Vanadium interferes with siderophore-mediated iron uptake in *Pseudomonas aeruginosa*

Christine Baysse,1 Daniel De Vos,1† Yann Naudet,1 Alain Vandermonde,1 Urs Ochsner,2 Jean-Marie Meyer,3 Herbert Budzikiewicz,4 Matthias Schäfer,4 Regine Fuchs4 and Pierre Cornelis1

Author for correspondence: Pierre Cornelis. Tel: +32 2 3590221. Fax: +32 2 3590390. e-mail: pcornel@vub.ac.be

Vanadium is a metal that under physiological conditions can exist in two oxidation states, V(IV) (vanadyl ion) and V(V) (vanadate ion). Here, it was demonstrated that both ions can form complexes with siderophores. *Pseudomonas aeruginosa* produces two siderophores under iron-limiting conditions, pyoverdine (PVD) and pyochelin (PCH). Vanadyl sulfate, at a concentration of 1–2 mM, strongly inhibited growth of *P. aeruginosa* PAO1, especially under conditions of severe iron limitation imposed by the presence of non-utilizable Fe(III) chelators. PVD-deficient mutants were more sensitive to vanadium than the wild-type, but addition of PVD did not stimulate their growth. Conversely, PCH-negative mutants were more resistant to vanadium than the wild-type strain. Both siderophores could bind and form complexes with vanadium after incubation with vanadyl sulfate (1:1, in the case of PVD; 2:1, in the case of PCH). Although only one complex with PVD, V(IV)–PVD, was found, both V(IV)– and V(V)–PCH were detected. V–PCH, but not V–PVD, caused strong growth reduction, resulting in a prolonged lag phase. Exposure of PAO1 cells to vanadium induced resistance to the superoxide-generating compound paraquat, and conversely, exposure to paraquat increased resistance to V(IV). Superoxide dismutase (SOD) activity of cells grown in the presence of V(IV) was augmented by a factor of two. Mutants deficient in the production of Fe-SOD (SodB) were particularly sensitive to vanadium, whilst sodA mutants deficient for Mn-SOD were only marginally affected. In conclusion, it is suggested that V–PCH catalyses a Fenton-type reaction whereby the toxic superoxide anion O₂⁻ is generated, and that vanadium compromises PVD utilization.

Keywords: *Pseudomonas*, vanadium, siderophores, oxidative stress

INTRODUCTION

Vanadium is a transition metal which, at neutral pH, can exist in two oxidation states, V(IV) (vanadyl ion; cationic species, VO²⁺), and V(V) (vanadate ion; anionic species, H₂VO₄⁻) (Rehder, 1991, 1992). As a result, vanadium can affect diverse biological processes. Vanadate resembles phosphate (HPO₄²⁻), and consequently can take its place in phosphate-metabolizing enzymes (Rehder, 1991, 1992). Another important biological characteristic of both vanadate and vanadyl ions is that they can form complexes with carboxylate, catecholate and hydroxamate ligands, which are present in different siderophores (Keller et al., 1991; Rehder, 1991). Although this last characteristic is well known, surprisingly, there has been no study done so far on the influence of vanadate or vanadyl ions on the siderophore-mediated iron(III) uptake systems in Gram-negative bacteria. Such an investigation is justified if one...
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 27853</td>
<td>Source of type II PVD</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>PA6</td>
<td>Source of type III PVD</td>
<td>Meyer et al. (1997)</td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type; source of type I PVD</td>
<td>ATCC 15692; Meyer et al. (1997)</td>
</tr>
<tr>
<td>PAO 6609</td>
<td>PVD’PCH’</td>
<td>Hohnadel et al. (1986)</td>
</tr>
<tr>
<td>PAO 66024</td>
<td>PVD’PCH’</td>
<td>Hohnadel et al. (1986)</td>
</tr>
<tr>
<td>PAO 6284</td>
<td>pchA (PVD’PCH’Sal⁺)</td>
<td>C. Reimann (University of Lausanne, Switzerland)</td>
</tr>
<tr>
<td>PAO 6285</td>
<td>pchDΩ (PVD’PCH’Sal⁺)</td>
<td>Serino et al. (1997)</td>
</tr>
<tr>
<td>PAO 128-6</td>
<td>pvdB pchA (PVD’PCH’Sal⁺)</td>
<td>C. Reimann (University of Lausanne, Switzerland)</td>
</tr>
<tr>
<td>PAO sodA::Gm’</td>
<td>Gm’ sodA mutant of PAO1</td>
<td>This study</td>
</tr>
<tr>
<td>PAO sodA::Gm’AsodB::Tc’</td>
<td>Gm’ Tc’ sodAsodB double mutant of PAO1</td>
<td>This study</td>
</tr>
<tr>
<td>FRD1</td>
<td>Wild-type cystic fibrosis isolate</td>
<td>Goldberg &amp; Ohman (1984)</td>
</tr>
<tr>
<td>FRD1 sodA</td>
<td>sodA mutant of FRD1</td>
<td>Hassett et al. (1997b)</td>
</tr>
<tr>
<td>FRD1 sodB</td>
<td>sodB mutant of FRD1</td>
<td>D. J. Hassett (University of Cincinnati, USA)</td>
</tr>
<tr>
<td>FRD1 sodAsodB</td>
<td>sodAsodB mutant of FRD1</td>
<td>Hassett et al. (1997b)</td>
</tr>
<tr>
<td>E. coli</td>
<td>Host for subcloning of plasmids F− lacZΔM15</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>DH5α-MCR</td>
<td>recA1 hsdR17 supE44 Δ(lacZYA argF)</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SM10</td>
<td>Km’, mobilizer strain</td>
<td>Schweizer &amp; Hoang (1995)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCRII-2.1</td>
<td>Ap’ Km’ TA cloning vector for PCR fragments</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pEX100T</td>
<td>Ap’oriT mob sacB</td>
<td>Bethesda Research Laboratories</td>
</tr>
<tr>
<td>pBR322</td>
<td>Ap’ Tc’, E. coli cloning vector</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap’ColE1, E. coli cloning vector</td>
<td></td>
</tr>
</tbody>
</table>

considers the importance of siderophores for iron acquisition by pathogenic bacteria (Crosa, 1997). Furthermore, it is known that, at neutral pH, V(IV) can be readily oxidized to V(V), generating the toxic superoxide radicals via the Fenton reaction.

Iron uptake in P. aeruginosa is controlled at different levels via a complex interaction of regulators (Vasil & Ochsner, 1999). The ferric uptake regulator (Fur) is a general regulator, which, together with Fe₃⁺ as corepressor, represses the transcription of the pvdS gene, which encodes a sigma factor needed for the transcription of PVD biosynthetic genes. Fur also controls the transcription of pchR, a gene that encodes an activator for the transcription of the PCH biosynthetic genes. Using an elegant approach, Ochsner & Vasil (1996) found that Fur regulates a large array of genes in P. aeruginosa, including genes for putative siderophore receptors, haem uptake, alternative sigma factors, two-component regulatory systems, regulators and other unknown genes. Another gene repressed by Fur is sodA, which encodes the Mn-superoxide dismutase (SOD) of P. aeruginosa (Hassett et al., 1997a). Two SODs are produced by P. aeruginosa, a Mn-SOD induced under low-iron conditions, and an Fe-SOD, which is the predominant form (Hassett et al., 1993). These enzymes catalyse the dismutation of O₂⁻ to H₂O₂ (hydrogen...
peroxide) and O$_3$; the peroxide, in turn, can be converted to H$_2$O and O$_2$ by catalases. Both reactive oxygen species are the product of the Fenton reaction catalysed by free Fe$^{3+}$. Knowing that vanadium can substitute for iron in siderophores and in some iron-proteins such as transferrin (Keller et al., 1991; Saponja & Vogel, 1996), we investigated whether vanadyl ions could interfere with siderophore-mediated iron uptake systems and/or induce an oxidative stress in _P. aeruginosa_.

**METHODS**

**Strains and growth conditions.** The _P. aeruginosa_ strains used in this study are listed in Table 1. Cultures were grown in Luria–Bertani medium (LB) or in the iron-poor Casamino-acids medium (CAA) (Cornelis et al., 1992). Cultures were grown in a Bioscreen apparatus (Life Technologies) using the following parameters: shaking for 10 s every 3 min; reading every 20 min; temperature, 37 °C; volume of culture, 300 µl. As inoculum, an overnight culture of PAO1 in CAA was stock solution and kept at 4 °C using a 1:1 V–PVD complex.

A vanadium(CAS) detection of complex formation be-

absorption coefficient, ε$_{500}$, of 2 × 10$^{4}$ M$^{-1}$ cm$^{-1}$ (Höfte et al., 1993) and normalized to the OD$_{500}$ value of the culture.

To determine the effect of vanadium on PVD production, PAO1 cells were grown for 12 h in CAA containing 0, 0.5, 1.0 or 1.5 mM VOSO$_4$ and then diluted to start a new culture in CAA for 48 h at 37 °C using the Bioscreen apparatus. Each experiment was performed in triplicate. The PVD content in the supernatant was determined by measuring A$_{380}$.

Detection and quantification of PVD in culture supernatants. PVD was detected in culture supernatants by measuring the absorbance at 400 nm. The concentration of PVD was estimated spectrophotometrically at 400 nm using a molar absorption coefficient, ε$_{500}$, of 2 × 10$^{4}$ M$^{-1}$ cm$^{-1}$ (Höfte et al., 1993) and normalized to the OD$_{500}$ value of the culture.

To determine the effect of vanadium on PVD production, PAO1 cells were grown for 12 h in CAA containing 0, 0.5, 1.0 or 1.5 mM VOSO$_4$ and then diluted to start a new culture in CAA for 48 h at 37 °C using the Bioscreen apparatus. Each experiment was performed in triplicate. The PVD content in the supernatant was determined by measuring A$_{380}$.

Chrome azurol (CAS) detection of complex formation be-

between siderophores and vanadium. A vanadium/CAS sol-

ution was prepared by a method adapted from the original protocol described by Schwyn & Neilands (1987). Six millilitres of 10 mM hexadecyltrimethylammonium bromide (HDTMA) was added to 20 ml of water, to which 5.5 ml of 0.5 mM VOSO$_4$, 5H$_2$O (in 10 mM HCl) was added. This solution, 7.5 ml of 2 mM CAS was added slowly under constant agitation. Finally, 35 ml pipervaine solution were added (4.3 g pipervaine in 30 ml water and 5 ml pure HCl) and 5-sulfosalicylic acid to a final concentration of 4 mM. The final volume was adjusted to 100 ml. This purple vanadium/CAS solution could be poured as a gel after addition of 1% (w/v) agarose.

Siderophore and vanadium-siderophore complex puri-

fication. PVD (succinimide isoforn) from _P. aeruginosa_ PAO1 was isolated from CAA growth supernatants by the chloroform/phenol method followed by chromatography on CM-Sephadex and elution with 0.1 M pyrimidine/acetic acid buffer pH 5, as previously described (Hohnadel & Meyer, 1988). Eighty micromoles of the pure compound solubilized in 2 ml de-ionized water was supplemented with a slight excess of VOSO$_4$.5H$_2$O (100 µmol) and then filtered through a Sephadex G25 column (2.5 × 45 cm) eluted with de-ionized water. The major peak showing a brownish colour was collected and lyophilized to obtain the dark brown compound further analysed as a 1:1 V–PVD complex.

PCH was extracted with chloroform from pH3-acidified growth supernatants and purified by Sephadex-LH20 chromatography as described by Meyer et al. (1989). Complexation with vanadium was achieved by mixing 90 µmol PCH in methanol solution with 100 µmol VOSO$_4$.5H$_2$O. The red-brown complex that formed instantaneously was purified by chromatography on Sephadex-LH20 (1.5 × 30 cm column, elution with methanol).

Mass spectrometric analysis of vanadium-siderophore complexes. Mass spectra were obtained with a Finnigan MAT (Bremen) 900 ST instrument equipped with an electrospray ion (ESI) source, an electrostatic and a magnetic analyser, and an ion trap system.

Plate assay for sensitivity to paraquat. CAA or LB plates containing 0, 0.5 or 1.0 mM VOSO$_4$.5H$_2$O were inoculated with 10° cells (1 ml) from pre-cultures and dried for 1 h. Resistance to paraquat was analysed according to Hassett et al. (1995).

SOD activity measurements. Total SOD activity present in _P. aeruginosa_ crude extracts was measured using the pyrogallol auto-oxidation inhibition assay described by Marklund & Marklund (1974). Briefly, 30 µl pyrogallol (20 mM), 30 µl diethylenetriaminepentacetic acid (DTPA; 0.1 M) and 100 µl freshly prepared crude cell extract (300 µg total protein) were added to 1.5 ml 0.1 M Tris/cacodylic acid pH 7.8; the final volume was adjusted to 3 ml with water. The change in A$_{550}$ was monitored at 25 °C for 20 min. In the control auto-

oxidation sample, the crude extract was replaced by 50 mM sodium phosphate buffer (cell lysis buffer; pH 7.7). _P. aeruginosa_ cell-free extracts were prepared from overnight cultures in CAA or in CAA containing 0.5 or 1.0 mM VOSO$_4$ as described by Clare et al. (1984) except that, instead of using a French press, cells were lysed by sonication.

Construction of _sodA_ and _sodAsodB_ mutants of _P. aeru-

ginosa_ PAO1. A 1150 bp fragment containing the _sodA_ region was PCR-amplified using the primers 5’-GATGTGGCGCT-GAAAAACAC and 5’-GCCAGTCGATACGTTGATAG. The PCR product was cloned into pCRII-2.1 (Invitrogen), and the PCR product was cloned into pCRII-2.1 poly-

merase into the unique HincII site within the _sodA_ gene. The 3.2 kb _sodA::Gm’_ construct was excised from the pCR11-2.1 poly-

linker with PvuII and ligated into the Smal site of the gene replacement vector pEX100T, which allows sacB counter-selection (Schweizer & Hoang, 1995). _Escherichia coli_ SM10 containing pEX100T-soda::Gm’ was used as the donor strain in a bi-parental mating with _P. aeruginosa_ PAO1. Trans-conjugants were selected on BHI agar containing gentamicin (75 µg ml$^{-1}$) and irgasan (50 µg ml$^{-1}$), and subsequently plated on LB agar containing gentamicin (75 µg ml$^{-1}$) and 5% (w/v) sucrose. Successful double-crossover events resulting in _soda::Gm’_ mutants were monitored by the loss of a pEX100T-encoded carbencillin resistance (Cb’) marker. The insertion of the Gm’ cassette into _soda_ was confirmed by PCR across the _soda::Gm’_ region using the primers above. This yielded a 2.8 kb product for _soda::Gm’_ mutants compared to a 1.5 kb product for PAO1 wild-type (data not shown). A 1380 bp PCR product containing the _sodB_ region was amplified with primers 5’-TATGTTGGCGACTGATGAG.
and 5'-ATGCCCCATTCCCGGCTCGAG and ligated into pCRII-2.1. The PCR product was excised with HindIII and XbaI and cloned into pUC19. A 540 bp NcoI–HindII fragment containing most of the sodB coding region was excised, the ends of the sodB flanking regions were filled in with Klenow enzyme and ligated to a 1.4 kb tetracycline resistance (Tc') cassette which had been obtained by cutting pBR322 with EcoRI and StyI followed by end-polishing. The 2.6 kb ΔsodB::Tc' construct was excised from the pUC19 polylinker with PstII and ligated into the Smal site of pEX100T. E. coli SM10 harbouring the resulting plasmid, pEX100T-ΔsodB::Tc', was used as the donor strain in a biparental mating with P. aeruginosa sodA::Gm'. The mating and the subsequent isolation of sodAsodB mutants were performed under anaerobic conditions using Campy Pak jars with palladium catalyst (Becton-Dickinson) in the presence of 1% (w/v) potassium nitrate as an alternative electron acceptor during anaerobic growth. Candidate sodA::Gm' sodB::Tc' double mutants were initially selected on BHI agar containing 1% (w/v) KNO₃, tetracycline (150 µg ml⁻¹) and irgasan (50 µg ml⁻¹), followed by sacB counter-selection on LB agar containing 5% sucrose, 1% KNO₃ and tetracycline (150 µg ml⁻¹). The successful replacement of the sodB gene by the Tc' cassette was verified by PCR across the sodB region using the above primers, resulting in a 2.2 kb PCR product for the sodA::Gm' sodB::Tc' double mutant compared to a 1.38 kb product for the sodA::Gm' single mutant (data not shown).

PCR was performed using Taq polymerase and custom-made primers (Bethesda Research Laboratories) in a Perkin-Elmer Cetus thermal cycler, with 30 cycles of denaturing (1 min, 94 °C), annealing (1 min, 54 °C) and extending (1 min per kb of DNA, 72 °C). The PCR products were purified in low-melting-point agarose gels, routinely cloned into pCR-II-2.1 (Invitrogen) and sequenced with Sequenase 2.0 (United States Biochemical) and M13 primers or custom-made 18-mer oligonucleotides.

RESULTS

Effect of iron on the growth of P. aeruginosa PAO1 in the presence of vanadium

Table 2 shows the effect of the addition of increasing concentrations of VO₅SO₄ (10, 1.5 and 2 mM) on the growth of PAO1 under iron-limiting or iron-sufficient conditions. The following parameters were analysed:

- the duration of the lag phase, the slope of the exponential phase (OD₆₅₀ min⁻¹), the maximal optical density (OD₆₅₀ max) reached, the time to reach the maximal OD₆₅₀ and the OD₆₅₀ after 48 h of growth. Addition of 10 µM FeCl₃ to the CAA medium resulted, as expected, in strong stimulation of growth as judged by the maximal OD₆₅₀ reached and the increase in the slope. When iron was present, the addition of increasing concentrations of VO₅SO₄ resulted in a concentration-dependent increase in the lag phase (up to 35 h for the highest concentration of 2 mM), but once growth resumed, both the slope and the maximal OD₆₅₀ reached were relatively unchanged. In the absence of iron, increased concentrations of VO₅SO₄ not only caused a prolongation of the lag phase, but also decreased the slope and decreased the maximal OD₆₅₀. In the presence of 2 mM VO₅SO₄, no growth was observed in CAA medium without added iron, even after 72 h.

Effect of Fe(III) chelators on the growth of P. aeruginosa in the presence of vanadium

The effects of adding the strong, non-metabolizable Fe(III) chelator EDDHA, the cognate purified PVD, and a heterologous PVD from P. aeruginosa ATCC 27853 for which PAO1 has no receptor (Hohnadel & Meyer, 1988), on the growth of PAO1 in CAA medium was tested after 24 h. As expected, addition of the cognate PVD (50 µM) in CAA medium stimulated growth (OD₆₅₀ units compared with 0.36 OD₆₅₀ units for the CAA control), whilst addition of EDDHA (0.5 mg ml⁻¹) or the heterologous PVD (50 µM) had little effect on growth (0.29 and 0.40 OD₆₅₀ units, respectively). In the presence of a subinhibitory concentration of vanadium (1 mM), the OD₆₅₀ after 24 h (0.33 OD₆₅₀ units) was reduced compared to the CAA control. Strikingly, the combination of 1 mM vanadium and EDDHA resulted in complete suppression of growth (0.09 OD₆₅₀ units), an effect which persisted even when incubation was extended to 48 h (results not shown). Interestingly, addition of the cognate PVD in the presence of 1 mM VO₅SO₄ did not stimulate growth (0.22 OD₆₅₀ units), whilst addition of the non-cognate PVD, as in the case of

| Table 2. Effect of vanadium on growth of P. aeruginosa PAO1 in CAA medium |
|-------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                               | 0               | 1.5             | 1.5             | 1.5             | 20              | 20              |
| VOSO₄ (mM)                    | 0               | 1.5             | 1.5             | 1.5             | 20              | 20              |
| FeCl₃                         | 0               | 1              | 0               | 1              | 0               | 1               |
| Lag phase (min)               | 230             | 300             | 350             | 700             | 1040            | 1490            | NG              | 2120            |
| Slope (OD₆₅₀ min⁻¹)           | 3.1 x 10⁻⁴      | 3.1 x 10⁻³      | 1.4 x 10⁻⁴      | 3.8 x 10⁻³      | 0.8 x 10⁻⁴      | 4.2 x 10⁻³      | 0.25            | 1.35            | 0.09            | 1.29            | 3.4 x 10⁻³      | 0.09            | 1.29            |
| OD₆₅₀ max                     | 0.43            | 1.56            | 0.38            | 1.36            | 0.25            | 1.35            | 0.09            | 1.29            | 0.09            | 1.29            |
| Time to OD₆₅₀ max (min)        | 3010            | 1030            | 3240            | 1580            | 3240            | 2420            | 3020            | 3020            |
| OD₆₅₀ after 48 h               | 0.43            | 0.95            | 0.38            | 1.16            | 0.23            | 1.25            | 0.09            | 1.25            |
Fig. 2. Growth of P. aeruginosa PAO1 wild-type (●), and PVD− mutants PAO 6609 (▲) and PAO 66024 (□) in CAA medium containing 1 mM VOSO₄. Growth was measured in a Bioscreen apparatus at 37 °C as described in Methods. Results shown are mean values from triplicate cultures.

Fig. 1 shows the growth curves of wild-type PAO1 and two PVD-negative mutants. These mutants are non-fluorescent under UV, do not produce PVD and are unable to grow in the presence of the Fe(III) chelator EDDHA. These two mutants grew in the presence of 1 mM vanadium only after a prolonged lag phase. Conversely, all mutants defective in the production of PCH showed an increased resistance to vanadium, compared to the wild-type, although to different extents (Fig. 2). The two PVD− PCH− mutants especially were found to be completely unaffected by the presence of 1.5 mM VOSO₄, a concentration that severely compromised the growth of the wild-type strain. The PCH− Sal+ and the PCH− Sal− mutants showed an intermediate level of resistance to vanadium. These results led us to conclude that the production of PVD contributes to resistance to vanadium whilst, conversely, the production of PCH enhances the toxic effect of vanadium.

### Effect of vanadium on PVD production

We wanted to investigate if pre-incubation of the cells with vanadium would affect PVD production since we had observed that the characteristic fluorescence due to PVD production disappeared when cells were grown in CAA medium containing 0.5 mM VOSO₄. Compared to the control condition (CAA medium, no VOSO₄, 100% PVD production), pre-culturing in the presence of 0.5, 1.0 and 1.5 mM VOSO₄ for 12 h caused a reduction in PVD production in CAA medium of 20, 40 and 42%, respectively, whilst cell growth, as determined by OD₆₀₀, was identical under each condition used. Conversely, PCH production was not affected by pre-exposure of the cells to 0.5 mM VOSO₄. The PCH content of supernatants from cultures of the PVD− mutant 6609 grown in CAA was determined by liquid CAS assay (Schwyn & Neilands, 1987) and was found to be identical whether the pre-culture was performed in the presence or in the absence of 0.5 mM VOSO₄.

### Vanadium complexation by PVD and PCH

Spent medium of PVD-producing strains grown in the presence of vanadium showed a characteristic brown colour that was not observed in spent medium of PVD-negative mutants. Furthermore, the fluorescence under UV, characteristic of free PVD, could not be detected in cultures of P. aeruginosa grown in the presence of 0.5 mM VOSO₄. This prompted us to investigate whether PVD and PCH could complex vanadium. For both purified siderophores, a very clear shift of the UV-visible peaks was observed after addition of 1 mM VOSO₄, an indication that a complex was formed. The following peaks were observed: purified free PVD at pH 5.2, 365 and 385 nm; PVD plus VOSO₄, 405 nm; purified PCH in methanol, 218 and 248 nm; PCH plus VOSO₄, 232 and 273 nm. Using another approach to detect vanadium binding by both siderophores, a vanadium-CAS assay was developed whereby iron was replaced by vanadium. Addition of both siderophores to a vanadium-CAS plate resulted in a rapid colour change due to the de-complexation of vanadium–CAS (Fig. 3).

### Mass spectrometric analysis of the vanadium–siderophore complexes

After binding with vanadium, the PCH− and PVD−V complexes were purified as described in Methods and
analysed by ESI-MS. In the case of PVD, a 1:1 complex with V(IV) was demonstrated, whilst in the case of PCH, a 2 PCH:1 V complex was found. Interestingly, for PCH, both V(IV) and V(V) were detected in the complexes.

The molecular ion region of the PVD (succinamide side chain)–V complex showed two peaks, viz. \( m/z \ 1397 \), corresponding to \([\text{PVD} - 2\text{H}^+ + \text{VO}^2+] + \text{H}^+ \) and \( m/z \ 1429 \), corresponding to \([\text{PVD} - 2\text{H}^+ + \text{V}^2+ + \text{CH}_3\text{OH}] + \text{H}^+ \). This is in agreement with the replacement of two \( \text{H}^+ \) by \( \text{V}(0) \). V(IV)O\(^{2+}\) has only four co-ordination sites free to accommodate two of the three bidentate ligands of PVD (catecholate and two \( \text{N-\text{formyl-N-hydroxy-Orn} } \)). One site is occupied by the oxygen atom. To complete the octahedral structure, the remaining site binds one molecule of \( \text{CH}_3\text{OH} \), the solvent used for the ESI measurements. In an aqueous medium it is probably replaced by \( \text{H}_2\text{O} \). That the mass difference of 32 units is actually due to \( \text{CH}_3\text{OH} \) and not, for example, to \( \text{O}_2 \) was confirmed by a determination of the exact mass difference between the two ions.

The PCH–V complex solution contains mainly (approx. 85%) ions with the composition \([\text{PCH} - 2\text{H}^+ + \text{VO}^{2+}] + \text{K}^+ (m/z \ 428) \) (form 1). Again V(IV)O\(^{2+}\) replaces two \( \text{H}^+ \). The neutral complex I is then ionized by attachment of \( \text{K}^+ \). The isotope pattern confirms the composition. A second component (approx. 9%) corresponds according to its exact mass and isotope pattern to \([\text{PCH} - 2\text{H}^+ + \text{VO}^{2+}]^- + \text{K}^+ (m/z \ 483) \) (form 2). In this case V(V) has been incorporated. The negative complex 2 needs the attachment of two \( \text{K}^+ \) to give a positive ion in the mass spectrometer. The last approximately 6% consisted of the uncomplexed anion of PCH, \([\text{PCH} - \text{H}^+]^- + 2\text{K}^+ (m/z \ 401) \) (form 3), again confirmed by exact mass measurements and the isotope pattern. As frequently observed in ESI mass spectrometry, cluster ions were also seen, such as \([1 + 3]^- + 2\text{K}^+ (m/z \ 790), [1 + 1] + \text{K}^+ (m/z \ 817), [1 + 2]^- + 2\text{K}^+ (m/z \ 872), [1 + 1 + 1] + \text{K}^+ (m/z \ 1206) \) and \([1 + 1 + 2]^- + \text{K}^+ (m/z \ 1261) \).

**Effect of V–PVD and V–PCH complexes on growth of *P. aeruginosa* PAO1**

Purified vanadyl-siderophores were tested for their inhibitory effect on growth of *P. aeruginosa* PAO1 in CAA medium. V–PVD up to 125 \( \mu \text{g mL}^{-1} \) did not affect the growth of PAO1, whilst, conversely, V–PCH had a very striking inhibitory effect on growth (Fig. 4). Addition of V–PCH resulted in a prolongation of the lag phase that lasted for more than 50 h before growth resumed.

**Effect of vanadium on resistance of *P. aeruginosa* to paraquat**

The strong inhibitory activity of V–PCH, and the fact that both V(IV) and V(V) were detected in association with PCH, were strongly suggestive of V–PCH undergoing a redox cycle which could result in the generation of toxic superoxide anions (\( \text{O}_2^- \)), as already described for ferripyochelin (Coffman et al., 1990; Britigan et al., 1997). On the other hand, it is well known that V(IV) can auto-oxidize in neutral aqueous solutions, yielding \( \text{O}_2^- \) and vanadate (Liochev & Fridovich, 1987). Cells grown in the presence of VOSO\(_4\) were found to be more resistant to the superoxide-generating agent paraquat, as expressed by the decrease in the diameter of the growth inhibition zone caused by paraquat on CAA or LB plates (Table 3). This resistance increased as a function of the concentration of vanadium present in the

---

**Fig. 4.** Growth of *P. aeruginosa* PAO1 in CAA (●), CAA plus 125 \( \mu \text{M} \) V–PVD complex (○) and CAA plus 50 \( \mu \text{M} \) purified V–PCH complex (□). Growth was measured in a Bioscreen apparatus at 37°C as described in Methods. Results shown are mean values from triplicate cultures.
Table 3. Effect of pre-incubation with VOSO₄ on the sensitivity of P. aeruginosa PAO1 to paraquat

The diameter of the growth inhibition zone around a drop of paraquat on a Petri plate (Hasset et al., 1995) was measured after 18 h (LB) or 48 h (CAA) incubation at 37 °C. Values are means (±0·2) of three separate experiments.

<table>
<thead>
<tr>
<th>Medium Pre-incubation with 0·5 mM VOSO₄</th>
<th>No VOSO₄</th>
<th>VOSO₄ (0·5 mM)</th>
<th>VOSO₄ (1 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAA</td>
<td>–</td>
<td>2·4</td>
<td>2·0</td>
</tr>
<tr>
<td>CAA</td>
<td>+</td>
<td>2·0</td>
<td>2·0</td>
</tr>
<tr>
<td>LB</td>
<td>–</td>
<td>1·9</td>
<td>1·6</td>
</tr>
<tr>
<td>LB</td>
<td>+</td>
<td>1·4</td>
<td>1·2</td>
</tr>
</tbody>
</table>

Table 4. Effect of pre-incubation with VOSO₄ on the growth of P. aeruginosa PAO1 in the presence of VOSO₄

Cells were grown overnight in CAA containing 1 mM VOSO₄ and 100 µM FeCl₃ at 37 °C and diluted to inoculate cultures in the Bioscreen apparatus as described in Methods. Results are shown only for cultures grown in CAA plus FeCl₃ (100 µM). Values are means of five experiments.

<table>
<thead>
<tr>
<th>VOSO₄ (mM)</th>
<th>Induction with 1 mM VOSO₄</th>
<th>Lag phase (min)</th>
<th>OD₆₀₀ min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>–</td>
<td>220</td>
<td>4·3 × 10⁻³</td>
</tr>
<tr>
<td>0</td>
<td>+</td>
<td>220</td>
<td>4·3 × 10⁻³</td>
</tr>
<tr>
<td>1·0</td>
<td>–</td>
<td>240</td>
<td>2·1 × 10⁻³</td>
</tr>
<tr>
<td>1·0</td>
<td>+</td>
<td>240</td>
<td>3·4 × 10⁻³</td>
</tr>
<tr>
<td>1·5</td>
<td>–</td>
<td>280</td>
<td>1·2 × 10⁻³</td>
</tr>
<tr>
<td>1·5</td>
<td>+</td>
<td>280</td>
<td>1·7 × 10⁻³</td>
</tr>
</tbody>
</table>

Fig. 5. (a) Growth of P. aeruginosa PAO1 wild-type in CAA (●), sodA mutant in CAA (■), sodAsodB mutant in CAA (▲). PAO1 wild-type in CAA plus 1 mM VOSO₄ (○), sodA in CAA plus 1 mM VOSO₄ (□) and sodAsodB mutant in CAA plus 1 mM VOSO₄ (△). (b) Growth of wild-type strain FRD1 (●), FRD1 sodA (○), FRD1 sodB (△) and FRD1 sodAsodB (□) in CAA plus 1 mM VOSO₄. Growth was measured in a Bioscreen apparatus at 37 °C as described in Methods. Results shown are mean values from triplicate cultures.

medium. Pre-incubation of the cells with 0·5 mM VOSO₄ also caused a decrease in the growth inhibition caused by paraquat. In addition, we noticed that on CAA plates containing 1 mM VOSO₄ a ring of increased growth was visible at the limit of the paraquat growth inhibition zone (not shown). Altogether, these results strongly suggest that vanadium can induce an oxidative response and a defence mechanism in P. aeruginosa. Since both paraquat and vanadyl ions can generate superoxides, it is most likely that vanadyl ions can induce a SOD activity in P. aeruginosa. To test this hypothesis, total SOD activity was measured by the inhibition of the auto-oxidation of pyrogallol in crude extracts of P. aeruginosa PAO1 cells before and after pre-incubation with 0·5 mM VOSO₄. The total SOD activity in the conditions used was 10% before and 20% after exposure to VOSO₄ (expressed as percentage of inhibition of pyrogallol auto-oxidation). Such a doubling of SOD activity was consistently observed, confirming that vanadyl ions indeed induce an oxidative stress and an adaptive response in P. aeruginosa.

Pre-exposure of bacterial cells to subinhibitory vanadium concentrations induced a higher level of resistance, resulting in an increased growth rate, but only when iron was present during the pre-culture and the culture (Table 4). Similar results were obtained in LB medium (results not shown).
We also observed that PAO1 cells plated on P-agar containing 1 mM VO$_3^-$ produced more pyocyanin than on P-agar alone, an indication that the level of Fe-SOD might be elevated in the cells exposed to vanadium (Hassett et al., 1995).

Effect of mutations in sodA and sodB genes on the resistance of P. aeruginosa to vanadium

P. aeruginosa produces two SODs, one Mn-co-factored (SodA), and another Fe-co-factored (SodB) (Hassett et al., 1993). As already mentioned, resistance to vanadium was higher in the presence of iron (Table 2), indicating that SodB could be the major contributor to the resistance since the production of this haem-containing enzyme was shown to be increased under conditions of iron sufficiency (Hassett et al., 1993). Furthermore, it is known that SodB contributes more to resistance to superoxide induced by paraquat than SodA (Hassett et al., 1995).

SOD mutants from PAO1 and a cystic fibrosis strain, FRD1 (Hassett et al., 1997b), were grown in CAA or in CAA containing 1 mM VO$_3^-$, The results are shown in Fig. 5(a) for strain PAO1 (wild-type, and sodA and sodAsodB mutants), and in Fig. 5(b) for strain FRD1 (wild-type, and sodA, sodB and sodAsodB mutants). The growth of both sodA mutants (PAO1 and FRD1) was relatively unaffected by the presence of 1 mM VO$_3^-$ compared to the respective wild-type strains. However, the growth of the sodB (FRD1) mutant was strongly inhibited in the presence of vanadium. The double sodAsodB mutants grew either very poorly (in the case of FRD1) or not at all (PAO1). These results again indicated that the resistance to vanadium-generated superoxides is largely due to SodB.

DISCUSSION

One of the main concerns for the treatment of bacterial infections is the increasing proportion of antibiotic-resistant strains. Metals, such as silver and cerium, are already used in topical applications because of their antibacterial activity (Klasen, 2000). Vanadium salts are suggested that PVD, as well as PCH, can complex copper, zinc or manganese (Poppe et al., 1987; Chen et al., 1994; Visca et al., 1992; Bouby et al., 1999). Visca et al. (1992) suggested that PCH could play a role in the acquisition of metals other than Fe(III), such as Co(II) and Mo(VI). Likewise, azotochelin, a catecholate siderophore, has been suggested to participate in the uptake of molybdenum by Azotobacter vinelandii (Duhme et al., 1998).

We demonstrate here that vanadium can indeed form complexes with the two siderophores of P. aeruginosa, PCH and PVD. This is not surprising since it is well established that vanadium can be liganded by hydroxamates, carboxylates and catecholates (Keller et al., 1991; Rehder, 1991). Others have also demonstrated the fact that PVD, as well as PCH, can complex copper, zinc or manganese. (Poppe et al., 1987; Chen et al., 1994; Visca et al., 1992; Bouby et al., 1999). Visca et al. (1992) also demonstrated that V–PCH complex, has a strong antibacterial effect. Such an effect was not observed by Visca et al. (1992) for PCH complexed with other metals, including Mo(VI) and Cu(II). Interestingly, two forms of V–PCH complexes were found, one with V(IV) and one with V(V). This result suggests that V–PCH can undergo an oxidative cycle that can result in the generation of superoxide radicals (O$_2^−$). It is well known that ferripyochelin can undergo such a redox cycle, resulting in the production of cell-damaging active oxygen species (Coffman et al., 1992; Bouby et al., 1997). Stern et al. (1992) also demonstrated that V(IV)–desferrioxamine has a redox-cycling activity and generates active oxygen species. In another study on A. vinelandii siderophores, Cornish & Page (1998) demonstrated that, among the three catecholate siderophores produced by this bacterium, only aminochelin, which has the lowest affinity for iron(III), was unable to sequester iron and prevent a Fenton reaction. Interestingly, the same authors also observed that increased aeration resulted in a parallel increase in the production all three catecholate siderophores of A. vinelandii, aminochelin, azotochelin and

The inhibitory effect of vanadium was strongly enhanced by the presence of non-utilizable iron(III) chelators such as EDDHA or a heterologous, non-cognate PVD. This could mean that vanadium interferes with siderophore-mediated iron uptake in P. aeruginosa. One way by which vanadium could affect iron uptake is via the formation of stable vanadyl– or vanadate–siderophore complexes that, eventually, could be taken up by the cell, increasing the intracellular concentration of vanadium. Alternatively, the vanadium–siderophore complexes could block the uptake of iron–siderophores in a competitive way. The competition of vanadium with iron for binding by siderophores could explain why the uptake of iron via the siderophores is compromised. However, we do not at this stage know whether the vanadyl–siderophores are actively taken up by the cells.
protochelin. Protochelin is a tri-catecholate with the highest affinity for iron and is produced only under conditions of extreme oxygen stress. Like protochelin, PVD from P. aeruginosa can form a 1:1 complex with iron and protect the cells from oxidative damage due to the Fenton reaction (Coffman et al., 1990). Indeed, these authors showed that $\text{O}_2^-$ could reduce and release iron bound to Fe–PCH but not Fe–PVD.

The fact that PVD-negative mutants are more affected by vanadium can be explained by their higher production of PCH (Höfte et al., 1993), which results in an increase in the toxicity of vanadium.

Another intriguing observation we report is the repression of PVD production when wild-type cells were grown in the presence of vanadium. We can assume that vanadium did not interfere with the normal Fur-mediated regulation since iron-repressed outer-membrane proteins are normally produced in CAA medium containing vanadium and their production is repressed when iron is also present (results not shown).

We propose that V–PCH molecules undergo an oxidative cycle, resulting in the production of reactive oxygen species that, in turn, induce a response in the form of increased SOD activity. We observed that a preincubation with vanadium salts increases the resistance of PAO1 cells both to vanadium and to the redox-cycling agent paraquat, and causes an increase in SOD activity. Analysis of sod mutants indicated that Mn-SOD, encoded by sodA, only marginally participates in the resistance towards vanadium in iron-limiting conditions, whereas SodB plays a major role. SodA is known to be regulated by the Fur repressor (Hassett et al., 1996, 1997a), whilst not much is known about the regulation of SodB production in P. aeruginosa except that its production is optimal under iron-sufficient conditions (Hassett et al., 1992). The pronounced growth inhibitory effect of vanadium on iron-starved cells could be the result of insufficient production of the iron-containing superoxide-detoxifying enzyme SodB under these conditions. Further studies should include experiments designed to investigate whether vanadium uptake by the cells is increased by siderophores. Also, the search for vanadium-resistant or vanadium-susceptible mutants in P. aeruginosa should provide useful information about the mechanisms involved in the homeostasis of this metal, and the interaction between siderophores and oxidative stress in this bacterium.

**ACKNOWLEDGEMENTS**

We thank Dr Cornelia Reimman for sending us the PCH mutants, and Dr Dan Hassett for sending us the FRD1 strain and the different sod mutants as well as for interesting discussions. We also thank Dr Theresa Pattery for carefully reading this manuscript. The Bioscreen apparatus was acquired thanks to the FWO (krediet aan navorsers). This work was also supported by the Jean and Alphonse Forton Fund.

**REFERENCES**


Received 23 March 2000; revised 20 June 2000; accepted 29 June 2000.