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EXPERIMENTAL ARTICLES

Occurrence of Nonmevalonate and Mevalonate Pathways for Isoprenoid Biosynthesis in Bacteria of Different Taxonomic Groups

S. M. Trutko*[1], L. V. Dorofeeva*, V. A. Shcherbakova*, N. A. Chuvil'skaya*, K. S. Laurinavichus*, V. I. Binyukov, D. N. Ostrovskii**, M. Hintz***, J. Wiesner***, H. Jomaa***, and V. K. Akimenko***

**Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*

***Bach Institute of Biochemistry, Russian Academy of Sciences, Leninskii pr. 33, 117071 Moscow*

****Institute of Biochemistry, Gissen University, Gissen, Germany*

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Abstract—The effect of fosmidomycin and mevinoline, inhibitors of the nonmevalonate and the mevalonate pathways of isoprenoid biosynthesis, respectively, on the growth of 34 anaerobic and 10 aerobic prokaryotic strains was studied. Fosmidomycin at the concentrations used was shown to inhibit the growth of 9 (of 10) representatives of the family *Microbacteriaceae*, 4 (of 5) strains of *Thermoanaerobacter*, and 11 (of 12) strains of *Clostridium*, whereas mevinoline inhibited the growth of lactobacilli (*Carnobacterium*), methanogenic and sulfate-reducing bacteria insensitive to fosmidomycin. During the late growth phase, four strains of actinobacteria (of nine) accumulated the compound, which, upon oxidation, generates a long-lived free radical; three strains synthesized 2-C-methyl-*D*-erythritol-2,4-cyclopyrophosphate (MEC). It was concluded that the difference in the sensitivity of the organisms to fosmidomycin and mevinoline might serve as a test to differentiate several representatives of the family *Microbacteriaceae*. The use of mevinoline for inhibiting methanogens in ecological investigations seems to be promising.

Key words: isoprenoid biosynthesis, the lysodextrose radical, MEC, fosmidomycin, actinobacteria, anaerobic bacteria, mevinoline, ^{1}H NMR and EPR spectrometry.

Isoprenoids exist in all organisms. In bacteria, they are involved in respiration and cell wall biosynthesis, are the constituents of a number of pigments, etc. Both in higher organisms and prokaryotes, the isoprenoid synthesis occurs via successive condensation of isopentenyldiphosphate monomers [1, 2]. In higher animals, in the cell cytoplasm of higher plants, and in certain prokaryotes, the monomer is formed by stepwise condensation of three acetyl-CoA molecules, and its biosynthesis is inhibited by mevinoline (the mevalonate pathway). In plastids of higher plants, in protozoa, and in most of the bacteria investigated, isopentenyldiphosphate is formed of pyruvate and 3-phosphoglyceric aldehyde (the nonmevalonate pathway). Fosmidomycin and active oxygen species inhibit this pathway, suppressing the activity of the enzymes DOXP and GcpE, respectively [3]; in this case, the cell wall biosynthesis, dependent on the bactoprenol concentration, is the most vulnerable [4]. Acetyl Co-A is formed by all bacteria; pyruvate and 3-phosphoglyceric aldehyde are the products of glucose metabolism, but they may also be synthesized in the anaplerotic pathways. Therefore, the conclusions as to the functioning of one or another pathway, based on the incorporation of 1-[^{13}C]acetate, 1-[^{13}C]glucose, [^{14}C]acetyl-CoA, and [^{14}C]mevalonate into isoprenoids of microorganisms [14], must be confirmed by other methods. Thus, the genes encoding the nonmevalonate pathway were found in the bacteria in which the experiments with [^{14}C]acetyl-CoA showed the functioning of the mevalonate pathway. Along with this, in *Streptomyces* sp. the genes of both biosynthesis pathways were revealed [5]. These data testify that the pathway of isoprenoid biosynthesis can be revealed from a complete analysis of the genome of a prokaryotic organism. Every year, taxonomists describe a large number of new bacteria, the comprehensive analysis of whose genomes seems to be a remote goal. Specific inhibitors may be used for analyzing the functioning pathways of isoprenoid biosynthesis. However, their use is limited either by poor solubility in water or the ability of the bacteria to destroy these compounds.

[1] Corresponding author. E-mail: trutko@ibpm.pushchino.ru

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Table 1. Influence of fosmidomycin on the growth of bacteria of the family *Microbacteriaceae*

Organisms	Inhibition of growth (%) and pigmentation in the presence of fosmidomycin					
	Control	+ fosmidomycin (mg/ml)				
		0.05	0.10	0.30	0.50	1.00
Agreia bicolorata VKM Ac-1804	Orange	25 O	25 O	50	50	75
Agreia sp. VKM Ac-1783	Yellow	0 Y	25 Y	75	75	75
Graminibacter sp. VKM Ac-1786	Yellow	–	0 Y	0 Y	0 Y	0 Y
Microbacterium sp. VKM Ac-2047		0	0	0	25	50
Microbacterium sp. VKM Ac-2049*	Yellow	25	50	50	50	75
Microbacterium sp. VKM Ac-2050	Yellow	0	0	25	50	75
Microbacterium sp. VKM Ac-2053	Yellow	50	50	50	75	100
Rathayibacter rathayi VKM Ac-1601	Yellow	0 Y	25	25	50	75
Rathayibacter tritici VKM Ac-1603	Yellow	0 Y	25*	25	50	50
Clavibacter michiganensis subsp. *michiganensis* VKM Ac-1403^T	Yellow	50	75	100	100	100

Note: The results are expressed as a percentage of the control. Abbreviations: Y, yellow; O, orange; the absence of letter, no coloration; *, the coloration is weak; –, not measured.

Therefore, the interpretation of the data obtained may be unambiguous only in the case of a positive effect.

The successful testing of fosmidomycin as a remedy for malaria [6], which kills millions of people every year, as well as the involvement of the nonmevalonate pathway intermediates in human immune reactions [7], offers new promise for this antibiotic and makes expedient testing its action on the broad range of microorganisms. The aim of this work is to determine, using inhibitors, the pathways of isoprenoid biosynthesis in a large group of anaerobic, facultatively anaerobic, and aerobic bacteria.

MATERIALS AND METHODS

The microorganisms considered in this study are listed in Tables 1, 2, and 3. The strains of bacteria and archaebacteria were obtained from the All-Russia Collection of Microorganisms. *Micrococcus luteus* VKM B-1314, *Corynebacterium ammoniagenes* ATCC 6872, and *Methanobacterium thermoautotrophicum* ΔH were used in the comparative experiments as controls.

The methods for cultivating actinobacteria and *C. ammoniagenes* ATCC 6872 were described earlier [8, 9]. The bacteria of the genera *Thermoanaerobacter, Thermobacteroides*, and *Clostridium* were grown in the PYG medium; *Carnobacterium*, in medium 141 [10]; and *Anoxybacillus pushchinoensis*, in medium 838 [10]; glucose or cellulose served as the source of carbon. For methane-forming archaebacteria, mineral medium PBBM [11] was used with $H_2 + CO_2$ (*Methanobacterium* sp.) or methanol (*Methanosarcina* sp.) as a source of carbon. Widdel and Pfennig's medium [12] with lactate (4 g/l) was used for sulfate-reducing bacteria. All anaerobic and facultatively anaerobic prokaryotes were grown in Hungate test tubes with a medium volume of 10 ml; the inoculum was introduced in an amount of 10 vol %.

The growth of bacteria was determined from the absorbance at 540 nm measured with a Spekol 221 spectrophotometer (Germany) or from the amount of accumulated methane measured chromatographically [13].

Actinomycete cells from the exponentially growing culture (18 h) were used for the cell extract preparation. The bacteria were sedimented for 15 min at 5000 *g* and 4°C and washed with 0.1 M NaCl solution. The biomass was lyophilized, treated with D_2O, and extracted with 50% methanol. 2-C-Methyl-*D*-erythritol-2,4-cyclopyrophosphate (MEC) and the saccharides generating long-lived free radicals passed into the extract. After removing the solvents in the rotary evaporator and redissolving the residue in D_2O, the presence of MEC and saccharides in cell extracts was determined by the method of ^1H NMR and EPR spectroscopy, respectively. ^1H NMR spectra were recorded with a Bruker WM-250 spectrophotometer (Germany) using 2,2-dimethyl-2-silapentane-sulfonic acid as the internal standard. The EPR spectra were recorded with an RE-1306 radiospectrometer (Russia) in a 50-μl quartz ampoule using Mn^{2+}/MgO as the external standard; its third and fourth spectral components are position on both sides of the signal of the substance investigated. Immediately before the measurements, $K_3[Fe(CN)_6]$ was added to the sample to a concentration of 1 mM.

Menaquinones were extracted from the lyophilized biomass with a chloroform–methanol mixture (2 : 1); the extract was applied on a Kieselgel 60 thin-layer plate (Merck) and separated in a hexane–diethyl-ether system (85 : 15). Menaquinones were eluted with chlo-

roform and analyzed using a Finnigan-mat 8430 mass-spectrometer (Germany).

The fosmidomycin concentration inhibiting the growth of actinobacteria was determined by the dilution method on the dishes with KBA medium containing 1.0, 0.5, 0.3, 0.1, and 0.05 mg/ml fosmidomycin. Inoculation was carried out by exhaustive streak or replica. The duration of incubation was 72 h. The fosmidomycin concentration inhibiting the growth of anaerobic bacteria was determined by the dilution method in test tubes containing 0.01, 0.1, 0.25, and 1.0 mg/ml fosmidomycin.

Fosmidomycin obtained from Jomaa Pharmaka GmbH and mevilonine obtained from Sigma were used in this work.

RESULTS AND DISCUSSION

The data obtained on the effect of fosmidomycin on aerobic actinobacteria are shown in Table 1. The antibiotic affected the growth of almost all tested representatives of the family *Microbacteriaceae*, which was indicative of the nonmevalonate pathway of isoprenoid biosynthesis. Since the synthesis of 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC) under the oxidative stress conditions could serve as a proof of nonmevalonate pathway functioning [9], we decided to grow actinobacteria under such conditions and determine MEC in the cells. The introduction of trace amounts of benzylviologen into the growth medium appeared to prevent the biomass accumulation of strain Ac-1786. Further experiments revealed growth instability of this strain cultivated in a liquid medium. In a number of cases, the growth stopped at low biomass levels and was accompanied by the loss of long-chain menaquinones. A reduction of medium aeration led to the recovery of growth and the quinone biosynthesis. This meant that, under the standard growth conditions, the cells of *Graminibacter* sp. were exposed to oxidative stress. Therefore, the biomass grown under such conditions could be tested for the presence of MEC. The results of ^{1}H NMR spectrometric analysis are shown in Fig. 1a. The comparison of the spectra of the control culture of *C. ammoniagenes* before (Fig. 1a, spectrum 10) and after (Fig. 1a, spectrum 11) the incubation with benzylviologen indicates that the oxidative stress resulted in the appearance of a new signal (darkened area) typical of MEC. In the same spectral region, the signal is also present in *Graminibacter* sp. VKM Ac-1786 (Fig. 1a, spectrum 1). Consequently, the nonmevalonate pathway of isoprenoid biosynthesis is also characteristic of *Graminibacter* sp. VKM Ac-1786 (Fig. 1a, spectrum 1).

A similar biomass analysis of other actinobacteria revealed the presence of MEC in *Microbacterium* sp. VKM Ac-2050 (Fig. 1a, spectrum 2) and *Microbacterium* sp. VKM Ac-2049 (Fig. 1a, spectrum 5), i.e., during the late logarithmic growth phase, three of the nine cultures studied were under oxidative stress conditions and synthesized MEC.

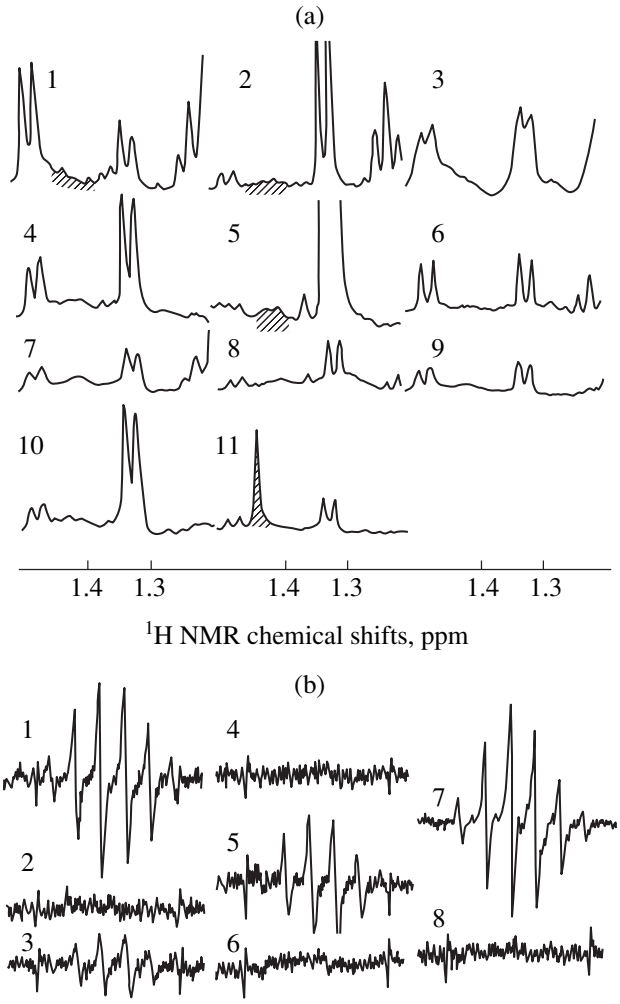

Fig. 1. ^{1}H NMR and EPR spectra of cell extracts of the bacteria of the family *Microbacteriaceae*. (a) The ^{1}H NMR and EPR spectra of (1) *Graminibacter* sp. VKM Ac-1786; (2) *Microbacterium* sp. VKM Ac-2050; (3) *Rathayibacter toxicus* VKM Ac-1600; (4) *Microbacterium* sp. VKM Ac-2053; (5) *Microbacterium* sp. VKM Ac-2049; (6) *Microbacterium* sp. VKM Ac-2047; (7) *Agreia* sp. VKM Ac-1783; (8) *Agreia bicolorata* sp. VKM Ac-1804; (9) *Rathayibacter rathayi* VKM Ac-1601; (10) *Corynebacterium ammoniagenes* ATTC 6872; (11) *Corynebacterium ammoniagenes* ATTC 6872 after incubation with benzylviologen. (b) The EPR spectra of (1) *Rathayibacter toxicus* VKM Ac-1600; (2) *Agreia bicolorata* sp. VKM Ac-1804; (3) *Agreia* sp. VKM Ac-1783; (4) *Microbacterium* sp. VKM Ac-2047; (5) *Rathayibacter rathayi* VKM Ac-1601; (6) *Microbacterium* sp. VKM Ac-2053; (7) *Graminibacter* sp. VKM Ac-1786; (8) *Microbacterium* sp. VKM Ac-2049.

The samples remaining after ^{1}H NMR spectrometry were analyzed for the presence of the hydroxylamine-type reducers. The accumulation of these compounds is supposed to indicate the functioning of an unknown metabolic pathway [14]. The results of the assays are shown in Fig. 1b. The addition of the acceptor

Table 2. Influence of fosmidomycin on the growth of anaerobic and facultatively anaerobic bacteria

Organisms	Inhibition of growth in the presence of fosmidomycin, %			
	0.01 mg/ml	0.1 mg/ml	0.25 mg/ml	1.0 mg/ml
Thermobacteroides acetoethylicus HTB2^T	15	15	15	–
Thermoanaerobacter lactoetanolicum ZE-1	0	11	45	91*
Thermoanaerobacter ethanolicus 39E	0	0	100	–
Thermoanaerobacter brokii HTD4^T	6	79–100	100	–
Clostridium sp. VKM B-2202	0	0	100	–
Clostridium sp. VKM B-2201	7	11	86	–
Clostridium thermosaccharolyticum DSM 571^T	0	20	100	–
Clostridium thermocellum VKM B-2203	0	34	95	–
Clostridium sporogenes 227	66	89–100	100	–
Clostridium sp. VKM B-2279	10	60	100	–
Clostridium sp. VKM B-2271	0	62		76
Clostridium frigoris DSM 14204^T	62	65		71
Clostridium lacusfryxellense DSM 14205^T	100	100		100
Clostridium bowmanii DSM 14206^T	6	8		15
Clostridium psychrophilum DSM 14207^T	71	72		68
Anoxybacillus pushchinoensis K1^T	0	26	59	–
Desulfotomaculum alcaliphilum S1^T	0	0	0	–
Desulfovibrio desulfuricans VKM B-1760^T	0	0	0	–
Carnobacterium funditum DSM 5970^T	0	0	–	11
Carnobacterium alterfunditum DSM 5972^T	0	0	–	0
Methanobacterium thermoautotrophicum ΔH^T	0	0	–	0
M. formicicum VKM B-1632	0	0	–	0
M. thermophilum VKM B-1786^T	0	15	–	22
M. bryantii VKM B-1629^T	0	0	–	0
M. ivanovii VKM B-1634^T	0	0	–	0
M. thermoflexum VKM B-1963^T	0	0	–	0
Methanosarcina mazei JL01	0	0	–	0
Methanosarcina mazei VKM B-2199^T	0	28	–	38
Methanosarcina barkeri VKM B-1635^T	0	0	–	10
Methanosarcina lacustris VKM B-2229^T	0	0	–	0

* The fosmidomycin concentration was 0.5 mg/ml.

($K_3[Fe(CN)_6]$) resulted in the appearance of a characteristic signal in the representatives of the genera *Rathayibacter* (Fig. 1b, spectra 1 and 5), *Agreia* sp. VKM Ac-1783 (Fig. 1b, spectrum 3), and *Gramini-bacter* sp. VKM Ac-1786 (Fig. 1b, spectrum 7). The EPR-spectra of the samples differed in intensity, and only the spectrum of the compound extracted from *Agreia* sp. VKM Ac-1783 had somewhat different parameters. The identity of the spectral characteristics of the reducer of hydroxylamine nature in genetically remote microorganisms virtually excluded its use for taxonomic purposes but pointed to the possibility of its use as a target in the search for new antibiotics.

Fosmidomycin inhibited the growth of *C. michiganensis* subsp. *michiganensis* VKM Ac-1403^T and *Microbacterium* sp. VKM Ac-2053 at a concentration of 0.3 and 1 mg/ml, respectively. The inhibition of growth of other actinobacteria varied depending on the antibiotic concentration (Table 1), but was always partial. The differences revealed might be due to the cell wall structure or switching to the second pathway of biosynthesis. However, mevinoline (the mevalonate pathway inhibitor) inhibited by 20–30% only the growth of *Gramini-bacter* sp. VKM Ac-1786, suggesting that only in this microorganism is the functioning of the two pathways of isoprenoid biosynthesis possible.

Table 3. Influence of mevinoline and fosmidomycin on the growth of anaerobic bacteria

Organism	Inhibition of growth, %		
	+ fosmidomycin	+ mevinoline	+ fosmidomycin + mevinoline
Methanobacterium thermoautotrophicum ΔH^T	0	90	86
Methanobacterium thermoalcaliphilum VKM B-1958^T	0	85	91
Methanosarcina barkeri VKM B-1629^T	0	63	57
Methanosarcina mazei S-6^T	30	67	67
Thermoanaerobacter brokii HTD4^T	72	38	100
Thermoanaerobacter thermohydrosulfuricus DSM 567^T	47	32	–
Clostridium stercorarium XCYB	89	32	–
Clostridium sporogenes 227	89	34	–
Carnobacterium funditum DSM 5970^T	0	57	45

Note: The concentration of each antibiotic was 0.1 mg/ml.

Note that, in our experiments, fosmidomycin inhibited by 50% the growth of *M. luteus*, for which the nonmevalonate pathway of biosynthesis is proven [9], at a significantly lower concentration (0.05 mg/ml) than the growth of the representatives of the family *Microbacteriaceae*.

The effect of antibiotics on the anaerobic organisms varied depending on their taxonomic identity. Thus, fosmidomycin (Table 2) did not influence the growth of the representatives of methanogenic (*Methanobacterium* and *Methanosarcina*) and sulfate-reducing (*Dm. alcaliphilum* S1 and *D. desulfuricans* VKM B-1760) bacteria utilizing mono- and tricarbon compounds but inhibited the growth of representatives of the genera *Thermoanaerobacter* and *Clostridium* metabolizing cellulose and/or glucose. In the latter case, the effective antibiotic concentration varied within wide limits depending on the strain, and two of them (*C. bowmanii* DSM 14206 and *T. acetoethylicus* HTB2) were even tolerant (10–15% inhibition) at the phosphonate compound levels used. The strain dependence of the fosmidomycin effect for *Thermoanaerobacter* and *Clostridium* may be explained by the variation in the cell wall structure, since the organisms studied live in different temperature ranges and ecological niches. However, the complete absence of sensitivity to fosmidomycin in lactobacilli of the genus *Carnobacterium* (also metabolizing glucose) suggested the functioning of the mevalonate pathway.

The effect of mevinoline was tested in a small group of microorganisms (Table 3). The introduction of the acetone solution of the antibiotic appeared to inhibit the development of methanogenic organisms by 60–90%. In the case of simultaneous introduction of both antibiotics, the degree of inhibition was the same as in the presence of mevinoline alone. This meant that only the mevalonate pathway functioned in methanogenic organisms. The sensitivity of *M. mazei* S-6 to fosmidomycin seems to be caused by the nonspecific action of the antibiotic. Mevinoline also inhibited the growth of *C. funditum* DSM 5970 both added alone and when introduced in combination with fosmidomycin. The addition of the latter did not affect the growth of this organism. Thus, the mevalonate pathway of biosynthesis is typical for *Carnobacterium.* In contrast to the microorganisms discussed above, the representatives of *Thermoanaerobacter* and *Clostridium* were sensitive to both antibiotics (Table 3). Fosmidomycin was more effective and generally decreased the biomass yield by 80–100%, whereas the degree of inhibition by mevinoline did not exceed 40% (Table 3), i.e., the nonmevalonate pathway of biosynthesis is characteristic for *Thermoanaerobacter* and *Clostridium.* The differences revealed in the action of the antibiotics on the organisms oxidizing the same carbon source reflect, in our opinion, the characteristic features of their metabolism. Clostridia are known to metabolize glucose via the fructosobisphosphate pathway and to possess both aldolase and triose phosphate isomerase [15]. The same enzymes function in homofermentative lactic acid bacteria. Heterofermentative lactic acid bacteria, also including the representatives of the genus *Carnobacterium*, lack the above enzymes; they realize the initial stages of glucose metabolism with the pentose cycle enzymes, forming acetylphosphate and glyceraldehyde-3-phosphate [16]. In other words, the nonmevalonate pathway of isoprenoid biosynthesis is typical of the vegetative cells of the anaerobic organisms that cleave glucose (or gluconic acid) into two three-carbon compounds. These metabolic pathways are also typical of the peptolytic clostridia, many of which are pathogenic to humans and animals. The nonmevalonate pathway of isoprenoid biosynthesis should also function in these microorganisms, and their growth should be inhibited by fosmidomycin. The high sensitivity of *C. sporogenes* to the inhibitor is evidence in favor of this conclusion (Table 2).

A large number of anaerobic bacteria tested were isolated from the methanogenic microbial communities inhabiting different ecological niches. In similar consortia, there exist multiple nutritive relations between microorganisms, impeding community investigation. The use of the inhibitors of the mevalonate and nonmevalonate pathways for biosynthesis of the isoprenoids exerting different effects on anaerobic bacteria might significantly facilitate the isolation of an individual organism and the study of its role in the community.

Thus, the nonmevalonate pathway of isoprenoid biosynthesis is characteristic of the representatives of the family *Microbacteriaceae* and the bacteria of the genus *Thermoanaerobacter* and *Clostridium*, whereas the mevalonate pathway was revealed in sulfate-reducing, methanogenic, and heterofermentative lactic acid bacteria. It suggested that the differences in the sensitivity of the organisms to fosmidomycin and mevinoline might serve as a test for differentiating several representatives of the family *Microbacteriaceae.* The use of mevinoline for suppressing methanogens in ecological investigations appears to be promising.

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