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EXPERIMENTAL ARTICLES

Reduction of Chromate, Selenite, Tellurite, and Iron (III) by the Moderately Thermophilic Bacterium *Bacillus thermoamylovorans* SKC1

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Abstract—A moderately thermophilic, facultatively anaerobic bacterium capable of reducing Cr(VI) (strain SKC1) was isolated from municipal sewage. Based on the analysis of the 16S rRNA gene nucleotide sequence and DNA–DNA hybridization data, strain SKC1 was identified as a representative of the species *Bacillus thermoamylovorans. B. thermoamylovorans* SKC1 is capable of reducing chromate with L-arabinose as an electron donor with an optimum at 50°C and neutral pH. The culture is able to reduce Cr(VI) at its initial concentration in the medium of up to 150 mg/l. In addition to chromate, strain SKC1 is capable of reducing selenite and tellurite, as well as soluble forms of Fe(III). It was shown that Cr(VI), Te(IV), and Se(IV) exert a bacteriostatic effect on strain SKC1, and the reduction of these anions performs the detoxification function. This is the first communication on the reduction of chromate, selenite, tellurite, and soluble Fe(III) species by a culture of thermophilic bacilli.

Key words: chromate reduction, selenite reduction, tellurite reduction, thermophilic microorganisms, *Bacillus thermoamylovorans.*

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As a result of the widespread industrial application of chromium, selenium, and tellurium compounds, they have become significant environmental contaminants. Chromium(VI), tellurium(IV), and selenium(IV) are more toxic than their less oxidized and less soluble forms [1, 2]. The technologies of wastewater purification using microorganisms for chromate, selenite, and tellurite reduction are economically and ecologically more efficacious than the physicochemical and electrochemical purification methods [3–5].

Microorganisms of various physiological and phylogenetic groups are capable of reducing Cr(VI), Te(IV), and Se(IV) [2]. Evidence of the reduction of chromium (VI), as well as selenite and tellurite, by thermophilic prokaryotes is of a fragmentary character. Growing cultures of representatives of the bacterial genus*Thermoanaerobacter* [6], as well as cell suspensions of *Thermus* sp., *Deinococcus geothermalis*, and the hyperthermophilic archaeon *Pyrobaculum islandicum* [7–9], are known to be capable of reducing chromate. *Tepidimicrobium ferriphilum* and *Thermosinus carboxydivorans* have a selenite-reducing capacity [10, 11]. Representatives of the genus *Thermus* are able to reduce both selenite and tellurite in growing cultures [12]. No reports on the use of Cr(VI) at the enrichment culture stage for obtaining chromium-reducing thermophiles are available.

The aim of this work was to search for new microorganisms capable of reducing Cr(VI), Te(IV), and Se(IV) at increased temperatures for potential biotechnological applications.

MATERIALS AND METHODS

Sampling. Wastewater from the municipal sewage system was sampled at the Kur'yanovskaya aeration station (Moscow). The samples had been kept in tightly closed jars at 4°C for 18 h before the enrichment culture was obtained.

Medium composition and cultivation. The medium for obtaining the enrichment culture of the chromium-reducing microorganisms had the following composition (g/l of distilled water): NH_4Cl, 0.33; KCl, 0.33; $MgCl_2 \cdot 6H_2O$, 0.33; $CaCl_2$, 0.33; KH_2PO_4, 0.33; $NaHCO_3$, 2; trace element solution [13], 1 ml; vitamin solution [14], 1 ml; yeast extract (Sigma), 0.2; sodium lactate, 1.5; K_2CrO_4, 0.25 mM. The media were prepared anaerobically, dispensed into Hungate test tubes under a CO_2 flow (100%), and sterilized by autoclaving at 2 atm for 1 h. The medium did not contain any reducing agents; the pH of the autoclaved medium was

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6.7–6.9 at 20°C. The medium with ferrihydrite was prepared as described earlier [13].

In order to obtain colonies, Petri dishes with nutrient agar (1.5%) were aerobically inoculated with tenfold serial dilutions. If not otherwise specified, cultivation was carried out at 50°C.

The type strain of *Bacillus thermoamylovorans* (DKP CNCM I-1378^T = LMG 18084^T) [15] was obtained from the University of Gent (Belgium) collection of microorganisms (BCCM/LMG Bacteria Collection) and cultivated either aerobically on nutrient agar or anaerobically on medium with the above-specified composition with L-arabinose (5 g/l) as the substrate.

Other methods. Observations and cell enumeration were carried out using a light phase-contrast microscope (MIKMED-1, LOMO, Russia). The isolation and purification of the genomic DNA, selective PCR amplification of 16S rRNA gene and the determination of its nucleotide sequence were carried out by the methods described earlier [13]. The Cr(VI), Te(IV), and Se(IV) ion concentrations were measured colorimetrically with diphenylcarbazide [17], diethyldithiocarbamate [18], and 2,2'-dipyridyl [13], respectively. The minimal inhibitory concentrations of Cr(VI), Te(IV), and Se(IV) were determined as the minimal initial concentration of the corresponding ion in the medium at which no cell growth was observed for 15 days. All the experiments on Cr(VI), Te(IV), Se(IV), and Fe(III) reduction were performed in three replicates.

RESULTS AND DISCUSSION

Isolation of the chromate-reducing microorganism. Wastewater samples (1 ml) were introduced into 10 ml of sterile anaerobic medium with sodium lactate and yeast extract as substrates and 0.25 mM K_2CrO_4 and incubated at 50°C. After 7–10 days of cultivation, discoloration of the initially slightly yellow medium and the formation of a greenish-white precipitate were observed; chemical analysis showed complete disappearance of Cr(VI). After three sequential 5% culture transfers onto the same medium, the enrichment culture stably reduced Cr(VI) and appeared to be a mixture of two or three morphological types of asporogenic rods. The growth of individual colorless 1- to -2-mm colonies was achieved after plating tenfold dilutions of liquid culture onto nutrient agar plates incubated aerobically. After five sequential passages onto nutrient agar, the culture was again incubated with chromate under anaerobic conditions. Cr(VI) reduction occurred after four days; microscopic observations showed the culture to be homogeneous and to appear as straight 3-to 5-μm rods with a diameter of 0.4–0.5 μm. This strain was designated as SKC1.

Identification of strain SKC1. The analysis of the 16S rRNA gene nucleotide sequence of strain SKC1 (1488 base pairs) showed a 99.5% similarity with *Bacillus thermoamylovorans.* The hybridization of the genomic DNA of strain SKC1 and the type strain *B. thermoamylovorans* DKP CNCM I-1378^T revealed 83% homology. Thus, strain SKC1 can unambiguously be identified as a representative of the species *Bacillus thermoamylovorans* [15]. Until now, only one (type) strain of this species, isolated from palm wine produced in Senegal, has been known.

Cr(VI) reduction by strain SKC1. Anaerobic growth and reduction of K_2CrO_4 (the initial concentration of 0.6 mM) were tested with the following substrates: glucose, fructose, cellobiose, xylose, L-arabinose (5 g/l each), and sodium lactate (1.5 g/l), as well as without the addition of any substrate to the medium. Despite the fact that the enrichment culture was obtained and maintained on lactate, the isolate growth did not exceed 1×10^7 cells/ml when the culture was cultivated in the lactate medium in the absence of chromium. The same growth was also noted in the medium without the addition of lactate; apparently, SKC1 growth in the enrichment culture occurred due to the yeast extract contained in the medium. When the culture was cultivated for two weeks in medium containing 0.6 mM of potassium chromate and sodium lactate or acetate as the electron donors, as well as without the addition of the electron donors, growth did not exceed 1×10^7 cells/ml in any of these cases. In all of the three variants and in the chemical control without cells, the same (20–40%) chromium reduction was observed. Thus, strain SKC1 does not utilize Cr(VI) as the terminal electron acceptor.

In the absence of chromate, cell growth (2×10^8 cells/ml) on all the sugars tested was observed as early as in 24 h. Good growth on sugars was also noted for the type strain of *Bacillus thermoamylovorans* [15]. The capacity of the type strain for Cr(VI) reduction has not been reported earlier; our studies showed that it is also able to reduce chromium with L-arabinose as an electron donor (data not shown). The table shows growth on chromate and its reduction during cultivation of strain SKC1 with different sugars. Microbial reduction of Cr(VI) by strain SKC1 occurred only when L-arabinose was used as the substrate. When xylose and fructose were used, chromium was chemically reduced by the substrate. When strain SKC1 was cultivated with glucose and cellobiose, Cr(VI) reduction was insignificant. In the absence of the substrate and yeast extract, neither growth nor Cr(VI) reduction was observed. In the experiments to follow, L-arabinose was used as the substrate for strain SKC1 growth and Cr(VI) reduction.

Chromate (0.6 mM) reduction on medium with L-arabinose was also observed upon addition to the medium of filtrates (Millipore, 0.2 μm) or cultures killed by autoclaving (2 atm, 1 h) in the amounts used for usual inoculation (5% of the medium volume). The amount of Cr(VI) reduced after six days (subtracting the chromate reduced in the chemical control) consti-

Biomass growth and chromate reduction during strain SKC1 cultivation with different electron donors

Electron donor	Growth, 10^7 cells/ml		Cr(VI) reduction, %	
	7 days	17 days	7 days	17 days
Arabinose	10	5	93.44	100
Control			43.3	80
Glucose	<0.1	2	31.9	62.1
Control			21.4	54.5
Xylose	<0.1	5	66.45	98.7
Control			54.6	89
Cellobiose	<0.1	0.8	19.3	41
Control			3.9	39
Fructose	<0.1	10	92.5	100
Control			91.4	98

Note: The control was uninoculated medium with the corresponding substrate.

tuted 74 and 35% of the amount of chromium reduced in the medium inoculated with living cells.

Strain SKC1 grew in the 6.0–8.0 pH range with the optimum growth at pH 6.8–7.0. The maximal growth temperature for strain SKC1 was 70°C; at 75°C, no growth was observed. The maximal temperature at which it was possible to observe the reduction of Cr(VI) by the SKC1 culture was 58°C; at 60°C and higher temperatures, its quick chemical reduction by the substrate occurred. The optimum temperature for chromium reduction coincided with the optimum growth temperature (50°C).

The increase in the initial Cr(VI) concentration resulted in the lag phase being increased, the growth rate being decreased, and the ultimate cell number being reduced. Cell growth began after virtually all Cr(VI) in the cultivation medium had been reduced (Fig. 1). In the noninoculated medium, chromium reduction over this time was significantly lower (Fig. 2). The minimal inhibitory chromium concentration in the cultivation medium was 150 mg Cr(VI)/l (3.0 mM chromate); 100% reduction of Cr(VI) was, however, observed in this case as well (Figs. 2, 3). Repeated passages of the culture on the chromate-free medium did not lead to the loss of chromium-reducing capacity.

Thus, as a result of the search for new thermophilic microorganisms capable of reducing Cr(VI), we obtained the *B. thermoamylovorans* SKC1 culture. The strain was isolated from the Moscow municipal sewer system collector, the chromium concentration in which at the moment of sampling was approximately 0.1 mg/l. It may be suggested that the resistance to Cr(VI) and the capacity for its reduction in strain SKC1 were due to the presence of chromium compounds in its natural surroundings. The capacity for Cr(VI) reduction has been shown for several mesophilic representatives of the genus *Bacillus*, in particular, for *B. subtilis*, *B. cereus*, and *Bacillus* sp. [1, 19]. The possibility of chromate reduction by thermophilic bacilli was not known earlier.

An important feature of strain SKC1 is its capacity for Cr(VI) reduction at relatively low cell concentrations (1×10^6 cells/ml), which allows the problem of the development of the technology of chromium removal under continuous cultivation conditions to be considered. Not many of the microorganisms for which Cr(VI) reduction was shown in cell suspensions are able to develop in Cr(VI)-containing media [1, 19].

Reduction of Se(IV), Te(IV), and Fe(III). Strain SKC1 was also capable of reducing some other inor-

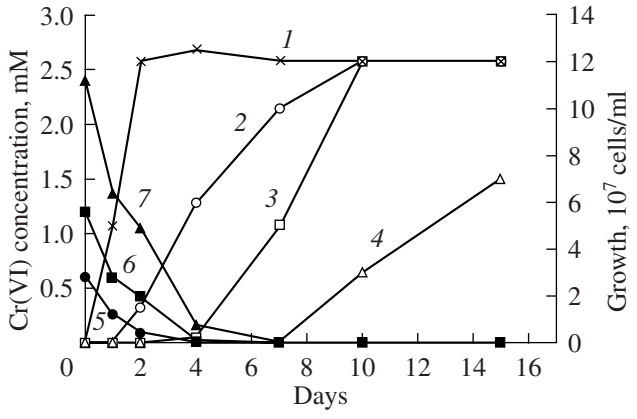

Fig. 1. Growth and reduction of chromium by strain SKC1 at different initial Cr(VI) concentrations: (*1*) cell growth, 0 mM Cr(VI); (*2*) cell growth, 0.6 mM Cr(VI); (*3*) cell growth, 1.2 mM Cr(VI); (*4*) cell growth, 2.4 mM Cr(VI); (*5*) reduction, 0.6 mM Cr(VI); (*6*) reduction, 1.2 mM Cr(VI); (*7*) reduction, 2.4 mM Cr(VI).

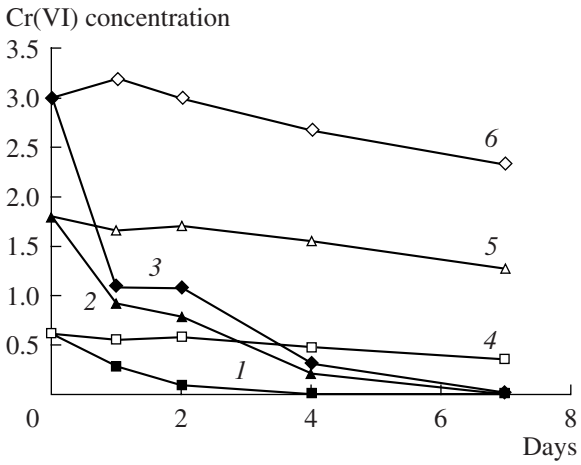

Fig. 2. Reduction of chromium by strain SKC1 at different initial Cr(VI) concentrations: (*1*) 0.6 mM; (*2*) 1.8 mM; (*3*) 3.0 mM; (*4*)–(*6*) noninoculated medium.

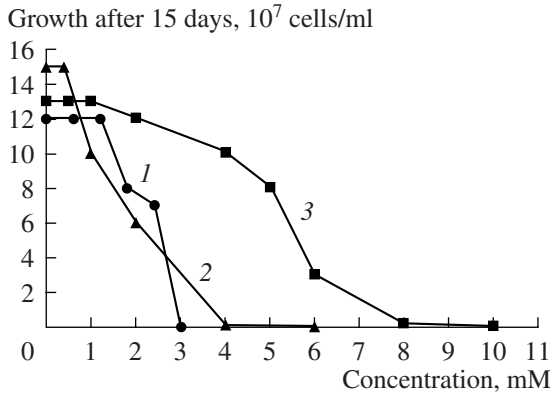

Fig. 3. Minimal inhibitory concentrations of different ions for *Bacillus thermoamylovorans* SKC1: (*1*) chromate; (*2*) tellurite; (*3*) selenite.

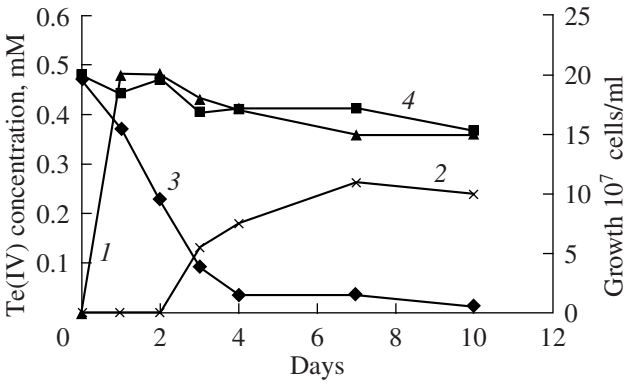

Fig. 4. Growth and reduction of tellurite by *Bacillus thermoamylovorans* SKC1: (*1*) cell growth, 0 mM Te(IV); (*2*) cell growth, 0.5 mM Te(IV); (*3*) reduction, 0.5 mM Te(IV); (*4*) reduction, 0.5 mM Te(IV), noninoculated medium.

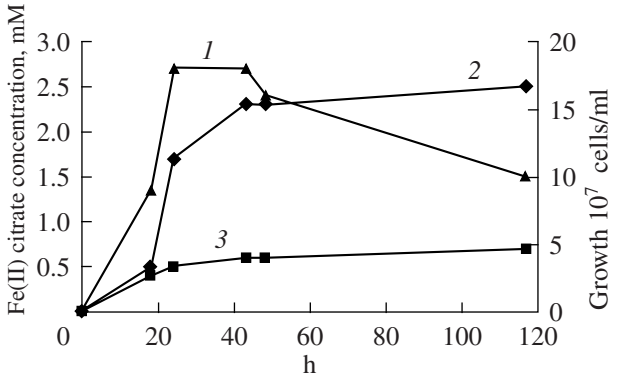

Fig. 5. Growth and reduction of Fe(III) citrate by strain SKC1 (initial Fe(III) citrate concentration was 4 mM): (*1*) cell growth; (*2*) Fe(II) formation; (*3*) Fe(II) formation in noninoculated medium.

ganic compounds, both toxic (selenite, tellurite) and nontoxic (soluble forms of iron (III)–citrate and the Fe(III)–EDTA complex). In the case of toxic anions, the minimal inhibitory concentration constituted 8 mM for selenite and 4 mM for tellurite (Fig. 3). Consequently, the most toxic for this organism is chromate followed by tellurite; selenite proved to be much less toxic.

Cultivation with selenite and tellurite was accompanied by a change in the medium coloration from colorless to red–orange and black, respectively, and precipitate formation. Cell growth in the presence of 0.5 mM potassium tellurite began after the reduction of 80% of Te(IV) (Fig. 4). Strain SKC1 did not reduce insoluble ferrihydrite (slightly crystalline Fe(III) oxide; the initial concentration was 90 mM Fe(III)); however, it was capable of reducing soluble Fe(III) forms—citrate (the initial concentration was 12 mM) and the Fe(III)–EDTA complex (the initial concentration was 10 mM). The reduction of nontoxic Fe(III) citrate occurred simultaneously with cell growth, without a markedly pronounced lag phase (Fig. 5). At the same time, when strain SKC1 was cultivated in the medium containing chromate, selenite, or tellurite, cell growth did not begin until these ions were reduced, irrespective of whether the reduction was chemical or microbial. Evidently, these anions exert a bacteriostatic effect on strain SKC1.

Despite the fact that the capacity for Cr(VI), Te(IV), and Se(IV) reduction has been shown for many microorganisms, the reductive function of these compounds in cell metabolism has not been unraveled for most microorganisms. In several cases, it was shown that Cr(VI) may be used as the terminal electron acceptor for growth [20, 21]. It was also suggested that Cr(VI) reduction may serve as a mechanism of resistance to this ion [22]. The absence of stimulation of growth by chromate in the experiments with nonfermentable substrates allows a conclusion to be made that strain SKC1 does not utilize Cr(VI) as the terminal electron acceptor for growth. Thus, detoxification is the main function of Cr(VI), Te(IV), and Se(IV) reduction for a given organism. The resistance to toxic compounds due to their conversion to a less toxic form may determine the strategy of survival of microorganisms under unfavorable conditions and of colonizing new ecological niches. The possibility of reducing the initially high chromium(VI) concentrations (up to 150 mg/l) at low biomass concentrations and the capacity for reducing other toxic compounds make *B. thermoamylovorans* SKC1 an attractive candidate for biotechnological application.

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