The interplay between classical and alternative isoprenoid biosynthesis controls γδ T cell bioactivity of *Listeria monocytogenes*

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Received 7 January 2004; revised 29 January 2004; accepted 30 January 2004

First published online 13 February 2004

Edited by Beat Imhof

Abstract **Isoprenoids are synthesised either through the classical, mevalonate pathway, or the alternative, non-mevalonate, 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway. The latter is found in many microbial pathogens and proceeds via (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), a potent activator of human Vγ9/Vδ2 T cells. *Listeria monocytogenes* is the only pathogenic bacterium known to contain both pathways concurrently. Strategic gene knockouts demonstrate that either pathway is functional but dispensable for viability. Yet, disrupting the mevalonate pathway results in a complementary upregulation of the MEP pathway. Vγ9/Vδ2 T cell bioactivity is increased in Δ*lytB* mutants where HMB-PP accumulation is expected, and lost in Δ*gcpE* mutants which fail to produce HMB-PP.**

Key words: HMG-CoA; *gcpE*; *lytB*; Hydroxymethylbutenyl pyrophosphate; Isopentenyl pyrophosphate; Listeriosis

1. Introduction

In humans, γδ T cells normally constitute 0.5–5% of peripheral blood T cells and comprise a number of subpopulations characterised by the distinct repertoire of their antigen receptors encoded by certain Vγ and Vδ gene segments, the majority of which express the Vγ9/Vδ2 T cell receptor. Elevated levels of Vγ9/Vδ2 T cells in the peripheral blood of patients are documented in a variety of microbial infections [1]. The responsible Vγ9/Vδ2 T cell-stimulating compound common to the respective pathogens is likely to be identical to the highly active non-peptidic compound, (*E*)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), initially isolated from *Escherichia coli* [2,3]. HMB-PP turned out to be the direct precursor of isopentenyl pyrophosphate (IPP) within the non-mevalonate, 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway of isoprenoid biosynthesis. In this respect, the two recently discovered enzymes HMB-PP synthase (GcpE) and HMB-PP reductase (LytB) play a key role in HMB-PP metabolism [4,5]. Homologues of the genes encoding the enzymes of the MEP pathway, including *gcpE* and *lytB*, are found in pathogenic organisms as diverse as *Brucella*, *Francisella*, *Mycobacterium*, *Pseudomonas*, *Salmonella*, and *Plasmodium*, and correlate with their Vγ9/Vδ2 T cell stimulatory capacity [6,7]. Bacterial species that lack the MEP pathway and synthesise IPP via the classical mevalonate pathway instead, such as *Streptococcus*, *Staphylococcus* and *Borrelia*, are unable to produce HMB-PP and consequently do not specifically activate lymphocytes through the Vγ9/Vδ2 T cell receptor.

Listeriosis, an opportunistic food-borne infection that primarily affects pregnant women and immunologically compromised individuals, is also associated with elevated Vγ9/Vδ2 T cell numbers [8]. The disease is characterised by septicaemia and eventually leads to infection of the foetus and abortion in pregnant women, or life-threatening meningitis in non-pregnant individuals. Mortality rates of common-source outbreaks often approach 30% [9,10]. The causative agent of human listeriosis, *Listeria monocytogenes*, is remarkably resistant to hostile environmental conditions and can grow at refrigeration temperatures and high salt concentrations [9]. This ability to sense and adapt to sudden changes in its environment is crucial for environmental growth and subsequent pathogenesis [11]. A number of genetic loci have been identified that play a role in survival of *L. monocytogenes* under extreme conditions experienced both during food production and host infection [12–15]. In particular, the ability of the pathogen to resist bile salts in the gastrointestinal tract is likely to be important for colonisation and pathogenesis in food-borne listeriosis [16,17]. A recent analysis of loci involved in bile tolerance resulted in the isolation of a bile-intolerant mutant that carries a disruption in the promoter region of the *lytB* gene suggesting a role for the MEP pathway of isoprenoid biosynthesis in bile resistance [16].

Previously, we demonstrated that *lytB* deficiency leads to significant HMB-PP accumulation and increased Vγ9/Vδ2 T cell-dependent immunogenicity in *E. coli* [18]. In the current study we investigated the effect of imbalanced isoprenoid biosynthesis in general, and *lytB* deficiency in particular, on the potential of *L. monocytogenes* to stimulate Vγ9/Vδ2 T cells, as an alteration in the production of the highly potent metabolite

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Abbreviations: HMB-PP, (*E*)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate; GcpE, (*E*)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate synthase; LytB, (*E*)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate reductase; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; IPP, isopentenyl pyrophosphate; LMW, low molecular weight; MEP, 2-*C*-methyl-D-erythritol 4-phosphate

0014-5793/04/$30.00
doi:10.1016/S0014-5793(04)00131-0

Table 1
PCR primers used in this study

Primer	Sequence (5′–3′)[a]
lmo0010F	AATCAAGAATTCGACTCGAA
lmo0010R	ATCGCCTTCTAGATGTGTC
lmo0010int	CTTAGCAGGAATCAAAGCA
lmo0825F	ACTCGTCGACACCCAAGA
lmo0825R	CGCCAATGGATCCCAAAC
lmo0825int	ATGATTGGTCAGATTGAGTT
lmo1441F	ACCCTACTGCAGACGGAA
lmo1441R	CATTGCGGAATTCGAGGAA
lmo1441int	GGTAACATTGGTCGCCGT
lmo1451F	GCAAAAGCTTAACGACAATC
lmo1451R	TTCGGGAATTCCCCCTTT
lmo1451int	ATCCATTGGTATTTATACAGT

[a]Nucleotides altered to contain restriction sites are underlined.

HMB-PP may affect the immune response during the course of infection in vivo.

2. Materials and methods

2.1. Genetic manipulations

Restriction enzymes were obtained from Roche (Mannheim, Germany). Polymerase chain reactions (PCRs) were carried out using a Hybaid PCR express system (Middlesex, UK) and products were purified with the QiaexII gel extraction kit (Qiagen, Hilden, Germany). Plasmids were isolated with the Qiagen QIAprep Spin Miniprep Kit.

2.2. Construction of pORI19 mutants

Individual mutants were reconstructed by gene disruption followed by a single crossover event, as described by Law et al. [19] for *Lactococcus lactis* and Begley et al. [20] for *L. monocytogenes*. Briefly, internal fragments of genes were generated by PCR with appropriate primers (forward and reverse), which were modified to contain restriction sites (see Table 1). These products were digested and ligated into similarly digested pORI19. The resulting plasmids were transformed into *E. coli* EC101 and then into *L. monocytogenes* harbouring the temperature-sensitive helper plasmid pVE6007. In each case, one transformant was grown overnight at 30°C in brain–heart infusion (BHI) supplemented with 5 μg/ml erythromycin, after which 10 μl was transferred to BHI broth pre-warmed to the non-permissive temperature of 42°C. Following overnight incubation aliquots were plated onto BHI containing 5 μg/ml erythromycin. This selected for chromosomal integration at the point of homology with PCR products. Site-specific plasmid insertions were confirmed by PCR analysis using primers for the plasmid (M13F/R) and primers that bind outside the gene regions amplified (*int* primers).

2.3. Sequence analysis

Genomic sequences from bacterial species, as well as the protein sequences derived thereof, were retrieved from the databases provided by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and the Institute for Genomic Research (http://www.tigr.org). Sequence homologies were calculated using EMBOSS/stretcher software for global pairwise alignments (http://bioweb.pasteur.fr), multiple alignments were performed using CLUSTAL W (http://www.ebi.ac.uk) and BOXSHADE (http://bioweb.pasteur.fr) software.

2.4. γδ T cell stimulation assays

Bacteria were harvested from fresh liquid cultures at an optical density $(OD)_{600}$ of 0.8, and sonicated in 1/10 volume phosphate-buffered saline, pH 7.4. Low molecular weight (LMW) fractions were obtained using Centriprep 3 kDa filters (Millipore, Eschborn, Germany). Flow cytometrical analysis of human peripheral blood mononuclear cells (PBMCs) was performed as described before [18,21]. LMW samples were tested at serial dilutions between 1:10 and $1:10^6$. Cells were harvested after 6 days, and analysed on an Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany), using CD3-PC5 and Vγ9-fluorescein isothiocyanate monoclonal antibodies. Bioactivity of bacterial extracts was determined using a standard curve of synthetic HMB-PP [3], and expressed as the stimulatory capacity comparable to an equivalent concentration of HMB-PP.

3. Results and discussion

3.1. The L. monocytogenes genome contains the genes of both pathways of isoprenoid biosynthesis

Of the bacterial genomes sequenced to date, the genomes of *L. monocytogenes* and certain *Streptomyces* species appear unique in containing the genes of both the classical mevalonate and the alternative MEP pathways of isoprenoid biosynthesis (Fig. 1, Table 2). In all other microorganisms examined thus far these pathways are mutually exclusive [22,23]. The presence of intact genes encoding enzymes from both pathways implies that *L. monocytogenes* may be capable of synthesising the isoprenoid precursor IPP via either route. This may increase the metabolic flexibility of *L. monocytogenes* relative to other bacterial species and ensure isoprenoid biosynthesis under conditions that would normally block a single pathway.

3.2. The presence of either pathway is dispensable for the survival of L. monocytogenes mutants

In an attempt to understand the significance of both complete pathways of isoprenoid biosynthesis in *L. monocyto-*

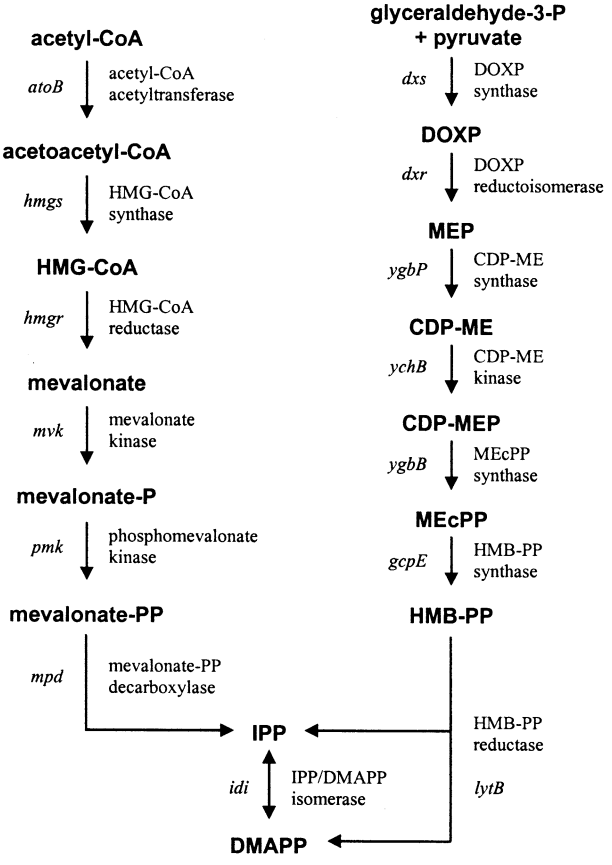

Fig. 1. Isoprenoid biosynthesis in *L. monocytogenes* via the classical mevalonate pathway (left) and the alternative MEP pathway (right). CDP-ME, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol; CDP-MEP, 4-diphosphocytidyl-2-*C*-methyl-D-erythritol 2-phosphate; DMAPP, dimethylallyl pyrophosphate; DOXP, 1-deoxy-D-xylulose 5-phosphate; MEcPP, 2-*C*-methyl-D-erythritol 2,4-cyclopyrophosphate; P, phosphate.

Table 2
Genes controlling isoprenoid biosynthesis in *L. monocytogenes* and *L. innocua*

Gene	*L. monocytogenes*[a]		*L. innocua*[b]		% homology of *L. monocytogenes* proteins to[c]		
	cds	no.	cds	no.	*L. innocua*	*E. coli*	*Streptococcus pyogenes*
dxs	1 387 381–1 389 210	lmo1365	1 394 166–1396 064	lin1402	84.2 (89.0)	44.8 (61.1)	–
dxr	1 342 682–1 343 824	lmo1317	1 349 456–1 350 598	lin1354	95.8 (98.7)	41.1 (57.9)	–
ygbP	255 800–256 498	lmo0235	278 872–279 570	lin0267	85.3 (94.8)	35.7 (49.0)	–
ychB	193 989–194 870	lmo0190	225 918–226 793	lin0229	95.9 (98.6)	29.4 (48.2)	–
ygbB	256 491–256 964	lmo0236	279 563–280 036	lin0268	90.4 (96.2)	61.2 (72.5)	–
gcpE	1 474 802–1 475 908	lmo1441	–	–	–	47.8 (66.4)	–
lytB	1 483 549–1 484 544	lmo1451	–	–	–	33.7 (53.6)	–
atoB	1 443 873–1 445 042	lmo1414	1 451 921–1 453 090	lin1453	92.3 (97.4)	51.5 (67.5)	51.1 (69.9)
hmgs	1 445 192–1 446 358	lmo1415	1 453 240–1 454 406	lin1454	93.3 (96.9)	–	49.7 (68.4)
hmgr	851 225–852 505	lmo0825	848 666–849 946	lin0821	88.5 (95.1)	–	45.2 (58.9)
mvk	12 918–13 886	lmo0010	12 919–13 887	lin0010	96.3 (98.4)	–	32.6 (49.5)
pmk	14 795–15 874	lmo0012	14 793–15 875	lin0012	98.9 (99.7)	–	26.4 (46.1)
mpd	13 843–14 814	lmo0011	13 796–14 815	lin0011	92.3 (93.5)	–	36.7 (58.5)
idi1?	395 602–396 111	lmo0368	169 535–170 044	lin0387	92.3 (95.9)	23.9 (39.1)	–
idi2	1 406 742–1 407 818	lmo1383	1 413 629–1 414 705	lin1420	91.1 (96.1)	–	37.7 (58.4)

[a]Coding region and gene number in *L. monocytogenes* strain EGD-e genome (accession number NC_003210).
[b]Coding region and gene number in *L. innocua* strain Clip11262 genome (accession number NC_003212).
[c]% identity (similarity) of *L. monocytogenes* proteins with the corresponding homologues of *L. innocua*, *E. coli*, and *S. pyogenes*, as calculated using the EMBOSS/stretcher software for global pairwise alignments.

genes, insertion mutants were created in the genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (*hmgr*) and mevalonate kinase (*mvk*) for the mevalonate pathway, and in the genes encoding HMB-PP synthase (*gcpE*) and HMB-PP reductase (*lytB*) for the MEP pathway (Fig. 2). Due to the large size of the inserted plasmid these disruptions are likely to impact downstream genes in polycistronic systems in addition to the targeted determinant. This is particularly relevant for the disruption in *mvk* (*lmo0010*) which is also likely to eliminate transcription of two further mevalonate pathway genes, *pmk* (*lmo0012*) and *mpd* (*lmo0011*).

Mutants were created efficiently and were stable (data not shown). None of the mutants displayed any impaired growth rates in BHI medium, showing that disrupting either pathway can be fully complemented by the action of the other. This confirms that *L. monocytogenes* is a convenient model system (and the only pathogenic bacterial species known) for investigating both pathways of isoprenoid biosynthesis in the same organism and under identical conditions. Previous mutation-based studies on the MEP pathway in *E. coli* were only possible once a reconstructed heterologous operon was introduced expressing three genes needed for the biosynthesis of the essential metabolite IPP from exogenously provided mevalonate [24]. This strategy did not allow for studies investigating the interdependence and cross-regulation of both pathways as can now be achieved in *L. monocytogenes* or in *Streptomyces* spp.

3.3. L. monocytogenes *but not* Listeria innocua *drives* $V\gamma9/V\delta2$ *T cell expansion in vitro*

In contrast to *L. monocytogenes*, the closely related but non-pathogenic species *L. innocua* possesses an incomplete MEP pathway. Indeed, the *L. innocua* genome contains genes encoding all enzymes of the MEP pathway with the exception of *gcpE* and *lytB* (Table 2), suggesting that *L. innocua* is incapable of synthesising and metabolising HMB-PP, and that isoprenoid biosynthesis in this species proceeds solely via the mevalonate pathway. As *gcpE* deficiency in *E. coli* causes an

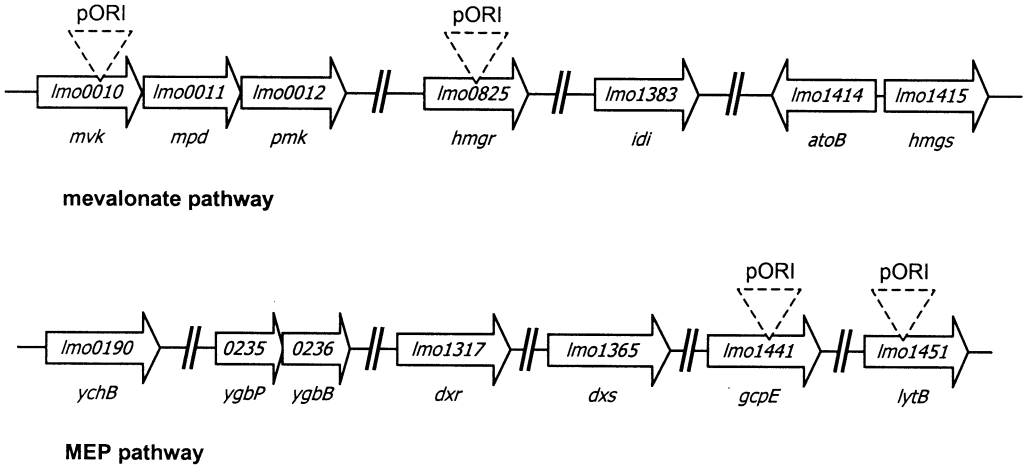

Fig. 2. Genomic organisation of the genes encoding putative enzymes of the mevalonate and MEP pathways in *L. monocytogenes*. pORI: sites of strategic knockout constructions.

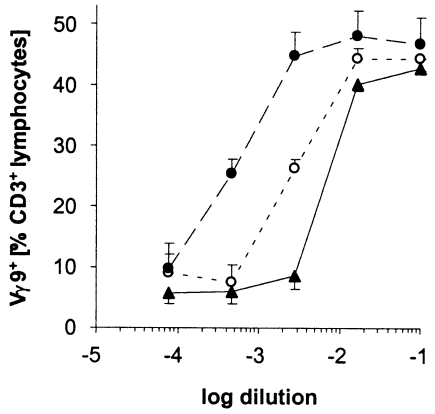

Fig. 3. Impairment of *lytB* function increases the γδ T cell bioactivity of bacterial extracts. Serial dilutions of LMW preparations from *L. monocytogenes* wild-type bacteria (filled circles), bile-sensitive *L. monocytogenes* BSM5 mutants with disrupted *lytB* promoter (empty circles), and *lytB*-deficient *L. monocytogenes* Δ*lmo1451* mutants (filled triangles) were added to PBMC cultures. Data shown for γδ T cell outgrowth are means and S.E.M. from two independently tested blood donors.

incapability to activate Vγ9/Vδ2 T cells [25], we investigated the natural immunogenicity of *L. innocua* in a model system using human PBMCs. Indeed, whilst wild-type *L. monocytogenes* preparations were highly potent in stimulating expansion of Vγ9/Vδ2 T cells in vitro, *L. innocua* did not display any significant γδ T cell bioactivity above the background (data not shown).

3.4. *Impairment of* lytB *function increases the Vγ9/Vδ2 T cell bioactivity of* L. monocytogenes

Deficiency in *lytB* leads to significant HMB-PP accumulation and increased Vγ9/Vδ2 T cell bioactivity in *E. coli* [18]. Here, we tested whether the same holds true for *L. monocytogenes* despite the complementary presence of the mevalonate pathway. Using a mutant with a disrupted *lytB* gene together with the previously described bile-sensitive BSM5 mutant with an insertion in the *lytB* promoter [16], we show that *L. monocytogenes* with impaired *lytB* function has an increased γδ T cell bioactivity compared to the wild-type (Fig. 3). This effect was more pronounced when the *lytB* coding region was affected by plasmid insertion, in contrast to the promoter mutant in which transcription of the *lytB* gene may occur at a reduced level. Thus, the results obtained with *L. monocytogenes* mirror the situation in *E. coli*, and although not formally proven on the molecular level, the phenomenon is likely to reflect a similar accumulation of the LytB substrate, HMB-PP.

3.5. *The balance between the mevalonate and the MEP pathways affects the Vγ9/Vδ2 T cell bioactivity of* L. monocytogenes

In order to obtain a greater insight into the contribution of both pathways to the Vγ9/Vδ2 T cell-dependent immunogenicity, we subsequently compared all *L. monocytogenes* isoprenoid biosynthesis mutants and calibrated their bioactivity against a standard of synthetic HMB-PP (Fig. 4). While the Δ*lytB* mutant showed a significant increase in γδ T cell bioactivity compared to the wild-type, the Δ*gcpE* mutant was much less potent in stimulating Vγ9/Vδ2 T cells than the wild-type. The γδ T cell bioactivity of the Δ*gcpE* mutant was actually similar to the naturally *gcpE*- (and *lytB*-) deficient species *L. innocua*. Strikingly, deficiency in either *mvk* or in *hmgr* led to an elevated γδ T cell bioactivity compared to the *L. monocytogenes* wild-type. This implies that a disruption of the mevalonate pathway can be compensated by a higher throughput via the alternative pathway in order to maintain a sufficient supply of isoprenoid precursor molecules, judged by increased concentrations of the intermediate HMB-PP. A fine-tuned cross-regulation between both pathways may also be the reason for the rather modest accumulation of HMB-PP by the *lytB*-deficient *L. monocytogenes* mutant when compared to previous studies with *lytB*-deficient *E. coli*. In the current study a 7-fold accumulation of HMB-PP occurs for the *L. monocytogenes* Δ*lytB* mutant relative to the wild-type whereas previous studies have demonstrated a 150-fold increase in bioactivity in the *E. coli* model [18].

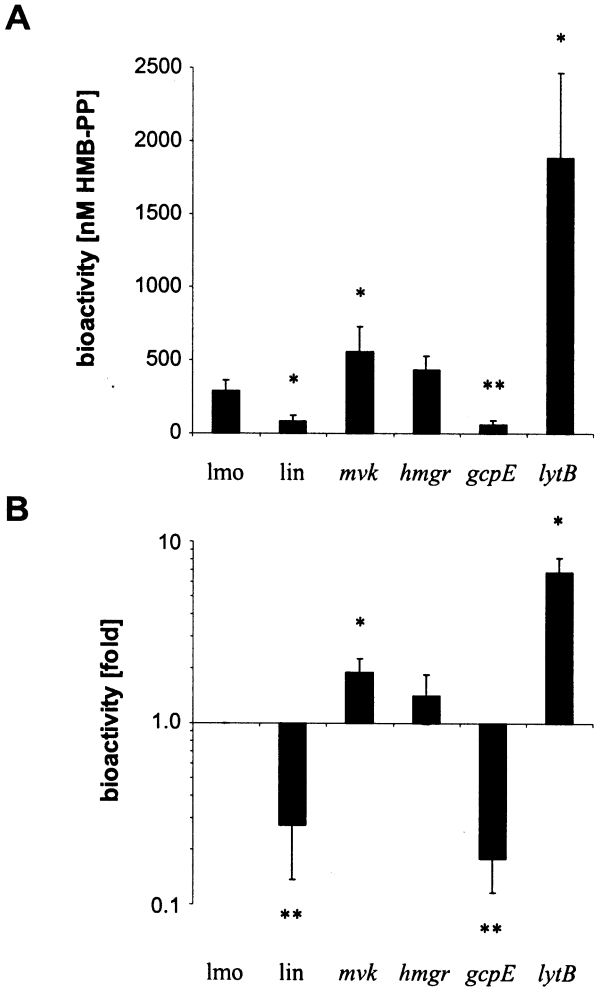

Fig. 4. Quantification of γδ T cell bioactivities of bacterial extracts. Bioactivity is expressed as absolute equivalents in nM HMB-PP (A), or as the relative change compared to the *L. monocytogenes* wild-type (B). Data shown are means and S.E.M. from six independently tested blood donors. lmo, *L. monocytogenes* wild-type; lin, *L. innocua* wild-type; *mvk*, mevalonate kinase (*lmo0010*); *hmgr*, HMG-CoA reductase (*lmo0825*); *gcpE*, HMB-PP synthase (*lmo1441*); *lytB*, HMB-PP reductase (*lmo1451*). $^{*}P < 0.05$, and $^{**}P < 0.002$, as determined by two-tailed Student's *t*-tests for paired data.

3.6. Implications of the interplay between the two pathways of isoprenoid biosynthesis

The above findings indicate that a blockade in one pathway of isoprenoid biosynthesis in *L. monocytogenes* can be fully complemented by the action of the other, when mutants are grown in complex media. Whether this holds true though under physiologic in vivo conditions during the course of host colonisation and infection remains to be investigated.

Mutants in one pathway of isoprenoid biosynthesis should be highly susceptible to specific inhibitors of the remaining pathway. The antibiotic fosmidomycin is a potent inhibitor of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (Dxr) [26], the second enzyme within the MEP pathway. However, this antibiotic did not inhibit the growth of either wild-type or mutant bacteria, when tested on agar plates and in liquid cultures at concentrations of up to 500 μM (data not shown). This finding was rather unexpected as survival of *Listeria* strains deficient in the mevalonate pathway (Δ*hmgr* or Δ*mvk*) should only be due to successful by-passing of the defect by a fully operative MEP pathway. Fosmidomycin is active against a range of Gram-negative bacteria including *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* ([27]; data not shown), as well as against malaria parasites [28]. However, it does not show any significant activity on *Burkholderia cepacia*, *Clostridium perfringens*, *Mycobacterium tuberculosis*, *Neisseria gonorrhoeae*, and others ([27]; data not shown), despite the presence of the MEP pathway in all these species [6]. It is possible that *L. monocytogenes* and other fosmidomycin-resistant bacteria possess mechanisms to exclude the antibiotic from the bacterial cytoplasm. In the absence of drug-dependent targeting of the MEP pathway, the construction of conditional double knockout mutants may be a more favourable approach in the future to provide formal proof that isoprenoid biosynthesis via either of the two pathways is required for in vitro and in vivo survival.

Our results using isoprenoid biosynthesis mutants are likely to have important implications for the pathogenesis and immunity in *Listeria* infections, as affecting the fine-tuned balance between the two pathways had profound consequences for the immunogenicity of the respective strain. While elevated levels of Vγ9/Vδ2 T cells are observed in many microbial infections including listeriosis, there is still a considerable debate whether this lymphocyte population has a role in clearance and protective immunity, or is rather involved in immune evasion of the pathogen and the establishment of chronic disease. Recent reports suggested that Vγ9/Vδ2 T cells are important mediators of resistance against extracellular bacterial infections [29]. However, the rapid and strong reactivity of Vγ9/Vδ2 T cells appears rather ineffective against certain other pathogens as many microbial species manage to escape the immune response nonetheless and establish chronic and debilitating infections, such as tuberculosis and malaria [6]. The mutants constructed in the present study provide a powerful tool to scrutinise the role of Vγ9/Vδ2 T cells in the infectious process by comparing HMB-PP-overproducing and HMB-PP-deficient strains. This will have to be done in a primate model as besides humans, the Vγ9/Vδ2 T cell subset and its response to non-peptidic antigens have so far only been observed in rhesus macaques (*Macaca mulatta*) and night monkeys (*Aotus nancymaae*) [30,31]. Other animals including rodents and cattle do not appear to have homologues to the Vγ9 and Vδ2 T cell chains and do not react towards non-peptidic antigens (e.g. [32]). However, uncoupling *Listeria* pathogenicity from the Vγ9/Vδ2 T cell reactivity might eventually lead to a thorough understanding of the role of Vγ9/Vδ2 T cells in microbial infection and have a fundamental impact on the creation of effective vaccine strains.

Acknowledgements: This work was supported by the Irish government under the National Development Plan 2000–2006, through funding of the Alimentary Pharmabiotic Centre by the Science Foundation of Ireland Centres for Science Engineering and Technology (CSET) scheme, the German Bundesministerium für Bildung und Forschung (BioChance 0312588), the European Commission (INCO-Dev ICA4-2000-10290), and the Fonds der Chemischen Industrie (SK 172/5). Blood samples from healthy donors were kindly provided by the Institut für Klinische Immunologie und Transfusionsmedizin, Universität Giessen. We gratefully acknowledge Armin Reichenberg for providing HMB-PP, Rosel Engel for excellent assistance, and Jochen Wiesner for critical discussion. This paper is dedicated to the lab of Ewald Beck on the occasion of the fire on 6 May 2003.

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