDe Δ*hmgR*Δ*gcpE* and EGDe Δ*hmgR*Δ*gcpE*::pPL2-lmo0825 (marked Δ*hmgR*Δ*gcpE* + *hmgR*). Three days postinfection the mice were sacrificed, and the average numbers of bacteria in the livers and spleens were determined. The presented experiment is representative of two independent experiments. (C) In vivo analysis of *L. monocytogenes* MEP and mevalonate pathway expression in the livers of BALB/c mice. Groups of two mice were inoculated through the intraperitoneal route with EGDe::pPL2*lux*-$P_{lmo0010}$, EGDe::pPL2*lux*-$P_{lmo0825}$, EGDe::*pPL2lux*-$P_{lmo1441}$, or EGDe::pPL2*lux*-$P_{lmo1451}$. Three days postinfection, mice were euthanized and bioluminescent imaging was conducted. The color bar indicates bioluminescence signal intensity in photons per s per cm^2. The presented experiment is representative of two independent experiments. Min, minimum; Max, maximum.

Initial work focused on using a bioinformatics approach to examine the *gcpE* and *lytB* genomic regions, and results obtained provide very strong evidence that these genes were lost in *L. innocua* during adaptation to a nonpathogenic life cycle. As this species also lacks the PrfA virulence cluster required for intracellular survival, we postulated that a complete MEP pathway may be required by *L. monocytogenes* during this stage of infection. Furthermore, previous studies imply that the MEP pathway may be important for the virulence potential of other pathogens. An in vivo expression technology screen revealed that *dxr* (DOXP reductoisomerase) was induced in vivo during *Klebsiella pneumoniae* infection of mice (21), the *Brucella abortus dxs* (DOXP synthase) gene was shown to be activated in RAW macrophages 4 h postinfection (12), and experiments by Shin et al. (31) revealed that a *gcpE* transposon mutant of *Mycobacterium tuberculosis* displayed a significant decrease in tissue colonization (liver, intestine) following intraperitoneal infection of BALB/c mice.

Experiments using the mouse model of infection support our hypothesis, as MEP pathway mutants were impaired in infection of mice via the intraperitoneal route. The precise role of the MEP pathway in bacterial survival in the host environment is unknown, but as mice do not have Vγ9Vδ2 T cells, the virulence defect observed cannot be attributed to immune modulation by HMB-PP. MEP mutants were not affected in macrophage culture-based experiments and remained unaffected in early (24-h) systemic growth in mice, indicating resistance to initial macrophage defenses in vivo (data not shown). In addition, microarray experiments performed by Chatterjee et al. (6) did not show altered expression of genes of either pathway of isoprenoid biosynthesis in *L. monocytogenes* cells isolated from infected macrophages compared to cells grown in broth. We suggest that the longer-term deficiencies in growth in vivo seen in our experiments may reflect an as-yet-unidentified shift in the nutritional environment of the pathogen during infection that necessitates an operational MEP pathway. Further work will be necessary to prove this hypothesis. The double pathway mutant was not recovered from the livers and spleens of mice 3 days postinfection, suggesting that the in vivo mevalonate and/or IPP levels are not sufficient to allow growth of a strain that cannot undergo isoprenoid biosynthesis.

Although *L. innocua* lacks a complete MEP pathway, our ability to restore the pathway by heterologous expression of *gcpE* and *lytB* demonstrates that the enzymes responsible for the synthesis of the first four intermediates of the MEP pathway are still functional. It is possible that these enzymes may feed into other pathways rather than having a role in isoprenoid biosynthesis. Taking all of our observations into account, we suggest that *L. innocua* has devolved to express only a single pathway for isoprenoid biosynthesis and has lost the functional MEP pathway required for in vivo growth. This may represent niche adaptation by the nonpathogenic species, which no longer involves colonization of the host. It is noteworthy that other researchers have also provided evidence that *L. monocytogenes* and *L. innocua* evolved from a common pathogenic ancestor but *L. innocua* has lost many of the genes required for infection (8, 33).

Furthermore, our experiments suggest that the stimulation of Vγ9Vδ2 T cells by *L. monocytogenes* but not *L. innocua* is conferred by the presence of a complete MEP pathway (*gcpE* and *lytB*) in the former species. It has been suggested that the function of Vγ9Vδ2 T cells is to initiate and regulate the mucosal immune system in order to limit a detrimental host response toward commensal bacteria (10). Indeed, a metagenomic approach by Gill et al. (13) revealed that MEP pathway genes are abundant in the indigenous microflora of the human distal gut microbiome, and genome sequence analyses have revealed that the common intestinal bacteria species of *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *E. coli*, and *Clostridium* all possess genes of the MEP pathway (10). It is therefore entirely possible that *L. monocytogenes* may deliberately exploit this surveillance system, thereby inducing a state of tolerance. Since *L. innocua* does not need to persist in the intestinal tract, for this bacterium the ability to interact with the hosts immune system may not be essential. As mice lack Vγ9Vδ2 T cells, rhesus macaques (*Macaca mulatta*) or night monkeys (*Aotus nancymaae*) will have to be employed to explore this hypothesis (10).

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