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ORIGINAL PAPER

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Anaerobic co-reduction of chromate and nitrate by bacterial cultures of *Staphylococcus epidermidis* L-02

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Abstract Industrial wastewater is often polluted by Cr(VI) compounds, presenting a serious environmental problem. This study addresses the removal of toxic, mutagenic Cr(VI) by means of microbial reduction to Cr(III), which can then be precipitated as oxides or hydroxides and extracted from the aquatic system. A strain of *Staphylococcus epidermidis* L-02 was isolated from a bacterial consortium used for the remediation of a chromate-contaminated constructed wetland system. This strain reduced Cr(VI) by using pyruvate as an electron donor under anaerobic conditions. The aims of the present study were to investigate the specific rate of Cr(VI) reduction by the strain L-02, the effects of chromate and nitrate (available as electron acceptors) on the strain, and the interference of chromate and nitrate reduction processes. The presence of Cr(VI) decreased the growth rate of the bacterium. Chromate and nitrate reduction did not occur under sterile conditions but was observed during tests with the strain L-02. The presence of nitrate increased both the specific Cr(VI) reduction rate and the cell number. Under denitrifying conditions, Cr(VI) reduction was not inhibited by nitrite, which was produced during nitrate reduction. The average specific rate of chromate reduction reached 4.4 μmol Cr 10^{10} cells^{-1} h^{-1}, but was only 2.0 μmol Cr 10^{10} cells^{-1} h^{-1} at 20 °C. The maximum specific rate was as high as 8.8–9.8 μmol Cr 10^{10} cells^{-1} h^{-1}. The role of nitrate in chromate reduction is discussed.

Keywords Chromate reduction · Nitrate reduction · Bacteria · *Staphylococcus epidermidis*

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Introduction

Chromium is a transition metal which is able to exist in several oxidation states. The most stable and common forms are the trivalent Cr(III) and hexavalent Cr(VI) species [6, 31]. Cr(VI) is considered to be the most toxic and carcinogenic form of Cr, and is usually associated with oxygen as chromate (CrO_4^{2-}) or dichromate ($Cr_2O_7^{2-}$) ions. By contrast, Cr(III) forms insoluble oxides and hydroxides above pH 5 [25], is much less mobile, and mostly exists bound to organic matter in soil and aquatic environments [18]. The use of chromium in leather-tanning, electroplating, paint pigment and dye production, automobile manufacturing, the steel industry and other industries has led to Cr(VI) being discharged into natural ecosystems. Since high levels of Cr(VI) may overcome the reducing capacity of the environment, it persists as a pollutant. Problems of incremental chromate pollution and the relatively low cost of biological methods of heavy metal recovery have encouraged interest in both Cr(VI)-reducing microorganisms and chromate-resistant bacteria. A wide variety of bacteria belonging to various systematic and physiological groups are involved in Cr(VI) reduction under anaerobic conditions [1, 3, 8, 10, 16, 20, 21, 23, 24, 32]. However, the reduction of CrO_4^{2-}—a terminal electron acceptor during anaerobic respiration—may not produce enough energy to enable bacterial growth [14, 15]. Consequently, the distribution of chromate reduction in the bacterial world is not specific and can be affiliated to various physiological processes, such as simultaneous reductive processes with alternative electron acceptors (nitrate, nitrite, sulphate). These compounds are present in contaminated systems and

often vary spatiotemporally. Cr(VI) reduction by bacterial consortiums has been shown to be related to sulphate and nitrate reduction [30]. It has also been suggested that the microbial reduction of nitrate, Cr(VI) and sulphate takes place consecutively [4, 13].

As far as bioremediation applications are concerned, it is important to know how various electron acceptors affect Cr(VI) reduction, and in turn how Cr(VI) affects other terminal electron-accepting processes. Hence, the goal of this work was to investigate the interaction of chromate and nitrate reduction by *Staphylococcus epidermidis* in model experiments.

Materials and methods

Microorganism

The pure bacterial culture, strain L-02, was isolated by the authors from the active chromium-reducing bacterial consortium obtained from the Grosskayna experimental wetland station in Merseburg, Germany [30]. The culture was identified at the German collection of microorganisms and cells (DSMZ). Its 100% similarity with the type strain of *Staphylococcus epidermidis* was determined by 16SrRNA sequencing [17, 26].

Reduction experiments

The basic medium for culturing *S. epidermidis* L-02 contained (g/L): KH_2PO_4, 0.9; $Na_2HPO_4{\cdot}2\ H_2O$, 1.2; NH_4Cl, 0.5; yeast extract, 0.9; Na pyruvate, 2.0; $NaHCO_3$, 0.2; $MgSO_4{\cdot}7\ H_2O$, 0.5 (pH 7). The high concentration of phosphates resulted in no change of pH during experiments. The experiments were performed in 55 ml Wheaton glass serum bottles (Sigma) with 25 ml of the medium. The bottles were purged with N_2, sealed with Wheaton butyl stoppers (Sigma), and sterilized in an autoclave at 105 °C for 20 min. Stock solutions of $K_2Cr_2O_4$ (10 g Cr(VI)/L) and KNO_3 (100 g/L) were sterilized separately and then added to the base medium either individually or combined to reach the final concentrations. All the supplements (inoculum, Cr (VI) and nitrate) were added by injection with sterile syringes. The ability of the isolated strain to reduce Cr(VI) was examined in cultures with 0.3 mmol (standard concentration) or 0.6 mmol (double concentration) Cr(VI) in the medium. The final working nitrate concentration was 3.2 mmol. Despite the strain's optimal temperature of 30–37 °C, our experiments were carried out at 20 °C to expand the process dynamics over time.

Analytical methods

Chromium (VI) concentration was estimated with diphenylcarbazide reagent (1% in acetone) and the resulting colour was measured at 540 nm using the standard method [30]. Nitrate and nitrite concentrations were analysed by ion chromatography using a Dionex 100 (AS4A-SC column/AG4A-SC column) with UV detection at 215 nm (NO_3^-/NO_2^-) [22]. Cell numbers were counted by direct microscopy in a counting chamber [34]. All the bottles were set up in duplicate.

Data analyses

Specific reduction rates were described as the relationship between the substrate concentration decrease per time unit and the number of bacterial cells. These specific rates can be expressed mathematically as: $V_{sp} = dC/N^*t$, where V_{sp} is the specific reduction rate in μmol Cr 10^{10} $cells^{-1}$ h^{-1}, dC the difference in the reduced substrate concentrations in micromole, N the cell concentration in 10^{10}/L, and t the time in hours. Analyses of parallel samples showed that variations never exceeded 5–8%.

Results and discussion

A representative of the *Staphylococcus* genus, namely *S. cohnii*, has already been reported to reduce chromate [27]. We found that our isolated *S. epidermidis* strain L-02 can also reduce chromate. Resistance to Cr(VI) has been shown for several species of the *Staphylococcus* genus including *S. epidermidis* [29]. Nevertheless, whether all the members of this genus possess chromate-reducing ability is yet unknown. Information on the reductive activity of Staphylococci suggests that one of the typical genus features is nitrate reduction. We tested the combined and separate effects of chromate and nitrate on the strain L-02 under anaerobic conditions in the presence of pyruvate, which provided an alternative possibility of growth with a fermentative process. In addition, pyruvate could be used as an electron donor in processes of nitrate or chromate reduction.

Changes in the cell numbers during the experiments showed that nitrate supplementation stimulated the growth of the culture. The increase in the cell numbers in the medium containing nitrate was faster than in the basic medium. Furthermore, cell numbers in the medium with nitrate and chromate increased faster than with just chromate (Fig. 1). By contrast, the presence of chromate inhibited the rate of multiplication and the cell yield. Thus, chromate depressed the growth of both *S. epidermidis* L-02 and various other bacterial species [2, 11].

During experiments with 0.3 mmol Cr (VI), the strain *S. epidermidis* L-02 totally reduced chromate in 3 days at 30 °C. The maximum specific reduction rate at 30 °C was 1.025 mmol Cr 10^{10} $cells^{-1}$ h^{-1}, meaning the strain can clearly be classified as an active chromate-reducer. Reported or calculated values of maximum specific Cr(VI) reduction available in the literature for other microorganisms under anaerobic conditions are shown in Table 1. No Cr (VI) reduction occurred in

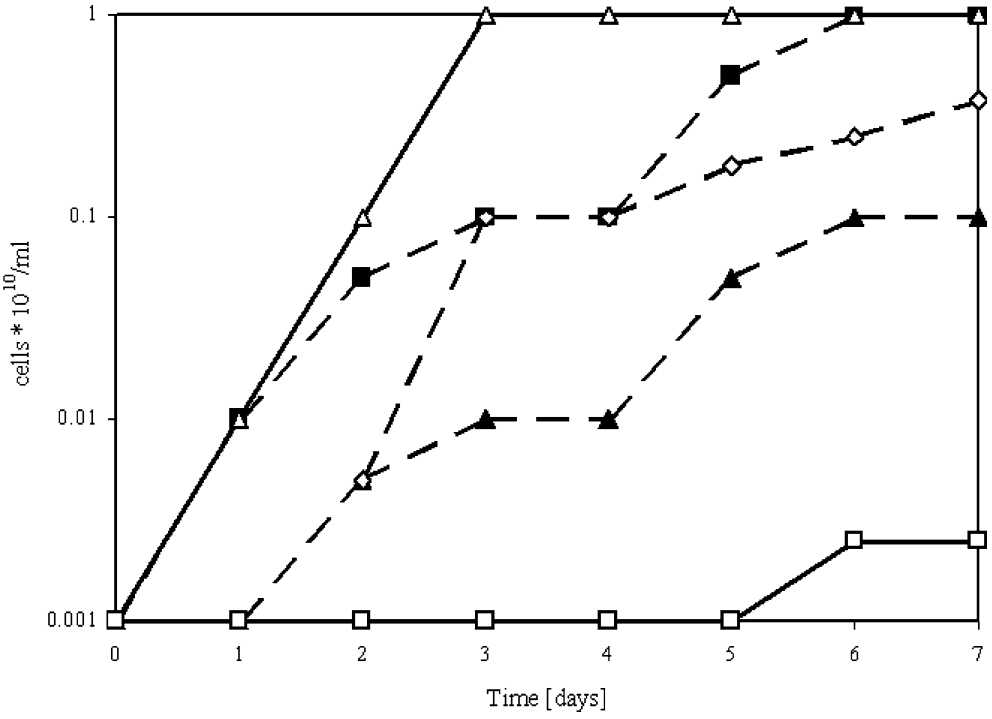

Fig. 1 Dynamics of *S. epidermidis* L-02 cell numbers during growth with various substrates. (*Filled square* medium without nitrate or chromate (fermentative growth), *open triangle* medium spiked with nitrate, *filled triangle* medium spiked with chromate, *open diamond* medium spiked with chromate and nitrate, *open square* medium spiked with nitrate and double concentration of chromate)

cell-free control experiments. The data presented were obtained at 20 °C to show differences between the various experiments in more detail. Although the change of temperature did not alter the basic regularities of the processes, it slowed down reduction: 0.3 mmol chromate was totally reduced in just 13 days (Fig. 2). When the initial Cr (VI) concentration was twice as high (0.6 mmol), the beginning of the process was observed later, even though the same amount of Cr (VI) was reduced within 13 days (data not shown).

The physiological features of the culture during the process of chromate reduction are characterized by the specific rate of reduction and its change in the experiments. The study on the specific rate of Cr(VI) reduction revealed a few stages of the process: (1) adaptation during the first 3 days (initial growth, enzyme induction), (2) a peak of activity on the fourth day (initial phase of active growth and the cell multiplication related to fast reductase formation as a protective mechanism), (3) a stable phase (5–13 days for the variant with no nitrate and 5–10 days for the variant supplemented with nitrate) of the reduction processes, (4) the appearance of a new peak of the specific nitrate reduction activity when chromate was nearly exhausted but nitrate was still present in the medium (only in the variant supplemented with nitrate). In the medium supplemented with nitrate, the biomass increase (Fig. 1) did not cause a corresponding increase in the specific Cr reduction rate. The same situation was observed in the medium supplemented with chromate only. (Fig. 2). Nevertheless, the specific rate of Cr reduction was fivefold higher in the absence of nitrate. This fact testifies to the fact of the nitrate competition as an alternative final electron acceptor to chromate. The chromate and nitrate reductions appear to be provided with the same reductase complex.

Table 1 Values of maximum specific Cr(VI) reduction as reported, or calculated from published data

Bacterial species	Maximum specific reduction rate, V_{max} (mmol Cr(VI) cells^{-1} h^{-1})
Escherichia coli ATCC 33456 [28]	$8.88*10^{-15}$ [a]
Desulfovibrio vulgaris ATCC 29579 [16]	$2.63*10^{-11}$ [a]
Enterobacter cloaceae HO1 [22]	$5.34*10^{-12}$ [a]
Shewanella oneidensis MR-1 [33]	$8.54*10^{-10}$
Staphylococcus epidermidis L-02 [This study]	$1.025*10^{-10}$
Pantoea agglomerans SP1 [7]	$1.87*10^{-10}$

[a]Assuming that 1 g dry-cell weight is approximately $7.3*10^{12}$ cells [33]

Nitrate was completely reduced within 4 days at 20 °C by the bacteria when Cr (VI) was not added to the medium (Fig. 3). Supplemented Cr (VI) exerted an influence on bacterial nitrate reduction by the strain: in experiments with 0.3 mmol Cr (VI), the nitrate concentration decreased drastically and vanished only after Cr (VI) had been completely reduced (Fig. 3).

Judged by the specific rate, the nitrate reduction process also showed different stages (Fig. 3). There was an increase at the beginning of growth and at the end of the stable phase when chromate was more or less exhausted (Fig. 2). The specific rate of nitrate reduction

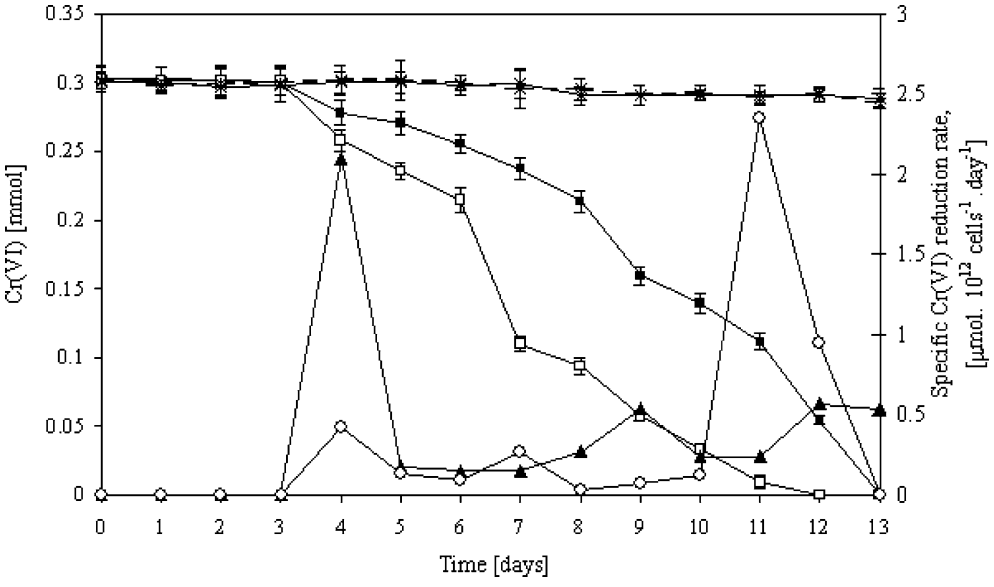

Fig. 2 Kinetics of chromate reduction by *S. epidermidis* L-02 depending on available electron acceptors. (*Filled rectangle* Cr(VI) concentration in the medium spiked with chromate, *open rectangle* Cr(VI) concentration in the medium spiked with chromate and nitrate, *filled triangle* specific rate of Cr(VI) reduction in the medium spiked with chromate, *open circle* specific rate of Cr(VI) reduction in the medium spiked with chromate and nitrate)

at log-phase was about 3.5 times higher in the absence of chromate (Fig. 3).

Nitrate reduction was accompanied by the appearance and accumulation of nitrite. In the final analyses, nitrite accounted for approximately 30% of the initial nitrate concentration at 20 °C and reached more than 60% at 30 °C. We discovered no relation between nitrite accumulation and chromate reduction (Fig. 4). It had been suggested that the interaction of nitrite with chromate can result in chemical chromate reduction associated with nitrite oxidation [19]. Then again, other authors [33] using a fixed initial Cr(VI) concentration of 0.04 mmol and varying the nitrite concentration in the range of 0–2 mmol have reported the inhibition of specific rates of Cr(VI) reduction with nitrite for *Shewanella oneidensis* MR-1. However, according to our experiments, this effect is only affiliated with their specific strain. None of these hypotheses were supported by our investigations into nitrite and chromate interaction in sterile medium. Neither our tests on *Paracoccus denitrificans* DSM 415 reducing nitrate via nitrite in the presence of chromate (data not shown) nor our sterile chemical experiments with nitrite and chromate in phosphate buffer significantly lowered the Cr(VI) concentration within a week. Moreover, the accumulation of nitrite in our experiments with *S. epidermidis* L-02 did not affect chromium reduction itself. We must thus conclude that our strain reduced chromate via a direct mechanism and not nitrite production. We tested the possibility of Cr(VI) reduction with biogenic nitrite: the bacterial cells were collected by centrifugation and removed from the liquid. This halted Cr(VI) reduction and the cultural cell-free medium did not enable the reduction process. These results also testified that the Cr(VI)-reducing enzymes were bound to the cells. Dmitrienko et al. [4, 5] studying chromate and nitrate reduction by *Pseudomonas* sps. reported that nitrate was reduced only after chromate reduction. This sequence may be

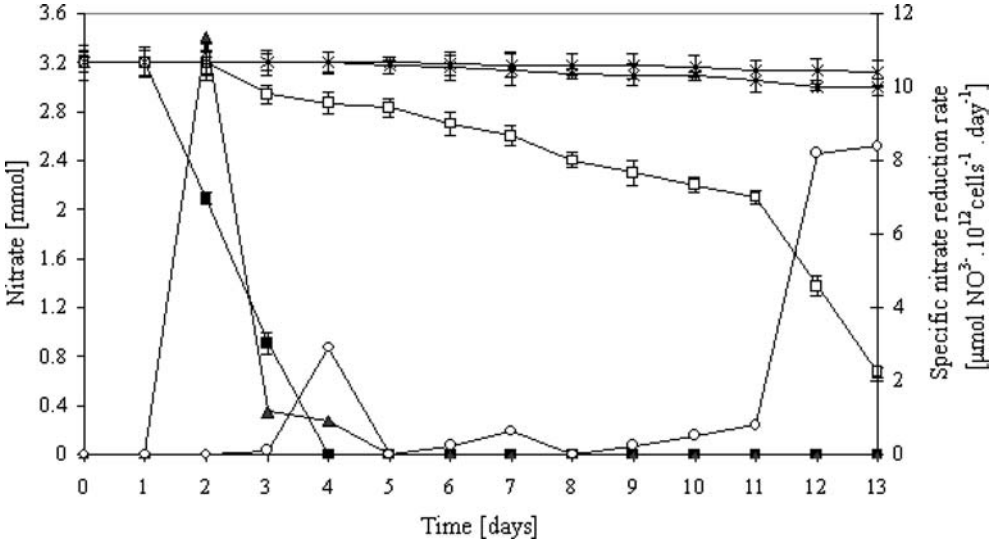

Fig. 3 Kinetics of nitrate reduction by *S. epidermidis* L-02 depending on available electron acceptors. (*Filled rectangle* NO_3^- concentration in the medium spiked with nitrate, *open rectangle* NO_3^- concentration in the medium spiked with chromate and nitrate, *filled triangle* specific rate of NO_3^- reduction in the medium spiked with nitrate, *open circle* specific rate of NO_3^- reduction in the medium spiked with chromate and nitrate)

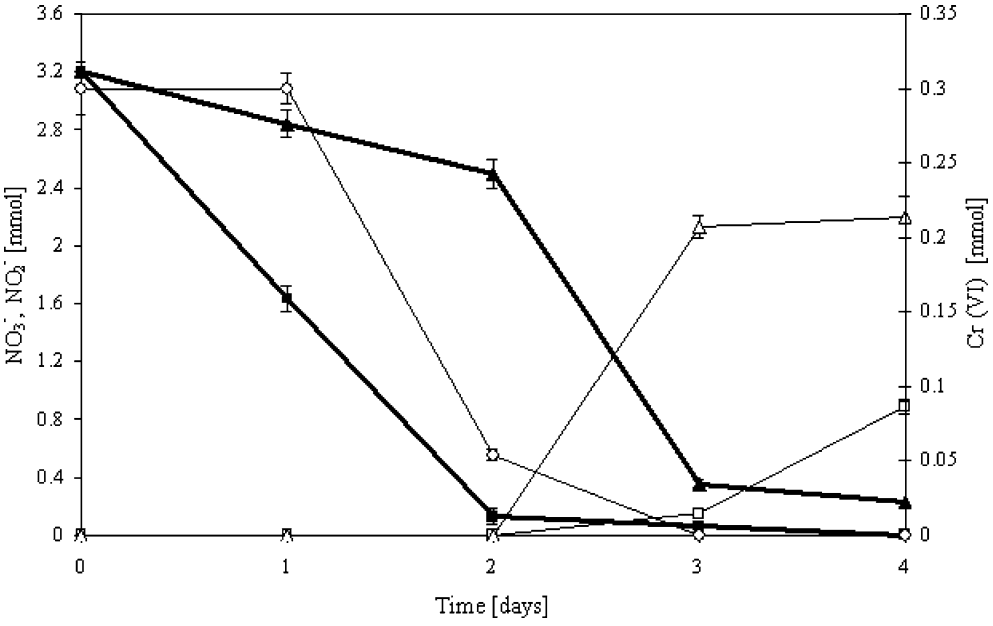

Fig. 4 Nitrite production by *S .epidermidis* L-02 depending on available electron acceptors at 30 °C. (*Filled rectangle* NO_3^- concentration in the medium spiked with nitrate, *filled triangle* NO_3^- concentration in the medium spiked with nitrate and chromate, *open rectangle* NO_2^- concentration in the medium spiked with nitrate, *open triangle* NO_2^- concentration in the medium spiked with nitrate and chromate, *open circle* Cr(VI) concentration in the medium supplemented with nitrate and chromate)

explained by the redox potentials of the anion groups mentioned. They suggested that bacteria used both chromate and nitrate as electron acceptors to gain energy, and that chromate tends to be used before nitrate. Our experiments with *S. epidermidis* L-02 showed that the strain reduced chromate and nitrate simultaneously. Similar data were published for *Bacilli* sps.: chromate reduction was not affected by a 20-fold excess of nitrate serving as an alternative electron acceptor [9]. However, differences between specific rates of reduction in the presence or absence of the second electron acceptor may be evidence of the partial alternative use of these compounds with the same or related enzymes. In addition, the slight increase in the NO_3^- reduction rate before Cr(VI) had been exhausted can be attributed to both the decrease in the Cr(VI)-inhibiting effect and the delivery of an additional nitrate reductase system already used for Cr(VI) reduction.

Conclusions

This paper describes the pattern of chromate reduction by *S. epidermidis*. The average specific reduction rate was 4.4 μmol Cr 10^{10} $cells^{-1}$ h^{-1} at 30 °C. The specific chromate reduction rate at 20 °C was generally only 2.0 μmol Cr 10^{10} $cells^{-1}$ h^{-1}, although at the maximum stages it reached 8.8 μmol Cr 10^{10} $cells^{-1}$ h^{-1} without nitrate supplements and 9.8 μmol Cr 10^{10} $cells^{-1}$ h^{-1} in the presence of nitrate. Nitrate also stimulated Cr(VI) reduction by *S. epidermidis* L-02 by increasing the cell numbers, too. The nitrite produced did not affect the process of Cr(VI) reduction under the experimental conditions. The mutual negative effect of nitrate and chromate on the specific reduction rate can be explained as the alternative use of the oxidizers by joint enzymes.

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