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VANADATE REDUCTION BY *PSEUDOMONAS AERUGINOSA* STRAINS

The ability of three Pseudomonas aeruginosa strains to reduce vanadate under microaerophilic conditions has been studied. It has been demonstrated that P. aeruginosa strains A17, A03 and C25a were able to reduce up to 38-60% pentavalent vanadium present in the medium. Vanadate reduction by P. aeruginosa strains A17, A03 and C25a was not associated with anaerobic respiration. It has been shown that vanadate reduction by P.aeruginosa strains is not the result of the spontaneous process. This is the first report of stimulation of vanadate reduction by ATPase activity inhibitor N-N-dicyclohexylcarbodiimide under aerobic conditions.

Key words: Pseudomonas aeruginosa, vanadate reduction, N-N-dicyclohexylcarbodiimide.

Microorganisms interact with metal ions in various ways. Many metal ions (copper, iron, zinc etc.) are essential microelements necessary for normal functioning of living organisms though they still possess toxicity at the elevated levels while other elements (cadmium, mercury, silver etc.) are highly toxic even at trace concentrations and usually do not play any role in the cell metabolism. Microorganisms can cope with metal toxicity in different ways: by exporting metal ions from the cell, forming metal complexes inside or outside the cell, adsorbing ions on the cell surface or changing the oxidation state of a metal ion [11].

Vanadium is widely used in metallurgical industry and may be discharged into the environment with petroleum products. Vanadium toxicity increases with its oxidation state and its pentavalent form (vanadate) is considered the most toxic. Vanadate toxicity may be attributed to its structural similarity to phosphate which could result in the negative effect on phosphate metabolism and inhibition of ATPase enzymes [9]. Microorganisms are reported to cope with vanadate either by accumulating it or reducing it to a less toxic tetravalent vanadyl form. Of great interest are microorganisms able to reduce vanadate. It has been reported that cell extracts [14] and intact bacterial cells [4] can reduce pentavalent vanadium. Mainly bacteria use vanadate as an electron acceptor during anaerobic respiration [4, 7, 12]. Aendekerk et al. reported that vanadate resistance in *Pseudomonas aeruginosa* was determined by the efflux system MexGHI-OpmD which also is responsible for antibiotic resistance [3]. Antipov et al. isolated *P. isachenkovii* from ascidia whose blood cells contained high levels of vanadium. This strain was tolerant to vanadate and was able to reduce pentavalent vanadium to V(IV) under anaerobic conditions [4]. However little to nothing is known about *P. aeruginosa* ability to reduce vanadate.

The aim of this work was to study the ability of *P. aeruginosa* strains to reduce vanadate under microaerophilic conditions.

Materials and Methods. *P. aeruginosa* strains used in this work were isolated from different natural sources [2]. Bacteria were routinely cultivated on the modified mineral medium M to decrease the risk of excessive metal complexation containing (g/l): KH_2PO_4 – 0.5; NH_4NO_3 – 0.5; $MgSO_4$ – 0.1, yeast extract – 0.5; sodium citrate – 10 [5].

The ability of bacteria to reduce pentavalent vanadium was determined by the colorimetric method based on the formation of the yellow complex between tetravalent vanadium and hydrogen peroxide at acidic pH [1]. Briefly, 1ml sample was mixed with 1 ml 3% hydrogen peroxide and 0.5 M H_2SO_4 was added, so the total volume of solution was 50 ml. The absorbance of solution was determined using photoelectrocolorimeter FEK-56 at wavelength 450 nm. The mixture containing all the reagents except vanadate served as a negative control. A standard curve was based on the serial dilutions of ammonium metavanadate.

Bacteria were grown in the flasks at 30 ^{0}C. Bacteria were inoculated into flasks filled with growth medium up to the top and fitted with rubber stoppers to prevent oxygen access. The inoculum of 5-6 x 10^7 CFU/ml and 1.5-1.8 x10^8 CFU/ml was used. Sterile controls were run under the same conditions. The cultivation lasted 26 days.

To confirm the biochemical nature of pentavalent vanadium reduction by the studied bacterial strains the bacterial suspension grown overnight on the rotor shaker at 240 rpm in the liquid medium M was centrifuged at 5000 rpm for 10 min and inoculum was added into flasks with medium M containing 1g/l V(V). Before inoculation one part of the centrifuged cells was heat killed by 80 ^{0}C treatment for 60 min.

To study the ability of *P. aeruginosa* strains to use vanadate as a sole electron acceptor the bacteria were inoculated into flasks containing phosphate buffer (20 mM Na_2HPO_4, pH 7.0) with vanadate as a sole electron acceptor and sodium citrate as a sole electron donor. Flasks were flushed with argon to create anaerobic conditions. Cultivation lasted 26 days.

The effect of the inhibitor N-N-dicyclohexylcarbodiimide (DCCD) at different concentrations (0.2, 0.4 and 1 mM DCCD) on the ability of bacteria to reduce vanadate under aerobic conditions was studied in the flasks which contained medium M with 1g/l V(V). Bacteria were cultivated on the rotor shaker at 240 rpm for 7 days.

The experiments were done in triplicate. The values represented are the means plus the standard deviation.

Results and Discussion. The ability of three *P. aeruginosa* strains A17, A03 i C25a to reduce vanadate has been studied under microaerophilic conditions during their cultivation in the liquid medium M containing 1g/l pentavalent vanadium (Fig. 1).

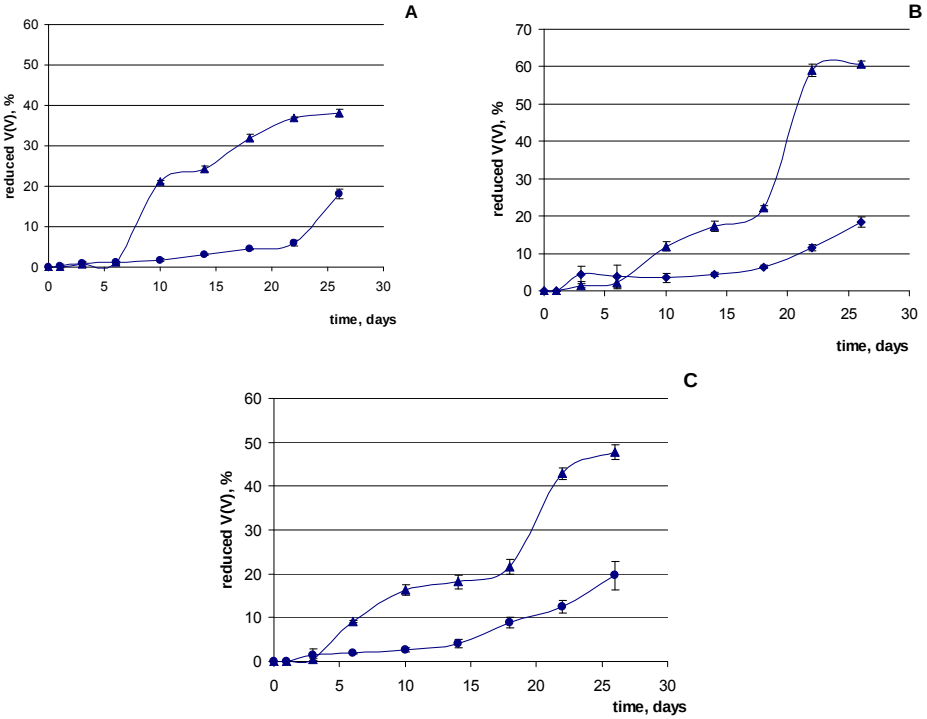

Fig. 1. Vanadate reduction (B, D, F) by *P. aeruginosa* strains A17 (A), A03 (B) and C25a (C) under microaerophilic conditions. Bacteria were inoculated into flasks filled with medium up to the top and fitted with rubber stoppers to prevent oxygen access. The inoculum size of 5-6x10^7 CFU/ml (●) and 1.5-1.8x10^8 CFU/ml (▲) was used.

When using inoculum of 5-6 x 10^7 CFU/ml the weak decrease in vanadate concentration was observed on the 7-10th day of cultivation. By the end of cultivation (26 days) strains A17, A03 and C25a reduced vanadate concentration by 18.1 %, 18.4 % and 19.6 %, respectively.

As the inoculum size was increased up to 1.5-1.8 x 10^8 CFU/ml the significant stimulation of vanadate reduction was observed. Vanadate reduction by *P. aeruginosa* strains A17, A03 and C25a under such conditions was more pronounced – 38.2 %, 60.6 % and 47.75 % of pentavalent vanadium present in the medium, respectively. The appearance of blue colour in the medium was observed on the 2-3rd day of cultivation though the noticeable decrease in vanadate concentration occurred only

after 5-10 days of cultivation. The increase in the inoculum size resulted in the stimulation of vanadate reduction. The similar effect was observed by Csotonyi et al. where the intensity of the colour change of the medium was much higher in cell suspensions of higher density [8].

It is well known that some gram-negative bacteria can use vanadate as an electron acceptor during anaerobic respiration [7, 12]. But it should be noted that the most intensive reduction of pentavalent vanadium by the studied strains did not coincide in time with the phase of active bacterial growth (data not shown) – the significant decrease in pentavalent vanadium concentration was observed after a week of cultivation or later. This could indicate that vanadate reduction by these bacteria is not associated with anaerobic respiration. Van Marwijk et al. reported that *Enterobacter cloacae* strain could reduce vanadate only while in the late exponential or stationary growth phase [13].

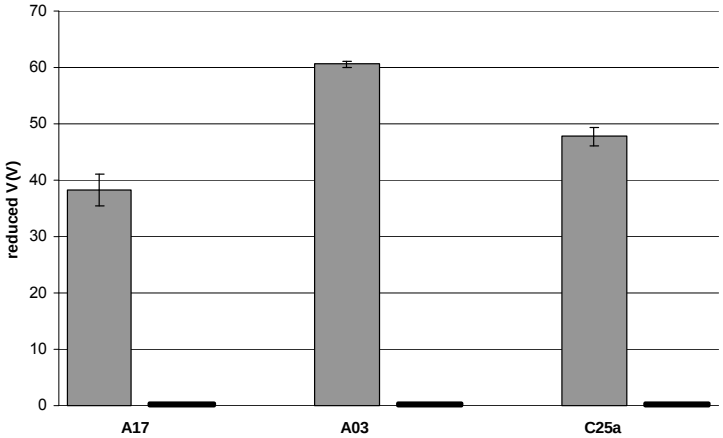

Fig. 2. Vanadate reduction by *P. aeruginosa* strains A17, A03 and C25a in the medium M (grey colour) and phosphate buffer (black colour).

To verify this assumption the flasks with phosphate buffer containing vanadate as a sole electron acceptor and sodium citrate as an electron donor were inoculated with *P. aeruginosa* strains A17, A03 and C25a. The flasks with medium M inoculated with these bacteria served as a control. Pentavalent vanadium concentration in the medium was determined after 26 days of cultivation (Fig. 2). While the decrease in vanadate concentration was observed in flasks with medium M, no pentavalent vanadium reduction occurred in phosphate buffer. The biomass increase in the flasks containing phosphate buffer was not detected. These findings confirm the speculation that vanadate reduction by A17, A03 and C25a strains is not associated with anaerobic respiration.

It is well known that some microorganisms cope with vanadate toxicity by reducing it to tetravalent vanadium. In yeasts glutathione is involved in vanadate reduction [10]. Siderophores produced by *P. aeruginosa* have been shown to form complexes with vanadate and vanadyl ions [6]. It could be presumed that the studied *P. aeruginosa* strains produce compounds with high reducing power resulting in vanadate reduction.

To exclude the possibility of vanadate reduction as a result of the spontaneous or chemical process flasks were inoculated with intact cells of *P. aeruginosa* strains A17, A03 and C25a, heat treated cells or left uninoculated. Heat killed cells of three *P. aeruginosa* strains did not possess the ability to reduce vanadate. The growth medium inoculated with untreated bacterial cells turned dark blue by 2-3rd day of cultivation while the medium containing inactivated cells did not change its colour (Fig. 3). So it can be concluded that vanadate is reduced by living cells of *P. aeruginosa* strains but this process is not associated with anaerobic respiration.

Previously we observed the appearance of blue colour in the medium containing vanadate and 200 μM DCCD – ATPase activity inhibitor – during cultivation of A17 strain under aerobic conditions, which indicated the presence of vanadyl-ion (V(IV)). The effect of different DCCD concentrations on vanadate reduction by A17 strain during aerobic cultivation has been studied (Fig. 4). No vanadate reduction under aerobic conditions was observed in the control medium (without DCCD). It has been demonstrated that the addition of DCCD to the medium resulted in 11.2-14.7 % reduction in pentavalent vanadium present in the medium by the given microorganism. The 5-fold increase in DCCD concentration from 200 μM to 1 mM did not significantly influence vanadate reduction.

A B C

Fig. 3. Vanadate reduction by living and heat-treated cells of *P. aeruginosa* strain A17. A – heat-treated cells, B – intact cells, C – negative control (uninoculated medium).

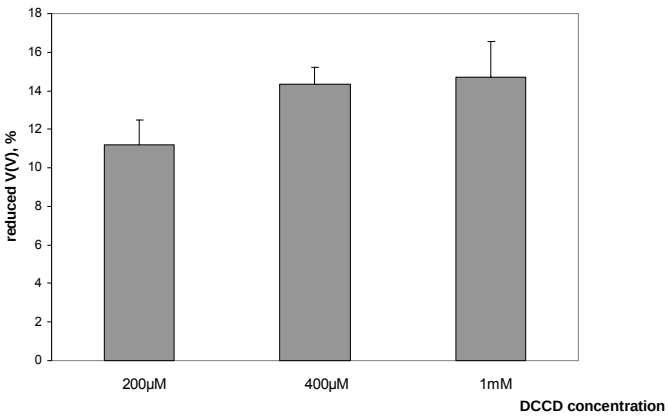

Fig. 4. Dicyclohexylcarbodiimide effect on vanadate reduction by *P. aeruginosa* strain A17 under aerobic conditions.

Aendekerk et al. [3] described efflux pump MexGHI-OpmD which was involved in vanadate resistance of *P. aeruginosa.* It could be hypothesized that A17 strain copes with pentavalent vanadium toxicity not only by reducing it to a less toxic vanadyl ion but also by expelling vanadate from the cell by the efflux mechanism. As a result inhibition of ATPase activity by DCCD could lead to suppression of one resistance mechanism (efflux) and intensification of the other (vanadate reduction). While bacterial vanadate reduction under aerobic conditions has been described [8, 13], this is the first report concerning vanadate reduction stimulation by ATPase inhibitor DCCD under aerobic conditions.

In summary, vanadate reduction by *P. aeruginosa* strains A17, A03 and C25a is a biochemical process conducted by living bacterial cells. Reduction has not been shown to be associated with anaerobic respiration. The stimulation of pentavalent vanadium reduction by the inhibitor DCCD under aerobic conditions has been shown for the first time.

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ВОССТАНОВЛЕНИЕ ВАНАДАТА ШТАММАМИ *PSEUDOMONAS AERUGINOSA*

Р е з ю м е

Изучена способность трех штаммов *Pseudomonas aeruginosa* восстанавливать ванадат в микроаэрофильных условиях. Показано, что штаммы *P. aeruginosa* A17, A03 и C25a восстанавливали до 38-60 % пятивалентного ванадия. Процесс восстановления ванадата штамами A17, A03 и C25a не был связан с

анаэробным дыханием. Установлено, что восстановление ванадата штаммами *P. aeruginosa* не является спонтанным процессом. Впервые показана стимуляция бактериального восстановления ванадата в аэробных условиях ингибитором АТФазной активности N-N-дициклогексилкарбодиимидом.

К л ю ч е в ы е с л о в а: *Pseudomonas aeruginosa*, восстановление ванадата, N-N-дициклогексилкарбодиимид.

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ВІДНОВЛЕННЯ ВАНАДАТУ ШТАМАМИ *PSEUDOMONAS AERUGINOSA*

Р е з ю м е

Досліджено здатність трьох штамів *Pseudomonas aeruginosa* відновлювати ванадат в мікроаерофільних умовах. Показано здатність штамів *P. aeruginosa* відновлювати до 38-60% п'ятивалентного ванадію. Процес відновлення ванадату штамами A17, A03 і C25a не був пов'язаний з анаеробним диханням. Встановлено, що відновлення ванадату штамами *P. aeruginosa* не є спонтанним процесом. Вперше показано стимуляцію бактеріального відновлення ванадату в аеробних умовах інгібітором АТФазної активності N-N-дициклогексилкарбодіімідом.

К л ю ч о в і с л о в а: *Pseudomonas aeruginosa*, відновлення ванадату, N-N-дициклогексилкарбодіімід.

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