Escherichia coli Produces Phosphoantigens Activating Human γδ T Cells*

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Human γδV9γδ2 T lymphocytes are suggested to play an important role in the immune response to various microbial pathogens. In contrast to αβ T cells, γδ T lymphocytes recognize small, non-protein, phosphate-bearing antigens (phosphoantigens) in a major histocompatibility complex-independent manner. Four different phosphoantigens termed TUBag1 to TUBag4 with a common 3-formyl-1-butyl-pyrophosphate moiety and isopentenyl-pyrophosphate have been isolated and identified from mycobacteria. However, natural occurring γδ T cell ligands from other bacterial species were not characterized so far. Here, we describe the structural identification of the two compounds responsible for the γδ T cell-stimulating capacity of Escherichia coli as similar to the mycobacterial phosphoantigens 3-formyl-1-butyl-pyrophosphate and its Ms 275 homologue TUBag2. In addition, E. coli phosphoantigens exert bioactivities on γδ T cells with similar potencies to the mycobacterial phosphoantigens at 5–15 nM concentration. Furthermore, our results clearly prove that the deoxyxylulose 5-phosphate pathway (also referred to as Rohmer metabolic route of isoprenoid biosynthesis) is essential for the biosynthesis of the phosphoantigens in E. coli. Because this pathway is absent from human cells, it proves an ideal target for focusing efficiently the antimicrobial selectivity of human γδ T lymphocytes.

Human γδV9γδ2 T lymphocytes are considered to play an important role in the immune response to various microbial pathogens. In contrast to αβ T cells, γδ T lymphocytes recognize small, non-protein, phosphate-bearing antigens (phosphoantigens) in a major histocompatibility complex-independent manner. These phosphoantigens are produced by different Gram-positive and Gram-negative bacteria as well as by eukaryote parasites (1). Mycobacterium tuberculosis produces four different phosphoantigens termed TUBag1 to TUBag4 wherein TUBag1 and TUBag2 are phosphatase-sensitive pyrophosphate monoesters, whereas TUBag3 and TUBag4 are nucleotide conjugates of TUBag1 (2). These compounds are active for stimulation of human γδ cells at 5–15 nM concentration and have been found not only in M. tuberculosis but also in most other mycobacterial species. Although complete identification of their structure was recently achieved by elucidation of their common 3-formyl-1-butyl-pyrophosphate (3bPP) moiety (3), isopentenyl pyrophosphate (IPP) had been characterized as another mycobacterial phosphoantigen from Mycobacterium smegmatis (4). IPP is a ubiquitous precursor for vitamins, steroids, and ubiquinones in all living organisms. However, in the case of Escherichia coli we found that the γδ T cell-stimulating capacity of IPP did not correlate with its amount in bacterial extracts (5). A different set of non-phosphorylated microbial metabolites stimulating human γδ T cells is composed of alkylamines, although their selective bioactivity is most frequently observed within millimolar concentration ranges (6).

Until recently, biosynthesis of IPP has been thought to rely solely upon the “classical” mevalonate pathway of isoprenoid biosynthesis. However, recent studies on the isoprenoid biosynthesis in bacteria, algae, and plants have led to the discovery of a second metabolic route, the so-called deoxyxylulose 5-phosphate (DOXP) or Rohmer pathway (7), which appears to be absent in all animals including man. Key metabolites of this pathway, e.g. deoxyxylulose 5-phosphate, 2-C-methylerythritol 4-phosphate, 4-diphosphocytidyl-2-C-methylerythritol or 2-C-methylerythritol cyclopyrophosphate (8–10) share a common carbon skeleton with the mycobacterial TUBag ligands (3). Furthermore, it was demonstrated that the presence of the Rohmer pathway in different bacterial species correlates with the ability of the metabolites to stimulate γδ T cells (5). These observations support the concept that γδ T cells discriminate between self and non-self through the recognition of specific biosynthetic routes and, therefore, contribute to the specific immune response to bacteria and parasites (11).

Although the presence of phosphoantigens was evidenced in Plasmodium falciparum (12), Francisella tularensis (13), Vis-

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cum album (14, 15), and Brucella suis (16), few non-mycobacterial phosphoantigens have been fully characterized so far. In the current study we focused on isolating and characterizing γδ T cell stimulating ligands from E. coli. We describe here the structural identification of these E. coli antigens as similar to the mycobacterial phosphoantigens. Furthermore, the relation of these bacterial antigens to the Rhomer metabolic pathway of isoprenoid biosynthesis is demonstrated by use of a selective inhibitor.

MATERIALS AND METHODS

Chemicals Reagents—Deoxyxylulose was synthesized and characterized using ¹H and ¹³C spectroscopy as well as gas chromatography/mass spectrometry as described previously (5). Prof. H. Seto, University of Tokyo, Japan, kindly provided 2-C-methylerythritol, 2-C-methylerythritol 4-phosphate, 5-deoxyxylulose 5-phosphate, 4-[(2-methyl-2-¹³C)Diposphorytidyl]-2-C-methylerythritol, 4-[(1,2,3,4,5)²⁵³C]Diposphorytidyl]-2-C-methylerythritol 2-phosphate, and 2-C-[¹³C]methylerythritol cyclophosphorylate were kindly provided by W. Eisenreich, TU München, Germany. Fosfomycin was synthesized according to published methods (17, 18). All intermediates were characterized using ¹H and ¹³C NMR spectroscopy; the final product additionally was analyzed by ESI-MS.

Bacterial Cultures—E. coli O:128 isolates were provided by Prof. J. Hacker, Institut für Molekulare Infektionsbiologie, Universität Würzburg; E. coli ATCC 11303 cultures were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ); lyophilized E. coli ATCC 11303 were obtained from Sigma. For feeding experiments with 2-C-methylerythritol and inhibition tests with fosfomycin, E. coli O:128 were cultured in 25 ml of minimal culture broth as described (5). To clearly purity the E. coli antigen(s), E. coli ATCC 11303 were grown in Luria-Bertani broth (10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl/1000 ml) at 37 °C. Bacteria were disintegrated in a French press; bacterial debris was removed by centrifugation, and the supernatant was filtered over 0.22-µm pore-size membranes.

Chromatographic Procedures—The E. coli extracts were fractionated on a 20 × 200-cm Q-Sepharose anion-exchange column (Amersham Biosciences, Inc.) eluted with a 50–500 mM ammonium acetate gradient. The active fractions were collected and further purified on a 10 × 17-cm diol column (Amersham Biosciences, Inc.) with an acetone/titrile/water gradient from 10 to 100% water. After removal of acetone, the active fraction was desalted by gel chromatography using a Bio-Gel P-2 column (Bio-Rad) and 200 mM ammonium acetate as eluent. The final step was an ethanol-range separation on a Hit-Gel 20/10 Q-Sepharose high performance column (Amersham Biosciences, Inc.) with a 50–1 mM ammonium acetate gradient. The active fraction was concentrated by lyophilization. The antigen content of the resulting solution was greatly enhanced by this procedure. Each chromatographic step was monitored by testing the collected fractions in the γδ proliferation assay.

The bioactive fraction isolated by the above procedure was then finally separated by HPAEC using a Dionex DX500 (Dionex, Sunnyvale, CA) system run with the Peaknet 5.1 chromatography software. A CD20 conductimeter was used downstream of an anion self-regenerat-

Liquid Chromatography-MS—HPLC-MS was carried out as described (19). In brief, chromatography was performed on a Nucleodex 1-Deoxyxylulose was synthesized and character-

HPLC-ESI-MS analysis was carried out on a triple stage quadrupole TSQ 7000 mass spectrometer with an ESI interface (Finnigan MAT, Bremen, Germany). Data acquisition and evaluation were performed on a personal DECstation 500/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). Nitrogen served as sheath gas, and argon served as the collision gas. Internal ESI parameters were used: negative mode, capillary voltage 4 kV, capillary temperature 200 °C, sheath gas (N₂) 60 psi, sample rate 1/20 to 500 in 1 s, molecular ion m/z of IPP (M–H) = 245. For precursor ion experiments, conditions were as follows: negative mode, daughter ions m/z 79 and m/z 97, CID 18 eV, collision gas 1.8 mTorr Ar, Q3 scan m/z 100 to m/z 50 in 1 s. Selected regions of the mass spectrum were examined in negative ion mode, precursor ion m/z of 36FP (M–H) = 261, daughter ion m/z 159 [HP(O)₆], CID 15 eV; precursor ion m/z of TUBag3 (M–H) = 567, daughter ion m/z 261, CID 25 eV, scan time 1 s.

Activation of Human γδ T cells by E. coli Phosphoantigens

Phenotype Analysis by Flow Cytometry—Cells were harvested on day 3 (for measurement of CD25 expression) or day 7 (for proliferation assay) and double-stained with fluorescein isothiocyanate- or phycoerythrin-conjugated monochromatic CD3, CD25, or TCR pan-γδ antibodies, respectively (Coulter ImmuneTech, Krefeld, Germany). 5 × 10⁵ cells from each sample were analyzed using a FACScan supported with CellQuest as acquisition and data analysis software (Becton Dickinson, Heidelberg, Germany). The lymphocytes were gated using forward/sideward scatter gating.

Measurement of TNF-α Secretion—TNF-α release from purified γδ T cells was measured by using a commercially available ELISA assay. Cells were incubated with 10 μl of the tested fractions in 100 μl of culture medium plus 25 units of interleukin 2/24 h for 24 h at 37 °C. 50 μl of the culture supernatant were then added to 50 μl of WEHI cells plated at 3 × 10⁵ cells/well in culture medium plus actinomycin D (2 μg/ml) and LiCl (40 mM) and incubated 20 h at 37 °C. Viability of WEHI cell was measured by a bioassay using the TNF-α ELISA kit (Bender MedSystems, Vienna, Austria).
bromide (MTT) assay; 50 μl of MTT (Sigma; 2.5 mg/ml in phosphate-buffered saline/well) were added, and after 4 h of incubation at 37°C, 50 μl of solubilization buffer (SDS 20%, dimethylformamide 66%, pH 4.7) were added, and absorbance (570 nm) was measured. For each sample tested in triplicate, TNF-α release was then deduced from a calibration curve obtained using human recombinant TNF-α (PeproTech, Inc, Rocky Hill, NJ).

RESULTS

E. coli Produces Specific Antigen-activating Human γδ T Cells—To assess the γδ T cell stimulatory capacity of E. coli, bacteria were grown to late-log phase, disintegrated in a French press, ultrafiltered (<3 kDa), and the extracts were subjected to a γδ T cell proliferation assay in presence of exogenous interleukin 2. E. coli low molecular weight preparations (LMWP) induced activation (CD25 expression) as well as outgrowth of γδ T cells (Fig. 1A). The antigenic activity could be totally abrogated by treatment with alkaline phosphatase (Fig. 1B). Therefore, the γδ T cell-stimulating bioactivity from E. coli LMWPs is solely due to phosphorylating activity, and/or nucleotide pyrophosphatase. As shown in Fig. 1B, the γδ T cell stimulatory activity in E. coli ATCC 11303 is sensitive to alkaline phosphatase alone, demonstrating the sole presence of an antigen composed of a terminal (pyro) phosphate group as in IPP or 3βIPP. Therefore, the bioactive LMWP component appeared chromatographically close to, but separated, from IPP. To selectively trace in the low molecular weight preparations any compound carrying a terminal phosphate, we applied a phosphate-specific precursor ion scan. Because we could not detect any unknown phosphate-bearing compounds co-eluting with the γδ T cell stimulatory activity (not shown), the bioactive antigen concentration in LMWP could be estimated at 10 nM or lower. This results are representative of several experiments with PBMC of different donors. B. E. coli LMWPs were treated with alkaline phosphatase and/or nucleotide pyrophosphatase. After each cleavage step, the enzyme was removed by ultrafiltration (<3 kDa). Activation of γδ T cells was assessed by flow cytometric analysis of CD25 and pan-γδ TCR expression on day 3; results are expressed as the percentage of CD25-positive cells of all γδ TCR-positive cells ± S.D.

![Image](https://example.com/image1.png)

**Fig. 1.** Stimulation of human PBMC with E. coli low molecular weight preparation leads to activation (CD25 expression) and proliferation of γδ T lymphocytes; treatment with alkaline phosphatase (AP) alone but not with nucleotide pyrophosphatase (PPhase) abolishes this activity. A, human PBMC were cultured with medium alone or E. coli LMWPs for 3 or 7 days. Lymphocytes were gated according to their side scatter and forward scatter parameters. The percentage of γδ TCR-positive cells was determined by 2-color flow cytometry using phycoerythrin anti-CD3 and fluorescein isothiocyanate anti-γδ TCR monoclonal antibody after 7 days of culture, and the percentage of CD25 expressing γδ T cells using fluorescein isothiocyanate anti-γδ TCR and phycoerythrin anti-CD25 monoclonal antibody was determined after 3 days. The results are representative of several experiments with PBMC of different donors. B. E. coli LMWPs were treated with alkaline phosphatase and/or nucleotide pyrophosphatase. As shown). By comparing to an LMWP sample spiked with 2 mM synthetic IPP (Rt 10.05 min, Fig. 2B), the γδ T cell-activating component appeared chromatographically close to, but separated, from IPP. Therefore, the bioactive LMWP component is distinct from IPP.

To selectively trace in the E. coli LMWPs any compound carrying a terminal phosphate, we applied a phosphate-specific precursor ion scan. Because we could not detect any unknown phosphate-bearing compounds co-eluting with the γδ T cell stimulatory activity (not shown), the bioactive antigen concentration in LMWP could be estimated at 10 nM or lower. This range of bioactivity is about 100 times superior to that of IPP in similar bioassays (data not shown) but appeared similar to these reported for the mycobacterial phosphoantigens (2, 3, 27, 28).

![Image](https://example.com/image2.png)

**Fig. 2.** HPLC-ESI-MS analysis and correlated biological activity of partially purified E. coli O:128 LMWP. A. γδ T cell-stimulating activity of HPLC-MS fractions of E. coli sample. Column eluates were split between the ESI interface and the collecting outlet (split rate 1:27), and 1-min fractions were collected and subjected to the γδ T cell proliferation assay. Activation of γδ T cells was assessed by flow cytometric analysis of CD3 and pan-γδ TCR expression on day 7; results are expressed as the percentage of γδ TCR-positive cells of all CD3-positive cells ± S.D. B. Mass chromatogram for m/z 245 ([IPP-H]+) in an E. coli sample spiked with 0.5 mg/ml IPP.
It has been shown recently that 3fPP (formerly referred to as TUBag1) has a molecular mass of 262 atomic mass units and yields a pyrophosphate fragment (m/z 159) upon tandem mass spectrometry (3). To probe whether a related compound was present in LMWPs, we conducted a selected reaction-monitoring experiment using this fragmentation pattern and detected a compound co-eluting with the γδ T cell stimulatory activity (data not shown). Thus, the active compound in E. coli could be related to 3fPP. However, an analogous selected reaction-monitoring experiment scanning for the nucleotidic derivative 3f-PPP-uridine (formerly TUBag3, selected reaction monitoring m/z 567/261) achieved no signal (not shown). Together these observations confirmed the aforementioned sensitivity of the LMWP bioactivity to degradation by alkaline phosphatase alone.

E. coli LMWP Antigens for γδ T Cells Are Identical to the Mycobacterial Phosphoantigens 3fPP and 3-Formyl-1-pentyl-PP (3fpePP)—Because evidence for small amounts of 3fPP in LMWPs was obtained from the above MS experiments, further chromatographic separation and enrichment of the bioactive component from LMWPs was needed. We undertook this by HPAEC with online detection by conductimetry, UV, and final collection of the fractions (every 3 s) to enable bioactivity-based detection. This was done through measurement of the TNF-α release by a γδ T cell line cultured 24 h with 1/10 of the final volume from the collected fractions 10-fold (Fig. 3A). Although the analyzed fraction appeared to comprise several well identified organic and inorganic anions such as acetate, chloride, and a major peak of N-acetylmannosaminuronic-1-phosphate (14 m/z in the sample), only a minor amount of remaining IPP was detected by conductimetry. However, the bioactivity for γδ T cells was separated into two consecutive fractions that eluted before IPP, as already observed in the former HPLC (see Fig. 2). These fractions were pooled, concentrated 50-fold in water, and analyzed by nanospray MS in the negative mode (Fig. 3B). Among several other ion species, only two signals, namely m/z 261 and m/z 275, corresponded to phosphorylated structures as revealed by phosphate losses in their subsequent MS2 spectra. These signals could identify 3-formyl-1-butylpyrophosphate, formerly discovered in mycobacteria and TUBag2 (3), if the observed signals corresponded to their pseudomolecular species in negative mode. This assignment was then confirmed by MS2 of these parent ions, which matched the reported spectra of pseudomolecular, fully protonated forms of the natural 3fPP (M, 262, formerly referred to as TUBag1) and TUBag2 antigens (M, 276) (3). Such acidic forms readily fragment in negative mode when using collision energies of around 18–25 eV. These fragmentation spectra harbor prominent pyrophosphate (m/z 159 and m/z 177)- and phosphate (m/z 97 and m/z 79)-derived signals in addition to a product ion corresponding to each respective anhydro derivative of the pseudomolecular ion (Fig. 4). The TUBag2 molecule had been detected in former studies about mycobacterial phosphoantigens. With the same spectroscopic properties as, but a higher (+14) molecular mass than 3fPP, it corresponds to its longer chain homologue. We tentatively position this -CH2-o n the end of its chain, as the other -CH2- is in the middle of the molecule. This results in a shorter chain with lower energy, as confirmed by the mass spectrometry results. The structure of the TUBag2 molecule is shown in Fig. 4B. The TUBag2 molecule has been identified as TUBag2, which is identical to the mycobacterial TUBag1 and TUBag2 antigens.
E. coli Pathway of Phosphoantigen Biosynthesis—We proposed earlier that the γδ T cell-stimulating antigens of E. coli and of other bacteria might be derived from the DOXP pathway (5). Thus we tested several known metabolites of this biosynthetic route for their capacity to elicit a γδ T cell response. DOXP, 2-C-methylerythritol 4-phosphate (2CMEP), 4-diphosphocytidyl-2-C-methylerythritol, 4-diphosphocytidyl-2-C-methylerythritol 5-phosphate, and 2-C-methylerythritol cyclopyrophosphate did not exert any γδ T cell stimulatory activity in both activation and proliferation assays (Fig. 5A). Therefore a direct action of these upstream intermediates of the Rohmer pathway on γδ T cell activation could be excluded.

However, our previous experiments show that supplementation of E. coli cultures with deoxyxylulose enhances their γδ T cell-stimulating potency (5), suggesting that compounds derived from the DOXP pathway are responsible for γδ T cell activation. To further probe the relation between the Rohmer pathway and production of phosphoantigens, we cultured E. coli in the presence of the dephosphorylated 2-C-methylerythritol (2CME) metabolite from the DOXP pathway. Bacterial growth rates and the γδ T cell stimulatory capacity of LMWPs from 2CME and control cultures were compared. No differences in bacterial growth rate and cell numbers were observed between the cultures with or without 2CME. Unlike the experiments using deoxyxylulose as a supplement, the bioactivity of 2CME LMWPs was not enhanced when compared with control (data not shown). Thus dephosphorylated 2CME is neither an obligate requirement for E. coli cell growth nor for phosphoantigen production.

In the Rohmer pathway, 2CMEP is formed from DOXP by the action of an essential enzyme, the DOXP reductoisomerase, which can be selectively targeted by the phosphonate inhibitor fosmidomycin (29). Thus E. coli were grown in culture broth with and without this inhibitor, and both growth rates and phosphoantigen production were measured. Although fosmidomycin concentrations as high as 20 μg/ml did not make any difference in cell growth rates, the LMWPs of E. coli cultured in the presence of fosmidomycin were totally devoid of activity on γδ T cells (Fig. 5B). This effect was not due to inhibition of γδ T cell proliferation by the antibiotic itself (data not shown).

**DISCUSSION**

γδ T cells are suggested to play a role as the first line of defense against various viral, bacterial, and parasitical pathogens. They recognize soluble phosphoantigens from different Gram-negative and Gram-positive bacteria as well as certain parasites (for review, see Refs. 30 and 31). To better understand the way of action of this still enigmatic T cell population, it is important to characterize these antigens. So far only five phosphoantigens, 3fbPP, TUBag2, 3fb-PPP-uridine, 3fb-PPP-thymidine, and IPP have been identified as natural occurring γδ T cell ligands (2, 28). These compounds were isolated and identified from several mycobacterial species (32), and they are presumably common to the entire mycobacterial genus. Yet, IPP represents a ubiquitous metabolic precursor of isoprenoids and prenylated proteins.

In the present study, we have isolated the two compounds responsible for the γδ T cell-stimulating capacity of E. coli and proved that these phosphoantigens are identical to the mycobacterial metabolite 3-formyl-1-butyl-PP and its M₇, 275 homologue TUBag2. By treating E. coli LMWPs with either alkaline phosphatase or nucleotide pyrophosphatase alone or with both enzymes sequentially, we demonstrated that no nucleotidic phosphoantigens such as the mycobacterial 3fb-PPP-uridine or 3fb-PPP-thymidine were present (Fig. 1B). Thus the γδ T cell-stimulating capacity of E. coli was entirely due to one or several compound(s) bearing a terminal phosphate, which therefore are phosphoantigens. Two lines of evidence demonstrated that these phosphoantigens do not comprise IPP. On the one hand, the γδ T cell-activating compound(s) was chromatographically separated from IPP, and on the other hand, the E. coli-derived IPP was not detected in quantities sufficient to exert its bioactivity on γδ T cells (Fig. 2), which is known in the micromolar range (4). This finding is in agreement with a previous quantification study showing that the IPP content of E. coli is not high enough to elicit a γδ T cell response (5).

The bioactivities of the E. coli phosphoantigen(s) were estimated in the nanomolar range on the basis of the titration of bioactivity in partially purified E. coli extracts as compared with the phosphate detection threshold of HPLC-ESI-MS². Therefore, these E. coli phosphoantigens exert bioactivities on γδ T cells with similar potencies to the mycobacterial phosphoantigens (2). Furthermore, a selected reaction-monitoring experiment scanning for a typical fragmentation reaction of
3-formyl-1-butyl-pyrophosphate (precursor ion, m/z 261 pseudo molecular ion; daughter ion, pyrophosphate moiety) resulted in a signal co-eluting with the γδ T cell-stimulating activity of the E. coli sample, indicating that the E. coli phosphoantigens could comprise a molecular isobar of 3fbPP.

The enrichment of E. coli phosphoantigens in a bioactive fraction from HPLC-separated LMWPs was undertaken by subsequent HPAEC monitored by bioassay. Here again, although a minute amount of IPP could be detected conductometrically, this product was separated from the phosphoantigens, which eluted earlier from the column. The direct MS analysis of the pooled bioactive fractions by nanospray MS indicated the presence of both 3fbPP (M, 262) and its longer chain homologue TUBag2 (M, 276). These identifications were confirmed by the complete MS² fragmentation map of these parent ions (Fig. 4). The lower mass compound was 3fpp, whereas the second phosphoantigen was tentatively identified as 3fpePP. As indicated by the above enzyme assays, no nucleotide analogue of phosphoantigens was present in these extracts, so E. coli phosphoantigens are only composed of 3fbPP and 3fpePP.

In the third part of this study, we studied the metabolic pathway involved in biosynthesis of these phosphoantigens by E. coli. Like mycobacteria, E. coli synthesize IPP and subsequent isoprenoids by the Rohmer pathway (33, 34). In agreement with several other reports, we demonstrate here that this metabolic route (also referred to as DOXP pathway) is responsible for phosphoantigen biosynthesis in E. coli. Because DOXP has also been described as a precursor for the biosynthesis of thiamin and pyridoxol (35, 36), the first specific metabolite of the Rohmer pathway is 2C-methylerythritol 4-phosphate (CMEP, CMEP, Fig. 6). Hence, we conducted E. coli feeding experiments using 2C-methylerythritol (CME) as a supplement of culture medium. Although bacterial culture supplemented with the dephosphorylated deoxyxylulose intermediate enhances the ability of E. coli LMWPs to stimulate γδ T cells (5) (indicated by reaction 1 in Fig. 6), we find here that similar experiments with 2C-methylerythritol do not cause such effects (data not shown; see reaction 2 in Fig. 6). These observations do not necessarily argue against phosphoantigen production through metabolites of the Rohmer pathway. Others have already reported extremely low incorporation rates of free 2CME as compared with deoxyxylulose in E. coli and in other species. This could be due to missing a specific kinase for 2CME, whereas another kinase able to phosphorylate deoxyxylulose seems to be present in bacteria (Ref. 10; see Fig. 6).

Because it was not possible to prove the involvement of 2CMEP in the biosynthesis of the γδ T cell-stimulating antigen by direct supplementation of 2CME, we attempted to inhibit DOXP reductoisomerase instead. This enzyme catalyzes the formation of 2CMEP from DOXP, probably through involvement of an aldehyde intermediate. The antibiotic fosfomycin inhibits this step of the DOXP pathway (8). Here we have found that addition of fosfomycin to E. coli cultures completely abolishes γδ T cell-stimulating activity (Fig. 5B). This clearly proved that formation of 2CMEP and, thus, the Rohmer pathway is essential for the biosynthesis of the phosphoantigens in E. coli. This conclusion, based on biochemical studies, is in perfect agreement with conclusions drawn by others from genetic lines of evidence. In recently published experiments, it is demonstrated that disruption of dxr, a gene coding for DOXP synthase, and of gcpE, a gene of the DOXP pathway with unknown function, abrogates the ability of E. coli extracts to stimulate γδ T cell proliferation (37).

The data presented here allow us to state that the E. coli phosphoantigens 3fbPP and 3fpePP are either authentic metabolites of the Rohmer pathway to IPP downstream of 2CMEP or that their formation branches somewhere from this route. Because the bioactive 3fbPP and 3fpePP found in E. coli were already characterized as mycobacterial phosphoantigens, it seems very likely that they were formed by the same way. However, the nucleotide-containing antigens of mycobacteria 3fb-PPP-thymidine and 3fb-PPP-uridine may most likely arise from the side reaction, as no corresponding compounds were found in E. coli so far. Such a side reaction is expected to bind 3fpp to a nucleotide moiety, most likely as an energetically favorable carrier. Interestingly enough, the E. coli gene ygbB, an ortholog of the microbial gene ygbB involved in terpenoid biosynthesis, was recently characterized as coding for an enzyme of the DOXP pathway that hydrolyzes the nucleotidic conjugate 2C-methyl-D-erythritol-2-phosphate into 2C-methyl-D-erythritol-2,4-cyclophosphate and CMP (38). In this regard, it is highly conceivable that the mycobacterial phosphoantigens 3fb-PPP-thymidine and 3fb-PPP-uridine arise from related enzymatic activities encoded by mycobacterial orthologues of some ygb genes missing from the E. coli genome.

A common feature of many microorganisms recognized by γδ T cells is their ability to synthesize isoprenoids via the DOXP pathway. Indeed, this pathway is absent from human or pri-
mate cells, which all share the TCR V\gammaVδ2-specific reactivity to phosphoantigens. Therefore, the Rohmer metabolic route proves an ideal target for focusing efficiently the antimicrobial selectivity of major histocompatibility complex-unrestricted human T lymphocytes.

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