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Research paper

Profiling of defense responses in *Escherichia coli* treated with fosmidomycin

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ABSTRACT

The mevalonate-independent isoprenoid biosynthesis pathway has been recognized as a promising target for designing new antibiotics. But pathogens treated with compounds such as fosmidomycin, a slow binding inhibitor of 1-deoxy-D-xylulose 5-phosphate reducto-isomerase, the second enzyme in this pathway, develop rapid drug resistance. In *Escherichia coli*, acquired resistance results mostly from inactivating the cAMP-dependent glpT transporter, thereby preventing import of the inhibitor. Such mutant strains are characterized by cross-resistance to fosfomycin, by susceptibility to efflux pump inhibitors, by disability to use glycerol 3-phosphate as a carbon source or by increased activity of the promoter controlling the expression of the *glpABC* regulon when grown in presence of fosmidomycin. The quite challenging task consists in conceiving new and efficient inhibitors avoiding resistance acquisition. They should be efficient in blocking the target enzyme, but should also be durably taken up by the organism. To address this issue, it is essential to characterize the mechanisms the pathogen exploits to defeat the antibiotic before resistance is acquired. Having this in mind, a 2-D Fluorescence Difference Gel Electrophoresis proteomic approach has been applied to identify defense responses in *E. coli* cells being shortly exposed to fosmidomycin (3 h). It seems that combined strategies are promptly induced. The major one consists in preventing toxic effects of the compound either by adapting metabolism and/or by getting rid of the molecule. The strategy adopted by the bacteria is to eliminate the drug from the cell or to increase the tolerance to oxidative stress. The design of new, but still efficient drugs, needs consideration of such rapid modulations required to adapt cell growth in contact of the inhibitor.

1. Introduction

The extensive use of antibiotics has raised the development of resistance to infectious diseases, which is a growing threat to human health even in developed countries. As a consequence, antibiotics are becoming increasingly useless, raising the need discovery of novel broad-spectrum antibiotics or novel targets. Most metabolic pathways in bacteria are similar to human ones and can therefore not be targeted by such antibiotics. However, other pathways, such as the alternative isoprenoid biosynthesis pathway, are distinct and are therefore predilections as targets. The biosynthesis of Δ^2- and Δ^3-isopentenyl diphosphate (DMAPP and IPP), the general C_5 isoprenoid precursors, proceeds *via* two pathways: The classical Bloch–Lynen mevalonic acid (MVA) pathway occurring in eukaryotes like Man, animals or fungi, and the alternative 2*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (for references see [1]) (Fig. 1). The development of many organisms (e.g. most eubacteria, the malaria parasite *Plasmodium falciparum*, algae and plastids of higher plants) is exclusively dependent on the prokaryotic MEP pathway, because they lack the MVA pathway (Fig. 1), at least for the biosynthesis of some specific primary metabolites [1]. In bacteria, most isoprenoids act as secondary metabolites, but molecules such as ubiquinones or menaquinones are crucial for

Abbreviations: 2-D DIGE, 2-D fluorescence difference gel electrophoresis; DMAPP, dimethylallyl diphosphate; Fos^R, fosmidomycin resistant strain; Fos^S, fosmidomycin sensitive strain; GFP, green fluorescent protein; IPP, isopentenyl diphosphate; LSM, laser scanning microscopy; MVA, mevalonic acid; MEP, 2*C*-methyl-D-erythritol 4-phosphate.

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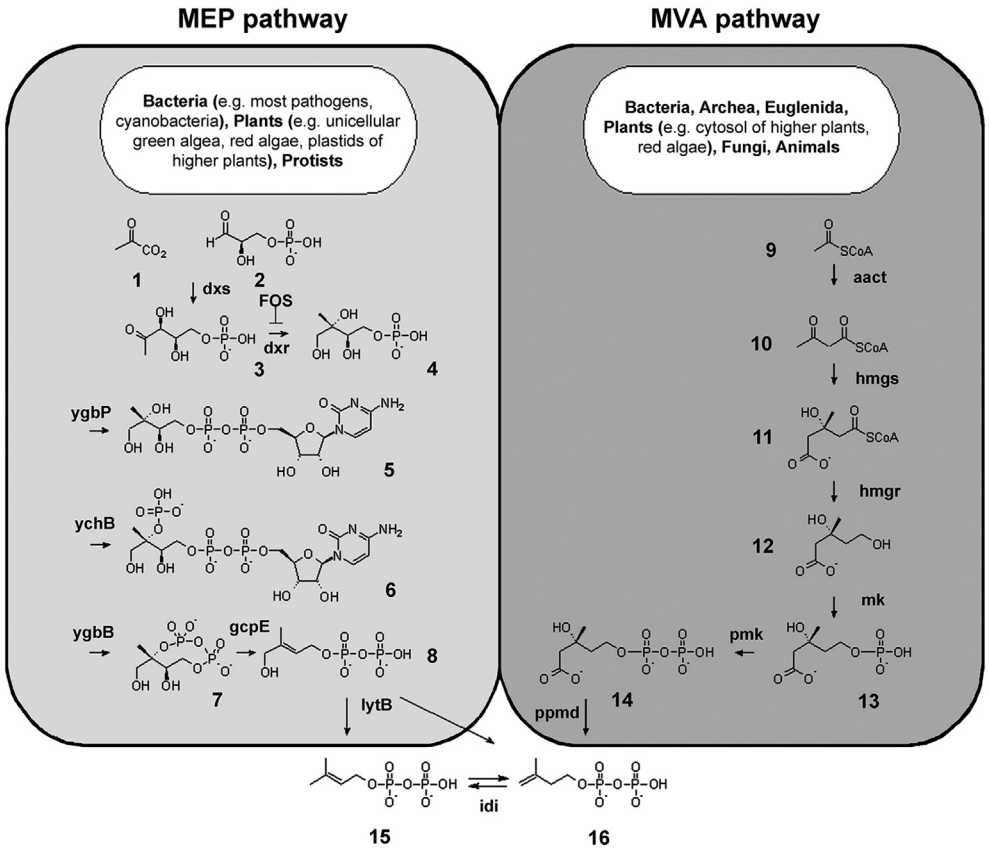

Fig. 1. Distribution in the living kingdom of two biosynthetic pathways for the biosynthesis of Δ^2- and Δ^3-isopentenyl diphosphate (DMAPP and IPP), the universal isoprenoid precursors. Distributions are described in more details by Lange et al. (2000) and Lombrand and Moreira (2010) [2,3]. Metabolites constituting the pathways are: 1: pyruvate, 2: D-glyceraldehyde 3-phosphate, 3: 1-deoxy-D-xylulose 5-phosphate, 4: 2*C*-methyl-D-erythritol 4-phosphate, 5: 4-diphosphocytidyl-2*C*-methyl-D-erythritol, 6: 2-phospho-4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol, 7: 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, 8: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate, 9: acetyl-CoA, 10: acetoacetyl-CoA, 11: 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), 12: mevalonic acid, 13: phospho-mevalonic acid, 14: diphospho-mevalonic acid, 15: isopentenyl diphosphate, 16: dimethylallyl diphosphate. Genes encoding the enzymes of the MEP pathway are: *dxs*: 1-deoxy-D-xylulose 5-phosphate synthase, *dxr*: 1-deoxy-D-xylulose 5-phosphate reducto-isomerase, *ygbP*: 4-diphosphocytidyl-2*C*-methyl-D-erythritol synthase, *ychB*: 2-phospho-4-(cytidine 5′-diphospho)-2*C*-methyl-D-erythritol kinase, *ygbB*: 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase, *gcpE*: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase, *lytB*: (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase, *idi*: isopentenyl diphosphate isomerase. Genes encoding enzymes of the MVA pathway are: *aact*: acetoacetyl-CoA thiolase, *hmgs*: HMG-CoA synthase, *hmgr*: HMG-CoA reductase, *mk*: mevalonate kinase, *pmk*: phosphomevalonate kinase, *ppmd*: diphosphomevalonate decarboxylase. Fosmidomycin (FOS) is blocking 1-deoxy-D-xylulose 5-phosphate reducto-isomerase.

growth. As a consequence, all seven enzymes constituting this prokaryotic IPP biosynthesis pathway can be exploited as potential targets for new anti-bacterial but also anti-parasitic antibiotic drugs or herbicides [4,5].

Highly active antibiotics are characterized by strong affinity to their targets, as well as by their efficient absorption to reach them. Limited efficacy often results from resistance acquisition. To overcome such a dilemma, intense research efforts are underway to design new synthetic inhibitors blocking specifically the activity of MEP pathway enzymes [6]. Two effective compounds have already been described: First, clomazone is widely used as a bleaching herbicide. Its oxidized metabolite oxo-clomazone was identified as inhibiting the first enzyme along the pathway, 1-deoxy-D-xylulose 5-phosphate synthase [7,8]. The second compound, fosmidomycin (Fig. S1), a phosphonic acid antibiotic, was isolated from *Streptomyces lavendulae* [9,10]. The molecular target 1-deoxy-D-xylulose 5-phosphate reducto-isomerase, the second enzyme in the MEP pathway also known as MEP synthase, had been identified by H. Seto and coworkers [11] (Fig. 1). Quite early, fosmidomycin was described as a highly efficient anti-bacterial compound [12,13], but unfortunately treated bacteria or parasites developed rapid resistance, mainly due to the lack of absorption of the chemical by various pathogens (Table 1 [9,14–21]). Thus, the search for novel active and efficient compounds relies on understanding their molecular mechanisms and the related bacterial responses in combination with a remarkable adaptive potential. Nonetheless, the question that still remains open is how do such microbes adapt to antibiotics, in particular to fosmidomycin?

2. Materials and methods

2.1. Fos^R strain selection and bacterial growth

Escherichia coli XL1-Blue cells (Stratagene) grown until reaching stationary phase (140 rpm, 37 °C) were subcultured into liquid Luria–Bertani (LB) medium (Sigma) supplemented with tetracycline (12.5 μg/mL) and containing increasing concentrations of fosmidomycin. This procedure was repeated 8 times with inhibitor concentrations starting from 0.01 up to 100 μM. The Fos^R resistant strain was selected on LB-agar plates supplemented with 200 μM fosmidomycin. The whole chromosomal sequence encoding the glpTQ and glpABC regulons (position 2347915–2354926) was sequenced using primer listed in Table S1 (Fig. S2). To verify that