Resistance to vanadium in *Pseudomonas fluorescens* ATCC 17400 caused by mutations in TCA cycle enzymes

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Abstract

Vanadium inhibits the growth of *Pseudomonas fluorescens* ATCC 17400 in the low-iron casamino acids medium and even more when iron is added to the medium. Analysis of transposon mutants allowed the isolation of two mutants with increased resistance to vanadium. One mutant had an insertion in the *idh* gene coding for the tricarboxylic acid enzyme isocitrate dehydrogenase. The second mutant had the transposon inserted into *acnD*, one out of three genes coding for a 2-methyl-isocitrate dehydratase (aconitase). In this mutant, there was a higher level of *acnB* aconitase transcripts while the levels of *acnA* transcripts were unchanged. A nonpolar *idh* mutant was obtained, which showed the same level of resistance against vanadium as the original transposon mutant.

Introduction

Vanadium is a transition metal, which, at neutral pH, can exist under two oxidation states: V(IV) (vanadyl ion, cationic species, VO$_2^+$), and V(V) (vanadate anion, H$_2$VO$_4^-$) (Rehder, 1991, 1992). Vanadate can replace phosphate (HPO$_4^{2-}$) in phosphate-metabolizing enzymes (Rehder, 1991, 1992). Both vanadate and vanadyl ions can form complexes with carboxylate, catecholate and hydroxamate ligands present in siderophores (Keller et al., 1991; Rehder, 1991, 1992; Baysse et al., 2000). Vanadium exerts a bacteriostatic effect on *Pseudomonas aeruginosa*, especially when the cells are grown under a condition of iron limitation (Baysse et al., 2000). Vanadium (IV) can be chelated by the two *P. aeruginosa* siderophores, pyoverdine (Pvd) and pyochelin (Pch), V-Pch being toxic for the cells (Baysse et al., 2000). Oxidation of V(IV) to V(V) generates the toxic superoxide anion (O$_2^-$), explaining the toxicity of V-Pch (Liochev & Fridovich, 1987). Resistance to oxidative stress generated by the superoxide anion via the production of the superoxide dismutase SodB is a factor contributing to the resistance of *P. aeruginosa* to vanadium (Baysse et al., 2000). In a recent study, we described the importance of a new efflux pump, MexGHI-OpmD, for the resistance of *P. aeruginosa* against vanadium (Aendekerk et al., 2002). Although expression of the components of this pump is needed to confer resistance to vanadium, it is not known yet whether this effect is direct or indirect, as it was shown that the pump is also needed for the production of quorum-sensing signal molecules (Aendekerk et al., 2002, 2005). We were interested to know whether the mechanisms of resistance to vanadium are the same in other pseudomonads and chose as a model organism *Pseudomonas fluorescens* ATCC 17400, which is known to produce two siderophores: pyoverdine and quinolobactin (Matthijs et al., 2004). In this study, we show, using a transposon mutagenesis approach, that inactivation of two enzymes involved in the tricarboxylic acid cycle results in higher resistance towards vanadium.

Materials and methods

Bacterial strains and media

*Pseudomonas fluorescens* ATCC 17400 was maintained in casamino acids (CAA) medium (Cornelis et al., 1992). Unless otherwise indicated, 50-mL cultures were inoculated from an overnight preculture and incubated at 28°C at 200 r.p.m. (New Brunswick Innova shaker). Growth was
studied in a Bio-Screen apparatus (Life Technologies, Finland) using the following parameters: shaking for 10 s every minute, reading every 20 min, temperature 28 °C. Antibiotics were added to P. fluorescens strains at the following concentrations: Km 200 μg mL⁻¹, Cm 300 μg mL⁻¹, Tc 100 μg mL⁻¹ and Gm 100 μg mL⁻¹. Escherichia coli strains were grown at 37 °C in Luria-Bertani with the appropriate antibiotics, Km 100 μg mL⁻¹, Ap 100 μg mL⁻¹, Cm 25 μg mL⁻¹ and Tc 15 μg mL⁻¹. Vanadyl sulfate (VOSO₄·5H₂O) was added from a freshly prepared, filter sterilized, 100 mM stock solution. Escherichia coli S17-1 (Tpr Smr recA, thi, pro, hsdR-M+ RP4:2-Tc:Mu: Km Tn7 λpir) was used to transfer suicide plasmids by conjugation (Simon et al., 1983).

**Transposon mutagenesis**

A mini-Tn5 mutagenesis was performed using a plasposon (transposon with an origin of replication, allowing the easy recovery of flanking sequences by plasmid rescue), pTnMod-OTc (Dennis & Zylstra, 1998). The mutants were screened for their growth in the presence of 0.25, 0.5 and 0.75 mM vanadium in order to detect V-resistant mutants. The DNA flanking the transposon was isolated and sequenced as described earlier (Aendekerk et al., 2002).

**RT-PCR analysis of acnA and acnB**

RNA was extracted (High Pure RNA Isolation Kit; Roche Diagnostics) from P. fluorescens ATCC 17400 wild type and the ATCC 17400 acnD mutant grown in CAA and CAA supplemented with 0.5 mM VOSO₄·5H₂O. cDNA was prepared with random primers, using the First-Strand Synthesis kit (Amersham Pharmacia). A PCR reaction was performed using primers AcnAfw (5’-CCTGCTGGAAAACCTGYT-3’) and AcnArv (5’-CGCCCATAGAACCCTCGACGA-3’) for the AcnA gene and AcnBfw (5’-ACAACACTGCGAGCCCTGTT-3’) and AcnBrv (5’-GTACCGTGCTTGAGACT-3’) for the acnB gene. The PCR was performed for 30 cycles with the following parameters: 30 s at 94 °C, 30 s at 52 °C and 1 min at 72 °C using Taq Polymerase (Qiagen). As a control for RNA contamination by DNA, the PCR reaction was performed on the same sample without first-strand cDNA synthesis. The oprL gene (De Vos et al., 1997) was used as a reference housekeeping gene during RT-PCR. As a control for the absence of DNA contamination, amplifications were performed using the purified RNA.

**Construction of an idh mutant of P. fluorescens ATCC17400**

A mutant within the isocitrate dehydrogenase gene (idh) was created using the pKnockoutG system (Windgassen et al., 2000). A 602-bp fragment of the idh gene was amplified by PCR with primers 7D9KOfw (5’-GTG GAA TTC AGA CCC TGG AGG ACA-3’) and 7D9KOrv (5’-GTG GTC GAC CCG ACA GCA GCA CGC CTT-3’) and the following parameters: 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C using Taq-polymerase (Qiagen). After restriction, the fragment was ligated into the EcoRI/Sall site of the pKnockoutG vector. Transformed E. coli S17-1 was used to mobilize the construct to P. fluorescens ATCC 17400. Selection for idh-knockout mutants was performed on CAA plates containing Gm (50 μg mL⁻¹); Sp (50 μg mL⁻¹) was used to counter-select for E. coli. Confirmation of the mutants was performed by PCR amplification using primers 7D9KOfw, 7D9KOrv and Z2 (5’-CGTCAAGGCGATTAAGTTG-3’), as only the combination of 7D9KOfw/7D9KOrv (and not 7D9KOfw/Z2) should result in the amplification of a 650-bp band for the nonpolar idh-knockout mutants.

**Results**

**Growth of different P. fluorescens siderophore mutants in the presence of vanadium**

The growth of P. fluorescens was followed using the multiwell Bioscreen apparatus. In CAA medium without iron, the lag phase increased as a function of the vanadium concentration. The biomass also increased as a function of the vanadium concentration, probably because of the presence of traces of iron in the vanadium sulfate solution (Fig. 1a). The wild-type cells could still grow in the presence of 1 mM VOSO₄, but only after a very long lag phase. When iron was present in the medium, growth of the wild type was completely inhibited by concentrations of vanadium above 0.25 mM (Fig. 2a). In this case, the biomass was not changed upon addition of vanadium to the medium. On solid CAA medium, the wild type could not grow at concentrations above 0.25 mM VOSO₄.

**Transposon mutants with increased resistance to vanadium**

After transposon mutagenesis, 3500 colonies were picked and screened for growth on solid CAA containing increasing concentrations of vanadium. Two mutants with increased resistance to vanadium were found: 22C3 and 7D9. Both had insertions in genes involved in the TCA cycle: 22C3 in acnD encoding a 2-methyl-isocitrate dehydrogenase ([Fe S] dependent aconitase, 89% identity at the nucleotide level with PFL1863 on 344 nucleotides), and 7D9 in the isocitrate dehydrogenase gene (idh, 84% identity at the nucleotide level with gene PFL3889 on 459 nucleotides). The EMBL accession numbers of these two partial sequences are AM292653 (acnD) and AM292654 (idh). Both mutants showed an increased resistance to vanadium and had a
shorter lag phase compared with the wild type in the presence of vanadium. Figure 1 shows that both mutants behaved like the wild type in CAA medium, except at the highest concentration of vanadium (1 mM) where they grew better than the wild type (Fig. 1b for 7D9 and 1c for 22C3). The resistance of the two mutants to vanadium was much more pronounced when grown in CAA plus iron (Fig. 2b and 2c for 7D9 and 22C3, respectively).
show any difference compared with the wild type for the production of pyoverdine, and for the sensitivity to superoxide or hydrogen peroxide (results not shown).

**RT-PCR analysis of aconitase transcripts**

The transposon insertion in mutant 22C3 occurred in a gene whose product has the highest identity with the putative aconitase. The closest homolog, found in the genome of *P. fluorescens* Pf5, is *acnD* or PFL_1863 (http://www.pseudomonas.com). Primers were designed based on the sequences of the two other aconitases of Pf5, *acnA* (PFL_1929) and *acnB* (PFL_2633), in order to detect whether their transcription is influenced by the mutation in *acnD* or by the presence of vanadium in the medium. Figure 3 shows that the levels of the *acnB* transcripts are higher in the *acnD* mutant compared with the wild type while the levels of the *acnA* transcript are unchanged. Vanadium does not seem to influence the transcription of these two genes.

**Making of an idh knock-out in *P. fluorescens* ATCC 17400**

An *idh* gene knock-out was obtained in *P. fluorescens* ATCC 17400, and its sensitivity to vanadium was compared with the wild type and with the original transposon mutant 7D9. As shown in Fig. 4, the mutant showed an increased resistance to vanadium compared with the wild type, similar to what is observed for the transposon mutant.

**Discussion**

*Pseudomonas fluorescens* ATCC 17400 behaved like *P. aeruginosa* in the presence of vanadium (Baysse et al., 2000) as, in both cases, addition of vanadium caused an increased lag phase. However, the presence of iron in the medium increased the sensitivity to vanadium as the wild type could not grow in the presence of 0.5 mM vanadium. The two mutants with increased resistance to vanadium had both an insertion in genes encoding enzymes from the TCA cycle involved in the conversion from citrate to isocitrate (aconitase in the case of 22C3) and isocitrate to κ-ketoglutarate (isocitrate dehydrogenase in the case of 7D9). In the TCA cycle, isocitrate can be converted either to κ-ketoglutarate with the production of NADPH or, via the action of isocitrate lyase (ICL), split into succinate and glyoxalate (glyoxalate shunt), which in turn can be degraded to oxalate (Fig. 5). The transposon insertion in mutant 22C3 occurred in a gene whose product has the highest identity with the putative aconitase AcnD. The homolog in the genome of *P. fluorescens* Pf5 is *acnD* or PFL_1863 (http://www.pseudomonas.com). The mutant with the highest resistance to vanadium, 7D9, had the insertion in the gene encoding the isocitrate dehydrogenase (*idh*, PA2624, PFL_3889), an NADP-dependent enzyme that converts isocitrate to κ-ketoglutarate and NADPH (Fig. 5). Interestingly, a similar reshuffling of the citrate–isocitrate pathway has been shown to occur in *P. fluorescens* ATCC 13525 when the cells use aluminum-citrate as the sole carbon source (Hamel & Appanna, 2001, 2003; Hamel et al., 2004; Middhaugh et al., 2005). When submitted to aluminum stress, *P. fluorescens* overproduces the two isocitrate-metabolizing enzymes, ICL and IDH, probably as a consequence of the degradation of [Fe–S] clusters in the aconitase via the production of reactive oxygen species (ROS) (Middhaugh et al., 2005). It has therefore been proposed that the flux from citrate to isocitrate can be increased despite the lower aconitase activity (citrate → isocitrate) due to the elevation of the production of the two enzymes that further degrade...
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isocitrate (Middhaugh et al., 2005). ICL produces glyoxalate and succinate with the further degradation of glyoxalate to oxalate, this last molecule being exported where it complexes aluminum (Hamel & Appanna, 2001, 2003; Hamel et al., 2004; Middhaugh et al., 2005). Although no pathway is known in pseudomonads for the conversion of glyoxalate to oxalate, such an activity, inducible by Al stress and associated with the inner membrane, has been described for *P. fluorescens* (Hamel et al., 1999). In our situation, we see that a mutant in one of the three aconitase genes is more resistant to vanadium. As this particular aconitase gene encodes an [Fe–S]-dependent activity, it is possible that this mutant will compensate for the absence of this activity by overproducing the other two aconitases, AcnA and AcnB, which could be less sensitive to oxidative stress (Varghese et al., 2004). We could observe that this is indeed the case for the *acnB* gene, which is more actively transcribed in the mutant. However, vanadium did not influence the rate of transcription of *acnA* or *acnB*. The high level of resistance of the *idh* mutant suggests that production of oxalate could be an important line of defense of *P. fluorescens* against vanadium toxicity.

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**References**


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