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SHORT COMMUNICATION

**D. Ostrovsky · G. Diomina · E. Lysak · E. Matveeva**
**O. Ogrel · S. Trutko**

# Effect of oxidative stress on the biosynthesis of 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate and isoprenoids by several bacterial strains

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**Abstract** In this study, the gram-negative bacteria *Xanthomonas campestris*, *Xanthomonas maltophilia*, and *Pseudomonas putida*, facultative parasites of plants and animals, were shown to accumulate 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC) in response to benzyl-viologen-induced oxidative stress. *Corynebacterium ammoniagenes* mutants capable of accumulating MEC in the absence of an exogenous oxidative stress inducer were obtained. Isoprenoid synthesis and MEC synthesis in these and other bacteria were shown to be alternative processes, while biosynthesis of brominated polyene xanthomonadin (an antioxidant pigment of *X. campestris*) increased concomitantly with the accumulation of MEC.

**Key words** 2-C-methyl-D-erythritol · *Xanthomonas* · *Pseudomonas* · *Corynebacterium* · Isoprenoid · Phosphate

## Introduction

Corynebacteria and mycobacteria are able to synthesize 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC) under oxidative stress conditions, which led us to suggest a connection between MEC metabolism and virulence of the pathogenic members of these genera, as well as MEC participation in the pathogenesis of tuberculosis (Ostrovsky et al. 1992, 1993). We observed that the bacteria tend to lose their pigmentation during oxidative stress induced by the presence of superoxide generators in cultivation medium. This loss could be explained by an influence of the reactive oxygen species upon the pigment itself, but recently published data on a newly discovered non-mevalonate pathway in the synthesis of isopenthenylpyrophosphate, the principal building unit of isoprenoids (Rohmer et al. 1996; Duvold et al. 1997; Kuzuyama et al. 1998), led us to postulate inverse dependence of MEC accumulation and isoprenoid accumulation upon oxidative stress. MEC was initially found in the gram-negative anaerobe *Desulfovibrio desulfuricans* (Turner et al. 1992) as a constitutive compound. However, there has been no report on MEC accumulation in gram-negative bacteria in response to oxidative stress. Therefore, we decided to investigate a range of gram-negative bacteria with special emphasis on parasitic ones.

D. Ostrovsky (✉) · G. Diomina · E. Lysak · O. Ogrel
Bakh Institute of Biochemistry RAS, Moscow;
117071 Leninsky Prospect, 33, Russia
e-mail: inbio@glas.apc.org, Fax +7-095-9542732

E. Matveeva
Institute of Phytopathology, Bolshie Viaziomy (Moscow region), Russia

S. Trutko
Institute of Biochemistry and Physiology of Microorganisms RAS, Pushino (Moscow region), Russia

## Materials and methods

### Strains, media, and growth conditions

Xanthomonas campestris pv. *campestris* N22, pv. *vasculorum* N1, pv. *malvacearum* N16, pv. *faseoli* 47, pv. *faseoli* 48, and pv. *vesicatorium* N324 were obtained from the collection of the Institute of Phytopathology (Bolshie Viaziomy, Russia). *Pseudomonas cepacea* N89 and *Pseudomonas aeroginosa* N303 were a gift from the Institute of Applied Microbiology (Obolensk, Russia). *Pseudomonas putida* VKM V-1301, *Pseudomonas fluorescens* VKM V-894, *Pseudomonas saccharophilia* VKM V488, *Pseudomonas aureofaciens* VKM V-1249 and *Pseudomonas* (*Xanthomonas*) *maltophilia* VKM V-591 were obtained from the Russian Collection of Microorganisms (Pushino). *Corynebacterium ammoniagenes* ATCC 6872 and *Micrococcus luteus* Flemming strain 2665 are from the collection of the Institute of Vaccines, Moscow, Russia. *Escherichia coli* strains B, C, C-600 str-2, K-235, CA-18, NF-58, NF-59, and DH-10B were a gift of I.A. Khmel (Institute of Molecular Genetics, Moscow, Russia).

Bacteria were grown aerobically in a liquid medium containing 1% peptone (Chemapol), 0.3% yeast extract (Sigma), and 0.5% NaCl. To investigate MEC accumulation, sterile glucose or saccharose up to a concentration of 1% was added to the cultivation medium. *Escherichia coli* was grown at 37 °C, *C. ammoniagenes* and *M. luteus* were grown at 30 °C, and *Xanthomonas* spp. and *Pseudomonas* spp. were grown at 28 °C. Artificial oxidative stress was induced by the addition of benzyl viologen dichloride (Sigma)

to the cultivation medium up to 50 μg/ml. Bacteria were harvested by centrifugation and were resuspended in water with 20% $D_2O$ up to 0.5–0.8 g (wet wt./ml$^{-1}$. The suspension was used for lipid extraction (Bligh and Dyer 1959) and for recording $^{31}$P-NMR spectra. In the latter case, EDTA (1 mM) and SDS (≅ 50 mg/ml) were added.

Analytical techniques

$^{31}$P-NMR spectra (average of 800–1000 scans) were recorded on a Bruker AM WB-400 spectrometer at a working frequence at 161.98 MHz at 20 °C. The external standard was triphenylphosphate in a $CCl_4$ solution (chemical shift, –18 ppm). Absorption spectra of lipids in methanol/chloroform cell extracts were recorded on a Specord (GDR) spectrophotometer. The total amount of pigments was determined by measuring the absorption at 450 nm.

For thin-layer chromatography of lipids, Silica gel G plates (Merck) and an eluent mixture of hexane/benzene/chloroform (1:1:1, by vol.) were used to separate naphthoquinones and ubiquinones, and chloroform/methanol/water (65:25:4, by vol.) was used to separate pigments. Routinely, quinones were visualized with leukomethylene blue reagent (Crane and Barr 1971) and compared with the standards ubiquinone Q10 and vitamin $K_2$. In several samples, the quionone content was quantitatively estimated by standard HPLC technique (Tamaoka 1986).

## Results and discussion

We screened 23 strains of gram-negative bacteria (*Pseudomonas* spp., *Xanthomonas* spp., and *E. coli* strains) for their ability to accumulate MEC under oxidative stress conditions. As can be seen from Table 1 and Fig. 1, all the *X. campestris* strains accumulated MEC under oxidative stress conditions, while only a few pseudomonads, e.g., *P. putida*, accumulated MEC. In suspensions of *P. cepacea*, *P. aeruginosa*, and *P. aureofaciens*, and in all the *E. coli* strains examined (B, C, C-600 str-2, K-235, CA-18, NF-58, NF-59, and DH-10B), MEC signals were not detected, except for a trace signal in *E. coli* XL-1 Blue (Stratagene).

**Table 1** Relative intensity of a $^{31}$P-NMR signal with a chemical shift of –14.8–15 ppm, characteristic of 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate (MEC) in bacterial cell suspensions grown aerobically in a medium supplemented with 50 μg benzyl viologen/ml. –, +, ++, +++, and ++++. Denote increasing content of MEC (*nd* not determined)

| Strain | | 1% Surcose medium | 1% Glucose medium |
|---|---|---|---|
| *Xanthomonas campestris* | | | |
| pv. *vasculorum* | 1 | + | +++ |
| pv. *malvacearum* | 16 | – | + |
| pv. *campestris* | 22 | + | ++ |
| pv. *faseoli* | 47 | + | + |
| pv. *faseoli* | 48 | nd | +++ |
| pv. *vesicatorium* | 324 | + | ++ |
| *Pseudomonas saccharophilia* | VKM B-488 | nd | + |
| *Pseudomonas putida* | VKM B-1301 | nd | + |
| *Pseudomonas maltophilia* | VKM B-591 | nd | ++++ |

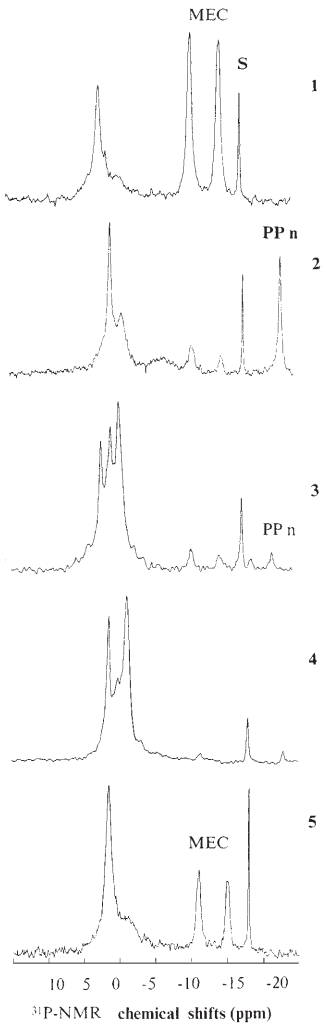


**Fig. 1** $^{31}$P-NMR spectra of cell suspensions of gram-negative bacteria grown aerobically. *1 Xanthomonas maltophilia* (medium plus glucose and benzyl viologen), *2 Pseudomonas putida* (medium plus glucose and benzyl viologen), and *3–5 X. campestris* pv. *campestris*; (*3* medium is supplemented with sucrose and benzyl viologen, *4* medium is supplemented with glucose only, and *5* medium plus glucose and benzyl viologen) (*S* external standard, *PPn* polyphosphates, *MEC* signals at –10.7 and –14.8 ppm due to 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate)

All the *X. campestris* strains examined are plant pathogens (Young et al. 1992), and they experience oxidative stress caused by a self-defense system of host plant tissues (Gay et al. 1996). However, biomass of *X. campestris* pv. *campestris* N22 isolated from black rot of cabbage stalks did not contain detectable amounts of MEC when examined by NMR. Bacterial cells may encounter oxidative stress in the living plant tissue, while persistence in dead tissue may lead to the utilization of preaccumulated MEC.

Benzyl-viologen-resistant *C. ammoniagenes* mutants were able to grow on solid medium supplemented with benzyl viologen (up to 100 μg/ml). All 25 mutants tested accumulated MEC when cultivated in benzyl-viologen-containing liquid medium, but failed to accumulate MEC in the absence of benzyl viologen. $^{31}$P-NMR spectra of cell suspensions of two benzyl-viologen-sensitive *C. ammoniagenes* mutants, E-1 and E-13, in different growth phases are presented in Fig. 2. As can be seen both mutants were able to accumulate MEC in the absence of the exogenous redox cycler. The highest level of MEC and other phosphates (possibly UDP derivatives) was observed in the exponential growth phase. In the stationary phase of mutant E1, the MEC concentration decreased to

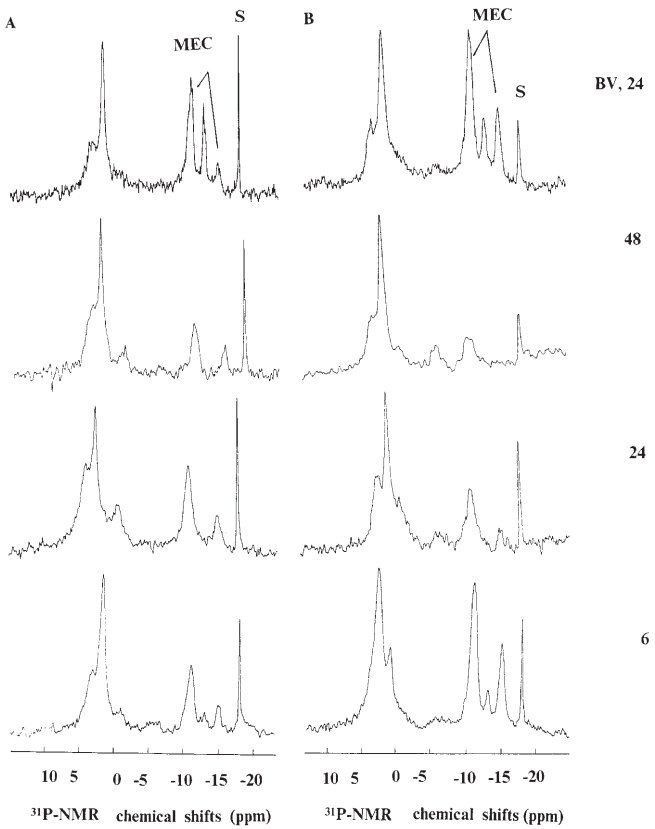


**Fig. 2** $^{31}$P-NMR-spectra of *Corynebacterium ammoniagenes* mutants E-13 (**A**) and E-1 (**B**). These mutants were selected for increased sensitivity to benzyl viologen (S external standard; *MEC* signals from 2-C-methyl-D-erythritol-2,4-cyclopyrophosphate at chemical shift –10.7 and –14.8 ppm; *6, 24, 48* hours of cultivation time; *BV,24* culture after 6 h of growth was supplemented with benzyl viologen and then incubated for a further 18 h). There was no glucose in the medium of mutant E-13

zero. The system used to neutralize superoxide may be damaged in these mutants, and a high rate of respiration in exponentially growing cultures is presumably responsible for superoxide accumulation to a level sufficient for activation of the MEC synthase.

The ratio of carotenoid content per biomass unit of *C. ammoniagenes* E13 grown in 50 or 400 ml medium per flask was 4, and that of MEC was 0.5. These results indicate that carotenoid biosynthesis and MEC biosynthesis are alternative processes. Indeed, when lipids were extracted from *C. ammoniagenes* wild-type and mutant E-13 cells [1 g (wet wt.) and 10 ml chloroform-methanol mixture], the $OD_{450}$ was 0.74 and 0.26, respectively. Thus, MEC is possibly accumulated at the expense of normal isoprenoid compounds. *M. luteus* showed the same effect. Incubation in the presence of benzyl viologen induced a sharp decrease in the carotenoid content (to 20% of the original level).

Carotenoid biosynthesis is strongly influenced by aeration (Nicolas et al. 1994). When oxygen is added to cultures of *Rhodopseudomonas spheroides*, carotenoid synthesis terminates. Synthesis starts again when $O_2$ is completely depleted (Goodwin 1981). However, in our studies, increased aeration achieved by decreasing the liquid culture level in shaken Erlenmeyer flasks led to the opposite effect, and this corresponds to other reported observations on the role of oxygen in carotenoid biosynthesis (Kobayashi et al. 1993).

*Xanthomonas* possesses a pigment that was thought to be a carotenoid and appeared to be a brominated polyene (Andrews et al. 1973). This gave us a rare opportunity to check the relationship between MEC and carotenoid synthesis. *X. campestris* pv. *campestris* N22 synthesized no MEC in the usual growth medium and a relatively large amount of MEC (comparable with the total amount of other phosphates; see Fig. 1 and Table 1) in the benzyl-viologen-supplemented medium. However, the pigment content did not decrease, but increased by 3–22% in the presence of benzyl viologen. The same pattern was observed with *X. campestris* pv. *faseoli* N 48 and *X. campestris* pv. *vesicatorium*.

Carotenoid synthesis in eukaryotes is regulated mainly by the desaturation level due to activity of the dehydrogenase (Goodwin 1981; Nicolas et al. 1994). The reaction of cells to reactive oxygen species may differ from this mechanism. To determine whether the earlier biosynthesis stage is regulated when isoprenoid chain building blocks are constructed, we tried to semi-quantitatively determine the amount of other isoprenoids, namely menaquinones, which are components of respiratory chains in *M. luteus* and *C. ammoniagenes* (Collins and Jones 1981). The diameters of spots of menaquinone on silica gel plates were compared with known quantities of vitamin $K_2$ as a standard. Menaquinones complexed with luminescent compounds can be detected in UV light and can also be identified by the leukomethylene blue reagent (Crane and Barr 1971). Using both these techniques, we showed that the menaquinone content of *C. ammoniagenes* and *M. luteus* after incubation with benzyl viologen and of mutants E-1 and E-13 grown in benzyl-viologen-free media, decreased two- to threefold in comparison with nonmutant cells. These data were supported by selected measurements of quinones of *C. ammoniagenes* separated on a reversed-phase HPLC column. The total menaquinone content for the nonmutant was 0.059 nmol (mg cell protein)$^{-1}$ and 0.023 and 0.021 nmol (mg cell protein)$^{-1}$ for mutants E-1 and E-13, respectively.

Recently the participation of 2-C-methyl-D-erythritol 4-phosphate (MEP) as an intermediate of the glyceraldehyde-3-phosphate/pyruvate pathway to isopentenyl diphosphate has been clearly shown (Kuzuyama et al. 1998). Most probably, MEP is withdrawn from the pathway due to inhibition of MEP dehydrotase under conditions of oxidative stress, leading to an accumulation of the dead-end product MEC and a concomitant depletion of the cells of the $C_5$ precursor for isoprenoid biosynthesis.

Carotenoids are good protectors against reactive oxygen species. Inhibition of their synthesis by reactive oxygen species places stress on bacterial cells. Substitution of carotenoids in their function as antioxidants by brominated polyene esters (xanthomonadine) in *Xanthomonas*

(Andrews et al. 1973; Rajagopal et al. 1997) may serve as an adaptation to maintain and increase the antioxidant pigment concentration under oxidative stress conditions in damaged plant tissues.

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