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Changes in the Nitrocellulose Molecule Induced by Sulfate-Reducing Bacteria *Desulfovibrio desulfuricans* 1388. The Enzymes Participating in This Process

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Abstract—The appearance of unsubstituted glucopyranose residues in nitrocellulose (NC) induced by *Desulfovibrio desulfuricans* was established by ^{13}C-NMR spectroscopy. After contact with bacterial cells, the degree of substitution by nitro groups in NC decreased from 2.59 to 2.40. The bacteria possess intra- and extracellular nitroesterase activities, which are responsible for denitration of the polymer. The presence of NC in the growth medium influences the extracellular nitroesterase activity. It is shown that inhibition of enzymatic activity in the presence of NC is caused by appearance of nitrates in the culture medium. Nitrate and nitrite reductases of dissimilatory type reduce nitrates. The data suggest consideration of bacteria belonging to the *Desulfovibrio* genus as the initial agent in utilization of an unnatural polymer—nitrocellulose—in a microbial consortium.

Key words: *Desulfovibrio desulfuricans*, nitrocellulose, nitroesterase, nitrate reductase, NMR spectroscopy

Transformation of unnatural polymers by microorganisms is defined by the biochemical potential of cells for sequential transformations of the initial compound into the final product. If it is complicated for one microorganism to use a xenobiotic as the substrate, an alternative pathway of degradation of the substrate by a mixed culture may be realized. The catabolic possibilities combine in a microbial consortium, and this allows mineralization of the initial compound.

Nitrocellulose (NC), a synthetic polymer containing more than 11% nitrogen, is stable to biological degradation. Several microorganisms are able to transform NC [1-4]. There are two possible pathways: the first is a cleavage of the β-1,4-glucoside bond of the carbon polymeric chain. This pathway of NC degradation is typical of some microfungi [4] and results in formation of nitrooligosaccharides of various lengths. The second pathway includes removal of the nitro groups, or denitration; the degree of substitution by nitro groups is thus decreased [2], and the polymer becomes accessible for hydrolysis by other microorganisms.

As shown by us earlier, sulfate-reducing bacteria of the *Desulfovibrio* genus are able to transform NC, decreasing its content in the medium [5]. When *D. desulfuricans* 1388 grew in the presence of NC, nitrates were detected in the culture medium [5]. Their subsequent disappearance and appearance of ammonium indicated possible utilization of nitrate by the culture. It was of special interest to clarify what changes occur in the NC molecule in the presence of *D. desulfuricans* and what enzymatic systems of the bacteria involve the poorly accessible nitrogen atoms of the NC nitro groups into cell metabolism.

In the present work the NC molecule was studied after its contact with *Desulfovibrio desulfuricans* 1388 by NMR spectroscopy and estimation of nitroesterase and nitrate and nitrite reductase activities of cultures growing in the presence of NC.

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MATERIALS AND METHODS

Desulfovibrio desulfuricans 1388 strain (VKM 1388) was kindly donated by the Russian Collection of Microorganisms (Pushchino, Russia). Mineral culture medium Postgate B containing 3.5 g/liter calcium lactate and the procedures of anaerobic cultivation of sulfate-reducing bacteria were described by us earlier [6]. Bacteria grown on Postgate B medium without addition of NC were used as the control. In experimental samples, NC containing more than 11% nitrogen was added to the medium at concentration 10 g/liter.

To obtain extracts, cells in a certain growth phase were harvested using a K80 centrifuge (VEB MLW Medizintechnik Leipzig, Germany) (5000*g*, 30 min, 4°C) and washed with 0.02 M Tris-HCl, pH 7.6, with subsequent centrifugation under the same conditions. The pellet was resuspended in the same buffer, taking 2.0-2.5 g of raw biomass per 10 ml of buffer, and disintegrated using a UZDN-2T ultrasonic disintegrator (Russia) (22 kHz, 60 sec, 1 ml, 0°C). The suspension was centrifuged at 17,000*g* for 60 min at 4°C, and the supernatant was used in subsequent experiments.

Protein was determined according to a modified Lowry procedure [7] using crystalline bovine serum albumin (BSA) as the standard.

Cell fractions were obtained by the modified method of osmotic lysis of spheroplasts [8].

^{13}C-NMR spectra (75.43 MHz) of NC samples dissolved in $(CD_3)_2CO$ were recorded using a Unity-300 NMR spectrometer from Varian (USA) with a VTC-4 temperature-regulatory device (inner stabilization via 2H resonance line). Conditions: 20-30°-impulses and broadband isolation from protons; delays between impulses *dl* = 1-2 sec; maximal width of NMR spectrum *sw* = 200 ppm; number of signal accumulation *nt* up to 1000; digital exponential filtration with coefficient *lb* = 2-4 Hz. The samples (experimental and control) were 10% NC solutions. Chemical shifts were measured with respect to tetramethylsilane, accuracy 0.03 ppm. Integral intensities were measured at the 2% level of relative accuracy.

Evaluation of nitroesterase activity. The reaction mixture (3 ml) contained 0.05 M phosphate buffer, pH 7.4, 15 mg NC, 200 µl of cell extract or 500 µl of culture medium after removal of the cells. The reaction time was 15 min at 37°C. To stop the reaction, the mixture was heated for 5 min at 100°C and centrifuged to remove NC particles and denatured protein. The nitrate concentration in the supernatant was determined colorimetrically in the presence of salicylic acid [9]. Cell extract or culture medium heated for 10 min at 100°C were used as control. One nanomole of NO_3^- formed by 1 mg protein per 1 min was taken as the nitroesterase activity unit.

Nitrate and nitrite reductase activities were determined via removal of the substrate (nitrate or nitrite) from the reaction mixture [10] or oxidation of benzyl viologen reduced by sodium dithionite [11]. One micromole of NO_3^- or NO_2^- reduced by 1 mg protein per 1 min or 1 µmol benzyl viologen oxidized by 1 mg protein per 1 min were taken as the activity units.

The numerical values presented in this work are the averaged values of three independent experiments each repeated thrice.

RESULTS AND DISCUSSION

Changes in the NC molecule induced by the contact with *D. desulfuricans* 1388 were analyzed by ^{13}C-NMR spectroscopy. Chemical shifts were compared with the tabulated data for ^{13}C atoms of monomeric glucopyranose fragments of NC accounting for their increments on substitution [12]. Multiple ^{13}C signals of monomeric fragments are caused by the presence or absence of the ONO_2^- group in the neighboring monomeric fragments [13].

The ^{13}C-NMR spectrum of the initial sample is presented in Fig. 1a. The absence of the lines in the high-field area (62.0 ppm) indicates that there are no signals corresponding to the unsubstituted NC fragment in this spectrum. The absence of the signal in the low-field area corresponding to the C-1 of 6-monosubstituted NC could be explained by high degree of nitro-substitution of the initial sample—2.59 on average (Table 1). The relative

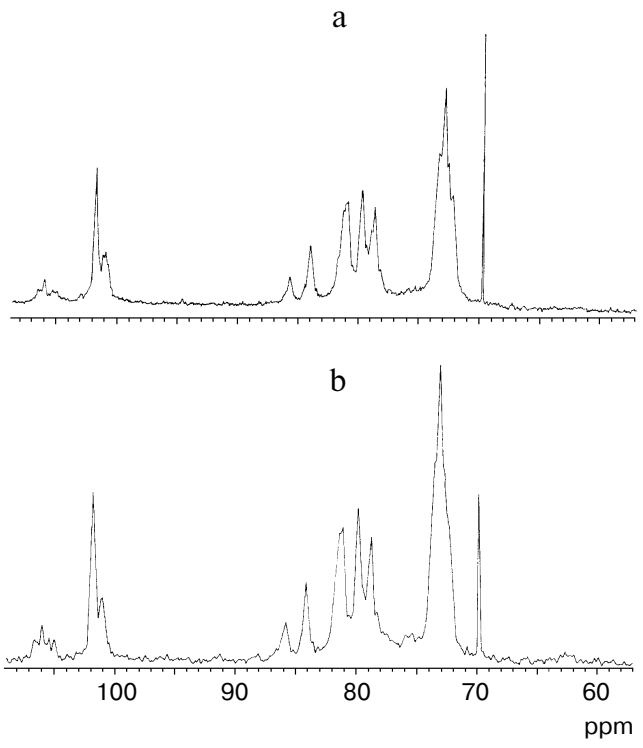

Fig. 1. ^{13}C-NMR spectra of the control (a) and experimental (b) samples of nitrocellulose.

Table 1. Content of NC monomers from the data of ^{13}C-NMR spectroscopy

Sample	Unsubstituted cellulose	6-MNC	2,6-DNC	3,6-DNC	2,3,6-TNC	Degree of NO_2^- substitution
Control	0	0	0.29	0.18	0.53	2.59
Experimental	0.05	0	0.26	0.19	0.50	2.40

Note: 6-MNC, 6-monosubstituted NC; 2,6-DNC, 2,6-disubstituted NC; 3,6-DNC, 3,6-disubstituted NC; 2,3,6-TNC, 2,3,6-trisubstituted NC.

contents of various monomeric fragments normalized to unity are presented in Table 1. The initial NC sample was a mixture of 2,6- and 3,6-disubstituted and 2,3,6-trisubstituted NC.

^{13}C-NMR data for the experimental sample (Fig. 1b) indicate that the biological denitration process includes all the nitro groups independent of their position in the glucopyranose residue. Appearance of signal in the high-field area (62.5 ppm) correlating with a signal from the C-1 atom in the low-field area (104.9 ppm) indicates that the signals corresponding to the unsubstituted fragment are present in this spectrum. The degree of substitution by the nitro groups of the experimental sample was 2.40—lower that that of the control (Table 1).

Thus, non-esterified fragments appear in the NC molecule by the action of *D. desulfuricans* 1388. This fact indicates that the nitro groups are cleaved from the poly-β-D-glucopyranose chain. Naturally, we suggested that appearance of nitrates in the culture medium on growing bacteria in the presence of NC is caused by the action of esterases. Esterases possess a relatively wide specificity and hydrolyze a wide variety of esters, catalyzing cleavage of the ester bonds.

We found that *D. desulfuricans* 1388 cells possess enzymatic activity hydrolyzing cellulose nitroesters. Study of cell growth in NC-free medium demonstrated that nitroesterase activity is a constitutive feature of the bacteria (Fig. 2). Production of nitroesterase is related with the logarithmic growth phase, whereas the maximal enzymatic activity is detected in the cells in the stationary growth phase. Nitroesterase activity was also detected in the culture liquid and reached 33% of the total cell nitroesterase activity at the late-logarithmic phase. The extracellular activity curve changed smoothly at various growth phases and reached 1.2-1.4 units at the stationary phase (not shown in the figure).

The presence of NC in the culture medium did not influence intracellular nitroesterase activity (Fig. 2), but caused a decrease in enzymatic activity of the culture liquid to 0.3 unit. Experiments on bacterial culture on Postgate B medium in the presence of nitrates (0.022 g/liter, which corresponded to the maximal level of nitrates in culture liquid on bacteria growth in the presence of NC) demonstrated that extracellular nitroesterase activity at the stationary growth phase decreased to 0.27 unit, whereas intracellular activity did not change. Thus, inhibition of extracellular nitroesterase activity of the cells growing in the presence of NC could be related with appearance of free NO_3^- anions in the culture medium.

Whether intra- and extracellular nitroesterase activities are defined by a single structural protein or by several structural analogs remains unclear. The inhibitory effect of nitrate suggests that enzymatic hydrolysis of the nitroester bonds in NC is performed involving nonspecific esterases sensitive to the presence of nitrates in the culture medium.

We failed to find literature data on nitroesterase activity of sulfate-reducing bacteria. Such nitro compounds as trinitrotoluene, nitrophenol, trinitrobenzene, etc. are subject to transformation by bacteria of the *Desulfovibrio* genus via direct reduction of the NO_2^- group with subsequent deamination or cleavage of the aromatic ring [14, 15]. But other enzymatic systems probably participate in these processes.

As shown by us earlier, nitrates liberated as a result of hydrolysis of the nitroester bonds of NC do not remain in

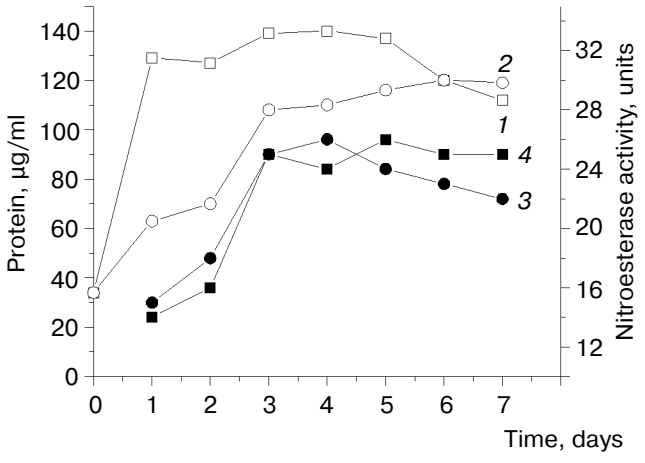

Fig. 2. Effect of nitrocellulose (10 g/liter) on protein formation (without (*1*) and with nitrocellulose (*2*)) and intracellular nitroesterase activity of *D. desulfuricans* 1388 (without (*3*) and with nitrocellulose (*4*)).

Table 2. Localization of nitrate- and nitrite reductases in *D. desulfuricans* 1388 cells

Fraction	Nitrate reductase activity, %	Nitrite reductase activity, %
Periplasm	15	56
Cytoplasm + membranes	85	44

the culture liquid but are absorbed by the *D. desulfuricans* cells [5]. Two pathways of reduction of nitrates by sulfate-reducing bacteria are known: the first (denitrification) suggests successive reduction of nitrates to gaseous nitrogen, and the second (dissimilation) to ammonium [16]. Appearance of ammonium during *D. desulfuricans* growth in the presence of NC detected by us earlier [5] indicated the action of nitrate and nitrite reductases of dissimilatory type. Studying intracellular localization of these enzymes, we found that 85% of the nitrate reductase activity is related with the "cytoplasm + membranes" fraction, and nitrite reductase activity is evenly distributed over the cell fractions (Table 2). Nitrate reductase activity of cell extracts grown in the presence or in the absence of NC was 0.2-0.3 unit. We did not detect nitrite, the product of reduction of nitrate, in the reaction medium; this possibly was related with high activity of nitrite reductase (1.5-2.0 units).

So, the ^{13}C-NMR spectroscopy data indicate formation of unsubstituted glucopyranose fragments in the NC molecule as a result of denitration of the polymer by the action of bacteria of the *Desulfovibrio* genus. The data suggest that sulfate-reducing *Desulfovibrio* bacteria can act as the initial agents in utilization of the polymer *in situ* in a microbial consortium. Nitrates appearing in the medium as a result of hydrolysis of the nitroester bonds in NC are either metabolized by bacteria possessing nitrate and nitrite reductase activities, or can be a source of nitrogen or the electron acceptors for other members of the microbial consortium. Cellulose thus formed can be attacked by microorganisms possessing cellulolytic activity.

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