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EXPERIMENTAL ARTICLES

The Successive Reduction of Cr(VI) and NO_3^- or Mn(IV) Ions Present in the Cultivation Medium of Denitrifying Bacteria

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Abstract—A study of the reduction of chromium (VI) and manganese (IV) (or nitrate) ions present in the cultivation medium of denitrifying bacteria of the genus *Pseudomonas* showed that Cr(VI) ions are reduced first. The rate of Cr(VI) reduction was found to be independent of the presence of Mn(IV) or nitrate ions in the medium.

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Key words: reduction, elements with a variable degree of oxidation, terminal electron acceptors, redox succession, denitrifying bacteria, dichromate, manganese dioxide, nitrate.

It is believed that microbial populations reduce terminal electron acceptors in the following succession: oxygen, oxidized nitrogen compounds, metal oxides, sulfate ions, and carbonate ions [1]. The ability of aerobic bacteria with oxidative metabolism to utilize nitrate is used for classifying bacterial genera and species into strictly aerobic and those capable of anaerobic respiration [2].

Recently, much attention has been given to the study of the ability of bacteria to utilize elements with a variable degree of oxidation as terminal electron acceptors. Bacterial strains capable of reducing inorganic compounds are typically isolated from ecological niches that are contaminated with these compounds [3]. In some cases, bacterial isolates were assigned to particular species based on their ability to reduce elements with a variable degree of oxidation [4–7]. As far back as the 1980s, Gvozdyak et al. found that nonadapted collection strains of nitrate-reducing bacteria can reduce chromium (VI) [8]. Later, other researchers showed that not only nitrate-reducing, but also nonnitrate-reducing bacteria are able to utilize chromium (VI) and manganese (IV) as terminal electron acceptors [9, 10]. The ability of bacteria that are commonly classified as strict aerobes to utilize Mn(IV) and Cr(VI) as terminal electron acceptors was explained by the fact that the standard redox potentials E^0 of Mn(IV) and Cr(VI) (1228 and 1333 mV, respectively) are close to that of O_2 (1228 mV).

This work was undertaken to study the successive reduction of Cr(VI) and Mn(IV) (or NO_3^-) ions present in the cultivation medium of the collection strains of denitrifying bacteria of the genus *Pseudomonas*.

MATERIALS AND METHODS

Experiments were carried out with the following denitrifying bacteria of the genus *Pseudomonas*: *P. aeruginosa* P-1, *P. fluorescens* var. *pseudo-iodinum* P-11, *P. mendocina* P-13, and *P. stutzeri* P-19, which were obtained from the collection of microorganisms at the Department of Microbiology of the Dumanskii Institute of Colloid and Water Chemistry.

The bacteria were cultivated at 28°C in 120-ml flat-bottom flasks containing 100 ml of a liquid nutrient M9 medium supplemented with Cr(VI), Mn(IV), and NO_3^- ions in various combinations. Cells for inoculation were grown at 28°C on complete nutrient agar in Petri dishes. The initial concentration of biomass in the liquid nutrient medium was 250–300 mg ADB/l (ADB stands for "absolutely dry biomass"). The surface of the medium was covered with a 2-cm layer of sterile mineral oil in order to prevent oxygen diffusion from the air. Oxygen dissolved in the medium was not removed.

The M9 medium (phosphate buffer, pH 7) contained the following ingredients (in g/l): KH_2PO_4, 3.0; Na_2HPO_4, 6.0; NH_4Cl,1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; and $CaCl_2$, 0.1. The medium was supplemented with a trace element solution in an amount of 1 ml per 1 l. The solution contained the following ingredients (in mg/l): $FeSO_4 \cdot 7H_2O$, 25.0; $MnSO_4 \cdot 2H_2O$, 5.0; $CoSO_4$, 1.0; $ZnSO_4$, 1.0; $CuSO_4 \cdot 6H_2O$, 0.1; H_3BO_3, 0.1; Na_2MoO_4, 25.0; $NiCl_2 \cdot 6H_2O$, 0.1. The medium was supplemented with 1.6 g/l glucose as the carbon and energy source.

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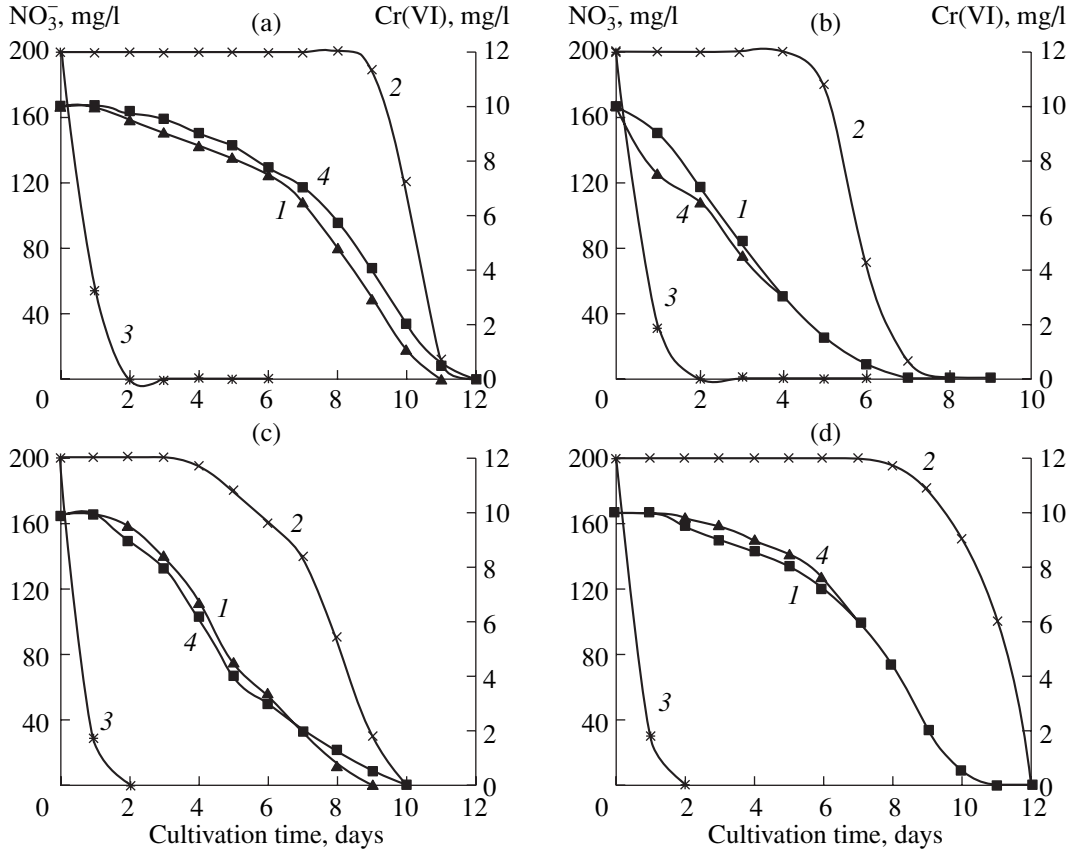

Fig. 1. Reduction of Cr(VI) and nitrate by the denitrifying bacteria (a) *P. aeruginosa* P-1, (b) *P. fluorescens* var. *pseudo-iodinum* P-11, (c) *P. mendocina* P-13, and (d) *P. stutzeri* P-19: (*1*) the dynamics of Cr(VI) ions in the presence of nitrate in the medium, (*2*) the dynamics of nitrate ions in the presence of Cr(VI), (*3*) the dynamics of nitrate ions in the absence of Cr(VI) in the medium, (*4*) the dynamics of Cr(VI) ions in the absence of nitrate.

Chromium (VI) was added in the form of potassium dichromate at a concentration of 10 mg/l. Nitrate ions were added in the form of KNO_3 at a concentration of 200 mg/l. Manganese (IV) was added in the form of insoluble MnO_2 powder at a concentration of 500 mg/l. The control flasks contained the same medium and the same additives (but did not contain bacterial cells) and were incubated under the same conditions.

Cr(VI), NO_3^-, and Mn(II) ions (the latter ions are the product of Mn(IV) reduction) were analyzed colorimetrically [11]. The standard redox potentials cited in this paper are taken from the handbook [12]. It should be noted that ambient temperature and pH may influence the absolute values of the redox potentials of particular compounds but not their relative values [13].

RESULTS AND DISCUSSION

Anthropogenically impacted econiches are typically contaminated by several inorganic compounds. The compounds that contain elements with a variable degree of oxidation can serve as terminal electron acceptors for bacteria inhabiting these econiches. The study of *Pseudomonas* strains isolated from soil contaminated by chromates, arsenates, and copper (II) showed that these strains were tolerant to Cr(VI) at a concentration of 520 mg/l and could reduce chromate ions in the presence of arsenate and copper (II) [14]. The rate of chromate reduction was found to be dependent on the concentration of the other contaminants present in the medium. The *Pseudomonas* isolates were also able to partially reduce Co(III) and U(VI). There are speculations that the inhibitory action of chromates on the reduction of nitrate and nitrite by the bacterium *Shewanella oneidensis* MR1 may result from the interaction of Cr(VI) with some components of the bacterial electron transport chain, such as nitrate reductase [15].

Based on our data and those of other researchers, we suggested that, when several elements with a variable degree of oxidation are present in the medium, the first elements to be reduced by microorganisms are those which have higher standard redox potentials.

It is known that bacterial denitrification occurs in two stages: nitrate is initially reduced to nitrite, and then nitrite is converted to N_2. Analysis of the standard redox potentials of reduced nitrogen compounds allowed the suggestion to be made that the first denitrification stage may have two steps: the formation of nitrogen dioxide (E^0 = 780 mV) and the formation of nitrite ions (E^0 = 880 mV).

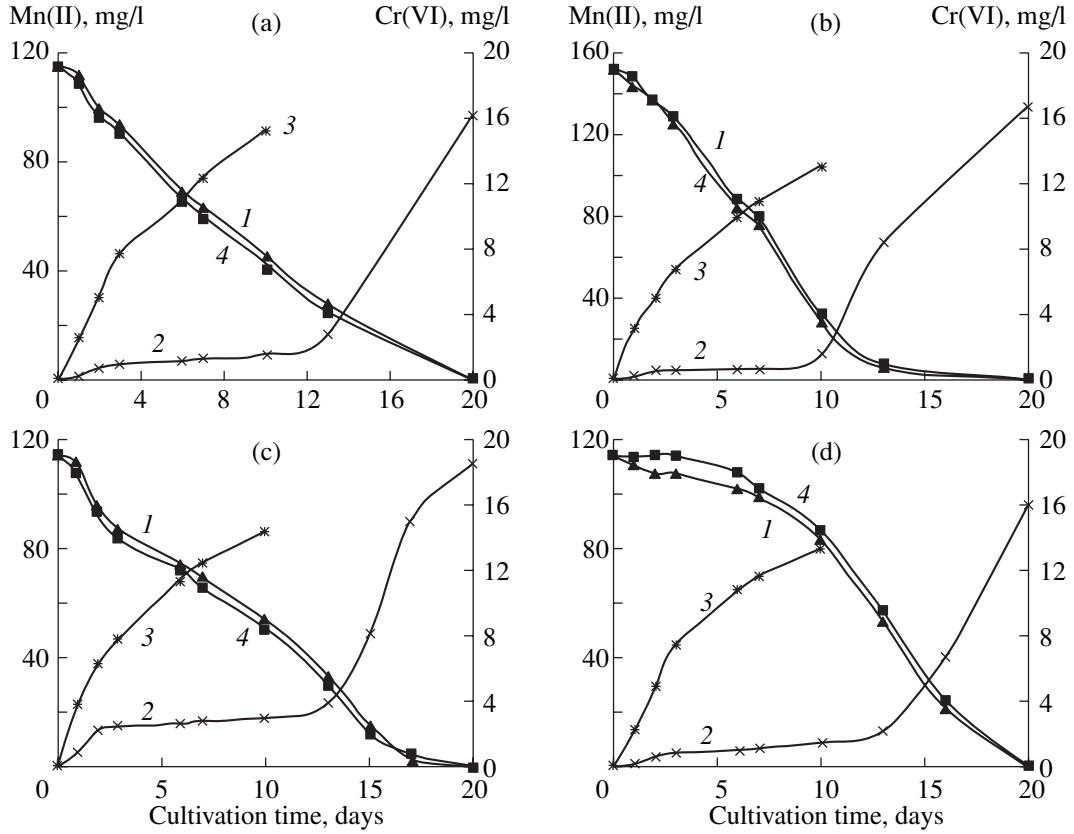

Fig. 2. Reduction of Cr(VI) and Mn(II) by the denitrifying bacteria (a) *P. aeruginosa* P-1, (b) *P. fluorescens* var. *pseudo-iodinum* P-11, (c) *P. mendocina* P-13, and (d) *P. stutzeri* P-19: (*1*) the dynamics of Cr(VI) ions in the presence of Mn(IV) in the medium, (*2*) the dynamics of Mn(II) ions in the presence of Cr(VI), (*3*) the dynamics of Mn(II) ions in the absence of Cr(VI) in the medium, (*4*) the dynamics of Cr(VI) ions in the absence of Mn(IV).

Taking into account the fact that the standard redox potentials of Cr(VI) and Mn(IV) reduction are 1333 and 1228 mV, respectively, the reduction of Cr(VI), Mn(IV), and nitrate may proceed in the following order:

$$Cr_2O_7^{2-} + 14H^+ + 6e^- \longrightarrow 2Cr^{3+} + 7H_2O, \quad E^0 = 1333 \text{ mV}; \tag{1}$$

$$MnO_2 + 4H^+ + 2e^- \longrightarrow Mn^{2+} + 2H_2O, \quad E^0 = 1228 \text{ mV}; \tag{2}$$

$$NO_3^- + 2H^+ + e^- \longrightarrow NO_2 + H_2O, \quad E^0 = 780 \text{ mV}; \tag{3}$$

$$2NO_2 + 2e^- \longrightarrow 2NO_2^-, \quad E^0 = 880 \text{ mV}; \tag{4}$$

To verify this suggestion, we studied the succession of ion reduction by the denitrifying bacteria of the genus *Pseudomonas* when Cr(VI) was present in the medium together with either NO_3^- or Mn(IV) ions. As is evident from Fig. 1a, the strain *P. aeruginosa* P-1 completely reduced 200 mg/l nitrate (curve *3*) and 10 mg/l Cr(VI) (curve *4*) in 2 and 12 days, respectively. The low rate of Cr(VI) reductions was probably due to the facts that the standard redox potential of Cr(VI) reduction into Cr(III) (E^0 = 1333 mV) is higher than that of water (E^0 = 1228 mV) and that the reduction of Cr(VI) ions requires 6 electrons and 14 protons (see reaction (1) above).

When both nitrate and Cr(VI) were present in the cultivation medium of *P. aeruginosa* P-1, Cr(VI) ions were reduced first (Fig. 1a, curve *1*), whereas the reduction of Mn(IV) began only after nine days of cultivation, when the concentration of Cr(VI) in the medium decreased to 3 mg/l (curve *2*). During the next four days of cultivation, the concentration of nitrate in the medium decreased to zero. Therefore, in the growing *P. aeruginosa* P-1 culture, chromate reduction precedes denitrification. A comparison of curves *1* and *4* in Fig. 1a shows that nitrate does not influence the rate of chromate reduction. In the control flasks, which contained no bacterial cells, the concentrations of Cr(VI) and NO_3^- ions did not change.

Similar data were obtained with the other denitrifying bacteria under study: *P. aeruginosa* var. *pseudo-iodinum* P-11 (Fig. 1b), *P. mendocina* P-13 (Fig. 1c), and *P. stutzeri* P-19 (Fig. 1d). For example, *P. mendocina* P-13 began to reduce nitrate when the concentration of Cr(VI) ions in the medium decreased to

4.5 mg/l. The reduction of nitrate became intense only when the concentration of Cr(VI) ions fell to 2 mg/l.

P. fluorescens var. *pseudo-iodinum* P-11 and *P. stutzeri* P-19 began reducing nitrate after, respectively, five and nine days of cultivation, when the concentration of Cr(VI) ions decreased to 0.5 mg/l. For comparison, when these strains were incubated in the medium containing no Cr(VI) ions, they completed denitrification in two days.

It should be noted that these data contradict the data of Zehnder and Svensson, who reported that the biological reduction of Cr(VI) ions follows the biological reduction of nitrogen oxides [1].

Figures 2a–2d illustrate the dynamics of Cr(VI) and Mn(IV) reduction. In the absence of Cr(VI) in the cultivation medium of *P. aeruginosa* P-1, the concentration of Mn(II) ions produced from the reduction of Mn(IV) ions reached 90 mg/l after ten days of cultivation (curve *3*). In the presence of 19 mg/l Cr(VI), the intense reduction of Mn(IV) began only after thirteen days of cultivation (curve *2*), when the concentration of Cr(VI) decreased to 4 mg/l. After twenty days of cultivation, the concentration of Mn(II) reached 96 mg/l. Therefore, although Mn(IV) ions are reduced by the *Pseudomonas* strains more efficiently than Cr(VI) ions, the biological reduction of chromate ions precedes the biological reduction of Mn(IV) ions when both chromate and Cr(VI) ions are present in the medium. The reduction of dichromate was not dependent on the presence of MnO_2 in the medium (curves *1* and *4*).

Similarly, in the presence of Cr(VI) ions, *P. fluorescens* var. *pseudo-iodinum* P-11 began reducing Mn(IV) ions only after thirteen days of cultivation (Fig. 2b), when the concentration of Cr(VI) in the medium decreased to 2 mg/l. In the absence of Cr(VI) ions, this strain produced 98 mg/l Mn(II) after ten days of cultivation. Two other strains, *P. mendocina* P-13 and *P. stutzeri* P-19, began reducing Mn(IV) when the concentration of Cr(VI) in the medium decreased to 4.5 mg/l (Figs. 2c, 2d). The reduction of Cr(VI) ions by these strains did not depend on the presence of MnO_2 in the medium (curves *1* and *4*). In the control flasks without bacterial cells, the concentration of NO_3^- did not change and the concentration of Mn(II) ions did not exceed 1 mg/l.

Thus, when the cultivation medium of the collection strains of denitrifying bacteria contain Cr(VI) and Mn(IV) (or nitrate) ions, the Cr(VI) ions are reduced first. The biological reduction of chromate ions does not depend on the presence of MnO_2 or nitrate in the medium. It can be suggested that the succession of biological reductive reactions is determined by the standard redox potentials E^0 of these reactions. Namely, in the presence of several electron acceptors in the cultivation medium, microorganisms first utilize those acceptors which have the highest standard redox potential.

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