Microbiology, Vol. 73, No. 2, 2004, pp. 139–142. Translated from Mikrobiologiya, Vol. 73, No. 2, 2004, pp. 175–179.

EXPERIMENTAL ARTICLES

Deuterium Oxide as a Stress Factor for the Methylotrophic Bacterium *Methylophilus* sp. B-7741

A. N. S. Newo*, A. B. Pshenichnikova*,[1], D. A. Skladnev,[1], and V. I. Shvets***

** Department of Biotechnology, Lomonosov State Academy of Fine Chemical Technology, pr. Vernadskogo 86, Moscow, 117571 Russia*

*** Laboratory of Genetics of Methylotrophs, State Research Institute for Genetics and Selection of Industrial Microorganisms, Pervyi Dorozhnyi proezd 1, Moscow, 113545 Russia*

Received July 7, 2003; in final form, July 31, 2003

Abstract—The adaptation of the methylotrophic bacterium *Methylophilus* sp. B-7741 to growth in highly deuterated media was studied. For the first time, we showed the cross adaptation of bacterial cells to deuterated media and oxidative and osmotic stresses. The activity of catalase in deuterated cells was higher than in the control cells. Deuterated cell-free culture liquids showed protective effects on the growth of *Methylophilus* sp. B-7741 in deuterated media, which was manifested as an increase in the deuterated biomass yield. These data and the data available in the literature suggest that the mechanisms of bacterial cell adaptation to heavy water and to oxidative and osmotic stresses are similar.

Key words: adaptation, deuterium oxide, oxidative and osmotic stresses, extracellular factors of adaptation.

Many microorganisms are able to grow in highly deuterated media containing up to 99.8% of deuterium oxide (heavy water, ^2H_2O, D_2O), although microbial growth in such media is slow and requires preliminary adaptation. The adaptation period and the growth parameters of microorganisms in deuterated media depend on the nature of the carbon source and the physiological characteristics of the microorganisms. Although the biological effects of D_2O have been studied in depth [1], the mechanisms of cell adaptation to heavy water remain poorly understood. Knowledge of the biological effects of deuterium is of great importance in the biotechnological production of deuterated biologically active substances [2], which find ever-increasing use in biochemical and biomedical research [3].

The effects of deuterium on cells can be divided into two main groups: substrate deuterium isotope effects and deuterium isotope effects of heavy water as a solvent. The isotope effects of the first group are due to the different energy potentials of C–^{1}H and C–^{2}H bonds in deuterated and ordinary substrates and the products of their metabolic conversion. Unlike heavy water, deuterated carbon sources do not dramatically affect microbial metabolism, due to which many microorganisms can utilize completely deuterated analogues of their substrates. The isotope effects of D_2O as a solvent are as yet poorly studied, although there are grounds to believe that they may be responsible for the adaptation of cells to growth in deuterated media.

[1] Corresponding authors. E-mails: biotechnology@mtu-net.ru and skladda@genetika.ru.

Since the physicochemical properties of ^2H_2O and ^1H_2O are different, the presence of heavy water in the culture liquid affects cell physiology, specifically, membrane permeability [4], membrane transport [5], and the activity of membrane enzymes [6]. Because of the lower chemical potential of heavy water as compared to ordinary water, solutions of salts in ^2H_2O are hyperosmotic in comparison with the analogous solutions in ^1H_2O. Consequently, heavy water induces an efflux of ordinary water from cells placed in deuterated media [7]. Andjus and Vucelic showed that heavy water induces osmotic shock and causes an efflux of intracellular potassium from algal cells [5]. It is known that *Escherichia coli* cells respond to hydrogen peroxide (oxidative stress) by increasing the activity of catalase and by excreting potassium cations [8].

The physiological role of rapid K^+ excretion in response to different stresses is not yet fully understood. The possible reasons for this excretion are cell envelope damage [9], membrane excitation [5], and glutathione-induced opening of K^+ channels [8]. There is evidence that the redox state of cells may control K^+ efflux through membrane channels [10]. The decreased level of intracellular potassium in *E. coli* cells exposed to oxidative stress may cause DNA relaxation and, hence, activate the DNA repair system [9]. Butler and Grist showed that D_2O-adapted cells of *Streptococcus pneumoniae* have higher Hex activity and repair DNA lesions better than do nonadapted cells [11]. Therefore, like other stress factors, heavy water may induce an adaptive response in cells. The question now arises of whether such phenomena as cross adaptation and pro-

0026-2617/04/7302-0139 © 2004 MAIK "Nauka/Interperiodica"

Table 1. The effect of the cultivation conditions of the inoculum on the growth parameters of *Methylophilus* sp. B-7741 in deuterated medium (99.8 vol % ^2H_2O, 0.1 vol % $C^2H_3O^2H$)

Cultivation conditions of inoculum	Growth parameters of *Methylophilus* sp. B-7741	
	Lag phase, h	Specific growth rate, h^{-1}
Ordinary medium without heavy water	49 ± 1	0.02 ± 0.01
Successive passages in media with 0, 75, 95, and 99.8% heavy water	13 ± 3	0.12 ± 0.01
Ordinary medium with 86 mM NaCl	14 ± 2	0.10 ± 0.01
Ordinary medium with 33 μM H_2O_2	13 ± 3	0.12 ± 0.01

duction of extracellular protectants [12, 13] are typical of cell adaptation to heavy water.

The aims of this work were to study (1) the effect of the cultivation conditions of the inoculum on the growth parameters of the methylotrophic bacterium *Methylophilus* sp. B-7741 in deuterated media, (2) the effect of deuterated media on catalase activity, and (3) the biological activity of deuterated cell-free culture liquid.

MATERIALS AND METHODS

Experiments were carried out with the strictly methylotrophic bacterium *Methylophilus* sp. B-7741, which has nonmotile rod-shaped cells 0.3–0.4 × 0.6–0.7 μm in size and produces round, even, white–cream colonies from 0.5 to 3 mm in diameter. The optimum growth temperature and pH are 28–30°C and 6.8–7.2, respectively. The bacterium was isolated from a mixed methanol-utilizing culture, which also contained a gram-positive bacterium of unknown taxonomic affiliation.

The bacterium was cultured in a synthetic medium containing (g/l) $NaNO_3$, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; K_2HPO_4, 1.5; and KH_2PO_4, 0.7. The medium was supplemented with a trace element solution (20 ml/l) of the following composition (mg/100 ml): $FeSO_4 \cdot 7H_2O$, 100; $MnCl_2 \cdot 4H_2O$, 1.5; $CoCl_2 \cdot 6H_2O$, 10; $ZnSO_4 \cdot 7H_2O$, 5; $CuCl_2 \cdot 5H_2O$, 5; Na_2MoO_4, 1.5; $NiCl_2 \cdot 6H_2O$, 1; and EDTA, 250. The medium was sterilized at 0.8 atm for 30 min (phosphate solutions were sterilized separately) and supplemented with 0.5 or 1 vol % methanol.

The deuterated medium had the same composition, except that it was prepared by using heavy water (99.85 at % ^2H) and supplemented with deuterated methanol $C^2H_3O^2H$ (99.7 at % ^2H).

The bacterium was adapted to heavy water by cultivating it at increasing D_2O concentrations (0, 75, 95, and 99.85%). To prevent the dilution of deuterium with protium of atmospheric moisture, cultivation in the medium containing 99.85% heavy water was performed in flasks furnished with traps that were filled with dry silica gel.

To obtain inocula adapted to osmotic and oxidative stresses, the bacterium *Methylophilus* sp. B-7741 was grown for 48 h (to the stationary phase) in the medium containing 0.5% (86 mM) NaCl or 0.0001% (33 μM) H_2O_2, respectively.

To assay catalase activity, bacterial cells were harvested by centrifugation, suspended in 50 mM phosphate buffer (pH 7.0), and disrupted by sonication at 0°C for a total of 3 min in 1-min bursts. The cell homogenate was centrifuged at 8000 *g* for 30 min, and the supernatant (referred to as cell-free extract) was used to assay catalase by the spectrophotometric method described by Beers and Sizer [14]. Catalase activity was expressed in nmol H_2O_2/(min mg protein).

Protein in cell extracts was quantified by the method of Lowry *et al.* The calibration curve was constructed with bovine serum albumin as the standard.

To evaluate the biological activity of deuterated culture liquid (CL), unadapted *Methylophilus* sp. B-7741 cells were grown in the medium containing 95% D_2O and 0.2% deuterated methanol for 24 h (OD_{600} = 0.1; the exponential growth phase) or for 72 h (OD_{600} = 0.3; the stationary growth phase). Then the cells were removed by centrifugation, and the supernatant (CL) was filter-sterilized. To check the sterilization efficiency, the CL was mixed with an equal volume of fresh medium, supplemented with 1 vol % methanol, and incubated at 30°C. The absence of growth was an indication of the efficiency of sterilization. The sterilized deuterated CL was mixed with 3 volumes of fresh nutrient medium (95% D_2O). The mixture was supplemented with 1 vol % methanol and inoculated with the unadapted *Methylophilus* sp. B-7741 cells.

All the experiments were conducted three or four times. The paper reports representative results.

RESULTS AND DISCUSSION

The effect of the adaptation of *Methylophilus* sp. B-7741 to increasing concentrations of heavy water in the cultivation medium on the lag phase duration and the specific growth rate is shown in Table 1. As can be seen from this table, the unadapted *Methylophilus* sp. B-7741 could barely grow in the deuterated medium, whereas the adapted cells exhibited good growth in this medium, with a high specific growth rate and a relatively short lag phase. These data are in agreement with the results of our earlier experiments with the facultative methylotrophic bacterium *Brevibacterium methylicum* [2].

If the cells used for inoculation were preliminarily adapted to osmotic stress (i.e., were grown in the medium with 86 mM NaCl) or oxidative stress (i.e., were grown in the medium with 33 μM H_2O_2), they

grew in the deuterated medium at the same rate as the cells that were preliminarily adapted to heavy water by successive cultivation in the media with increasing concentrations of D_2O (Table 1). In other words, *Methylophilus* sp. B-7741 cells exhibited cross adaptation to NaCl (osmotic stress), hydrogen peroxide (oxidative stress), and heavy water. This suggests that the adaptive mechanisms to these three types of stresses may be similar.

To verify this suggestion, we measured the activity of catalase, which is the major enzyme that is responsible for the decomposition of H_2O_2 in growing *E. coli* cells [8], in the *Methylophilus* sp. B-7741 cells grown in the ordinary and deuterated nutrient media. These measurements showed that the activity of catalase in the *Methylophilus* sp. cells grown in the deuterated medium was an order higher than in the cells grown in the ordinary medium (Table 2).

It is known that the activity of catalase in bacterial cells exposed to oxidative and osmotic stresses increases [8, 15] and that osmotic stress enhances the formation of reactive oxygen species in *E. coli* cells [15], which is likely to be due to the inhibition of the electron transport chain [8, 15]. It is also known that heavy water inhibits the respiratory chain of bacterial cells [16] and rat liver mitochondria [17]. This gives ground to believe that, like osmotic stress, heavy water can elevate the level of reactive oxygen species in cells and thereby induce antioxidant enzymes, particularly catalase and peroxidase. Indeed, the activity of peroxidase in winter rye seeds germinated in 99.77% D_2O was found to be higher than in control seeds germinated in ordinary water [18].

The analysis of the experimental data presented here and data available in the literature allow us to suggest that the processes of cell adaptation to D_2O-containing media and to other stressful conditions are similar. However, heavy water exerts multiple inhibitory effects on cells and, hence, their adaptation to D_2O-containing media may also involve additional mechanisms.

Table 2. The activity of catalase in the cell extracts of *Methylophilus* sp. B-7741 grown in the ordinary and deuterated media

Extract of cells grown in	Catalase activity, nmol H_2O_2/(min mg protein)
Ordinary medium	0.13 ± 0.03
Deuterated medium	1.38 ± 0.10

Nikolaev's experiments [12, 13] showed that *E. coli* cells synthesize extracellular factors of adaptation when they are exposed to unfavorable (or new) environmental conditions. Bearing this in mind, we studied the effect of deuterated culture liquids on the adaptation of *Methylophilus* sp. B-7741 cells to growth in the deuterated medium (Figs. 1, 2). The deuterated CL obtained from the exponential culture exerted a beneficial effect on the growth parameters (the growth rate and biomass) of *Methylophilus* sp. B-7741 in the deuterated medium (Fig. 1). When prepared from the stationary-phase culture, the deuterated CL also increased the growth rate and the biomass, although it extended the lag phase (Fig. 2).

Nikolaev's experiments also showed that *E. coli* cells exposed to heat shock produce the extracellular factor X_1, which promotes cell adaptation to various inhibitory agents, and the extracellular factor X_2, which decreases the growth rate and considerably enhances cell resistance to bactericidal concentrations of *N*-ethylmaleimide [13]. It should be noted that the effects of the factor X_1 and the deuterated CL are similar, which suggests that this CL may contain the factor

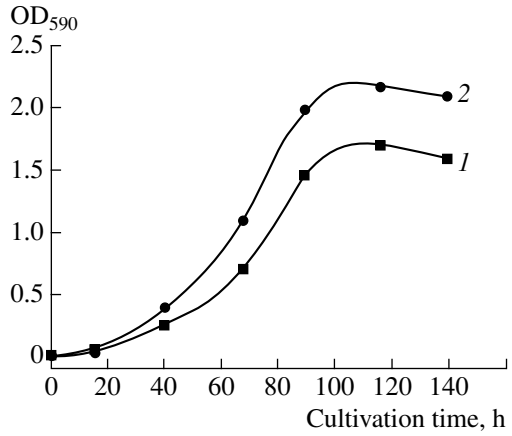

Fig. 1. Growth of *Methylophilus* sp. B-7741 in undiluted deuterated medium containing 95% heavy water and 1% deuterated methanol (curve *1*) and in the deuterated medium mixed with deuterated cell-free culture liquid in a proportion of 3 : 1 (curve *2*). The deuterated culture liquid was obtained from the *Methylophilus* sp. B-7741 culture grown in the deuterated medium to the exponential phase.

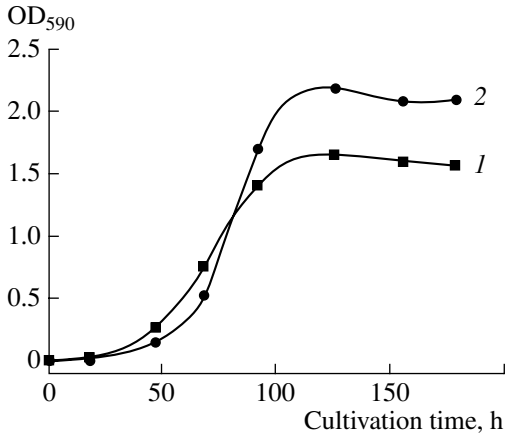

Fig. 2. Growth of *Methylophilus* sp. B-7741 in undiluted deuterated medium containing 95% heavy water and 1% deuterated methanol (curve *1*) and in the deuterated medium mixed with deuterated cell-free culture liquid in a proportion of 3 : 1 (curve *2*). The deuterated culture liquid was obtained from the *Methylophilus* sp. B-7741 culture grown in the deuterated medium to the stationary phase.

X_1 or other factors with similar properties. In other words, unadapted *Methylophilus* sp. B-7741 cells cultivated in deuterated media may produce extracellular substances that enhance cell resistance to heavy water.

Extracellular adaptive factors can be exemplified by low-molecular-weight alkyl hydroxybenzenes (AHBs), which act as chemical chaperones stabilizing the spatial structure of enzymes and increasing cell resistance to heat shock [19]. A similar action is typical of the molecular chaperones GroE, which stabilize deuterated ribulose bisphosphate carboxylase/oxygenase (EC 4.1.1.39) in *Chlorella ellipsoidea* [6], and of osmoprotectants that increase the stability of biopolymers (such as proteins) under denaturing conditions [20].

Thus, low-molecular-weight compounds such as AHBs and osmoprotectants possessing the properties of chemical chaperones may help bacterial cells retain high rates of metabolic reactions and growth in deuterated media. The data obtained in this study show that the mechanism of cell adaptation to heavy water is likely to be similar to the mechanisms of cell adaptation to other stress factors.

REFERENCES

1. Lobyshev, V.I. and Kalinichenko, L.P., *Izotopnye effekty D_2O v biologicheskikh sistemakh* (The Isotope Effects of D_2O in Biological Systems), Moscow: Nauka, 1978.
2. Mosin, O.V., Skladnev, D.A., and Shvets, V.I., Biosynthesis of ^{2}H-Labeled Phenylalanine by a New Mutant of RuMP Facultative Methylotroph *Brevibacterium methylicum, Biosci. Biotechnol. Biochem.*, 1998, vol. 62, no. 2, pp. 225–229.
3. Kushner, D.J., Baker, A., and Dunstall, T.G., Pharmacological Uses and Perspectives of Heavy Water and Deuterated Compounds, *Can. J. Physiol. Pharmacol.*, 1999, vol. 77, pp. 79–88.
4. Rastogi, R.P., Skukla, P.C., and Yadava, B., Membrane Permeability of Heavy Water, *Biochim. Biophys. Acta*, 1971, vol. 249, no. 2, pp. 454–461.
5. Andjus, P.R. and Vucelic, D., D_2O-Induced Cell Excitation, *J. Membr. Biol.*, 1990, vol. 115, pp. 123–127.
6. Yokogaki, S., Unno, K., Oku, N., and Okada, S., Chaperonin-Repairable Subtle Incompleteness of Protein Assembly Induced by a Substitution of Hydrogen with Deuterium: Effect of GroE on Deuterated Ribulose 1,5-Bisphosphate Carboxylase, *Plant Cell Physiol.*, 1995, vol. 36, no. 3, pp. 419–423.
7. Brooks, S.C., Osmotic Effects of Deuterium Oxide (Heavy Water) on Living Cells, *Science*, 1937, vol. 86, no. 2239, pp. 497–498.
8. Smirnova, G.V., Muzyka, N.G., Glukhovchenko, M.N., and Oktyabr'skii, O.N., Hydrogen Peroxide Modulates Intracellular Levels of Thiols and Potassium in *Escherichia coli, Mikrobiologiya,* 1998, vol. 67, no. 5, pp. 594–600.
9. Tkachenko, A.G., Pshenichnov, M.R., Salakhetdinova, O.Ya., and Nesterova, L.Yu., The Role of Putrescine and the Cellular Energy State of *Escherichia coli* in Regulation of DNA Topology during Adaptation to Oxidative Stress, *Mikrobiologiya*, 1999, vol. 68, no. 1, pp. 27–32.
10. Meury, J. and Robin, A., Glutathione-Gated K^+ Channels of *Escherichia coli* Carry Out K^+ Efflux Controlled by the Redox State of the Cell, *Arch. Microbiol.*, 1990, vol. 154, pp. 475–482.
11. Butler, L.O. and Grist, R.W., The Effect of D_2O on the Growth and Transforming Activities of *Streptococcus pneumoniae, J. Gen. Microbiol.*, 1984, vol. 130, pp. 483–494.
12. Nikolaev, Yu.A., Involvement of Exometabolites in Stress Adaptation of *Escherichia coli, Mikrobiologiya*, 1997, vol. 66, no. 1, pp. 38–41.
13. Nikolaev, Yu.A., Comparative Study of Two Extracellular Protectants Secreted by *Escherichia coli* Cells at Elevated Temperatures, *Mikrobiologiya*, 1997, vol. 66, no. 6, pp. 790–795.
14. Beers, I.F. and Sizer, W.I., A Spectrophotometric Assay for Measuring the Breakdown of Hydrogen Peroxide by Catalase, *J. Biol. Chem.*, 1952, vol. 196, pp. 133–140.
15. Smirnova, G.V., Muzyka, N.G., and Oktyabrsky, O.N., The Role of Antioxidant Enzymes in Response of *Escherichia coli* to Osmotic Upshift, *FEMS Microbiol. Lett.*, 2000, vol. 186, pp. 209–213.
16. Laser, H. and Slater, E.C., Effect of Heavy Water on Respiratory-Chain Enzymes, *Nature* (London), 1960, vol. 187, no. 4743, pp. 1115–1117.
17. Kalinichenko, L.P., Khristova, M.L., and Shnol', S.E., The Effect of D_2O, Glycerol, and Aliphatic Alcohols and the Possible Role of Hydrophobic Interactions in Electron Transport in Mitochondria, *Biofizika*, 1967, vol. 12, no. 5, pp. 824–828.
18. Siegel, B.Z. and Galston, A.W., Biosynthesis of Deuterated Isoperoxidases in Rye Plants Grown in D_2O, *Biochemistry*, 1966, vol. 56, pp. 1040–1042.
19. Il'inskaya, O.N., Kolpakov, A.I., Shmidt, M.A., Doroshenko, E.V., Mulyukin, A.L., and El'-Registan, G.I., The Role of Bacterial Growth Autoregulators (Alkyl Hydroxybenzenes) in the Response of Staphylococci to Stresses, *Mikrobiologiya*, 2002, vol. 71, no. 1, pp. 23–29.
20. Loiko, N.G., Kozlova, A.N., Osipov, G.A., and El'-Registan, G.I., Low-Molecular-Weight Autoregulatory Factors in the Haloalkaliphilic Bacteria *Thioalkalivibrio versutus* and *Thioalkalimicrobium aerophilum, Mikrobiologiya*, 2002, vol. 71, no. 3, pp. 308–315.