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Vanadium affects vacuolation and phosphate metabolism in *Hansenula polymorpha*

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Abstract

The yeast *Hansenula polymorpha* is able to grow on vanadate concentrations that are toxic to other organisms. Transmission electron microscopy analysis showed that *H. polymorpha* cells growing on a vanadate-containing medium undergo a significant increase in cell vacuolation and a thickening of the cell wall; the presence of small cytoplasmic vesicles and an increase in cristae at the level of the plasma membrane were also observed. These ultrastructural modifications were accompanied by a change in the intracellular polyphosphate level, as shown by in vivo ^{31}P-NMR. The involvement of these observed changes in vanadium detoxification is discussed.

Keywords: Vanadate resistance; Yeast; *Hansenula polymorpha*; Secretion; Vacuole; Polyphosphate; Vesicle

1. Introduction

Vanadium is a trace element essential for normal growth and development in plants and animals. It exhibits a wide range of stable oxidation states, V^{III}, V^{IV} and V^{V}, two of which, vanadate (V^{V}) and the less toxic vanadyl (V^{IV}), are considered to be predominant in living systems. It is also a cofactor for some marine brown algae haloperoxidases and *Azotobacter* nitrogenases [1]. Normally vanadium becomes toxic when present at intracellular concentrations above micromolar, although some species of marine tunicates can accumulate extremely high concentrations of this metal (up to 1 M) in specialised cells called vanadocytes [2]. Studies on *Neurospora crassa* and erythrocytes demonstrate that vanadate enters the cells through the phosphate transport system [3,4]. Once inside the cell, vanadate is likely to be reduced to vanadyl by glutathione, catechol and other cellular components [5]. Vanadate can also substitute for organic phosphate in key molecules of oxidoreductive and energy metabolism, reacting either with NAD to give NADV (an analogue of NADP), or with some diphosphate nucleotides to give ADPV and GDPV (analogues of ATP and GTP) [6].

Some resistance mechanisms to this metal have been hypothesized. In vanadate-resistant mutants of *N. crassa* [7] and *Candida albicans* [8] resistance to

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vanadium is due to vanadate exclusion from the intracellular compartment as a consequence of the inactivation of the phosphate transport system. Conversely, in *Saccharomyces cerevisiae*, vanadate-resistant mutants do not show alterations in the phosphate transport system, with vanadate resistance in this yeast probably being due to the extrusion of a toxic vanadate molecule formed intracellularly [9].

In the present study, we initially show that the thermotolerant yeast *Hansenula polymorpha* is able to grow in the presence of extremely high orthovanadate concentrations (>96 mM), thus exhibiting a tolerance to vanadium that is higher than that shown by many other organisms [7,8,10]. In order to study the interaction between *H. polymorpha* and vanadium, we have carried out a study of the vanadate effects on cellular morphology, ultrastructure and phosphate metabolism in *H. polymorpha* by means of transmission electron microscopy and in vivo ^{31}P-NMR spectroscopy.

2. Materials and methods

2.1. Strains and media

The *H. polymorpha* strains used were NCYC495 and 1-HPO65 (*ade2-88, ura3-1, met 4-220*), an auxotrophic derivative of CBS4732. Cells were grown at 37°C on GYNB (2% glucose; 0.7% yeast nitrogen base w/o a.a. Difco) or VGYNB (GYNB plus 50 mM sodium orthovanadate unless otherwise specified). Sodium orthovanadate (Sigma Chemical Co.) was added to the autoclaved medium from a filter sterilized 500 mM stock solution, pH 5.8. Amino acids were added as needed. YPD (2% glucose, 2% peptone, 1% yeast extract, 1.8% agar when needed) was used for viable counting.

2.2. Growth inhibition

VGYNB (at different vanadate concentrations, ranging from 1 to 96 mM) and GYNB (as a control) were inoculated with about 7×10^4 cells ml^{-1} from a pre-culture in GYNB. The cell density of each culture was checked just after the inoculum and after 48 h by plate counting (0.5 ml aliquots were collected, sonicated briefly to disrupt aggregates, and serially diluted in ice-cold YPD; 100 µl from suitable dilutions were plated in duplicate on YPD). The initial density varied between 3.0 and 4.5×10^4 CFU ml^{-1}.

2.3. Morphological and ultrastructural analysis

Cells grown to mid-exponential phase on GYNB or VGYNB were harvested by centrifugation, washed 3 times in H_2O and fixed in 1% potassium permanganate for 20 min at room temperature. Pellets were then dehydrated in a graded series of ethanol and embedded in Epon araldite. Ultrathin sections of the order of 30 nm were stained for 40 s in a solution of lead citrate and observed by transmission electron microscopy (Philips CM12 operating at 80 kV). Quantitative ultrastructural analysis was performed as follows: fields of cells in the sections were selected at random and photographed at a final magnification of 20 000×. Photographs were scanned (Epson GT 8000: EPSON SCAN version 1.30 I software) and analysed (Kontron KS200 imaging system; Kontron electronics) to determine vacuolar and cellular areas.

2.4. Spectroscopic measurements

Yeast cells in exponential phase of growth on GYNB or VGYNB were harvested by centrifugation, washed 3 times and resuspended in distilled H_2O to a concentration of $3–5\times10^9$ cells ml^{-1}. Aliquots (1 ml) were examined by ^{31}P-NMR spectroscopy after addition of D_2O (10% final concentration) to provide a lock signal. Chemical shifts were measured in ppm units from 85% orthophosphoric acid at 0 ppm. Upfield shifts were given a negative sign. NMR measurements were performed with a Varian VXR 300 spectrophotometer with 5 mm sample tubes operating at 121.4 MHz. A pulse angle of 60°C was used, and the repetition rates were 0.34 s. The peaks were identified using the assignments of Navon et al. [11] and den Hollander et al. [12].

2.5. Fluorescence microscopy

Yeast cells in exponential phase of growth on GYNB or VGYNB were harvested by centrifugation, washed 3 times in distilled H_2O, resuspended in 0.025 M Tris-HCl (pH 7.0) to a final concentra-

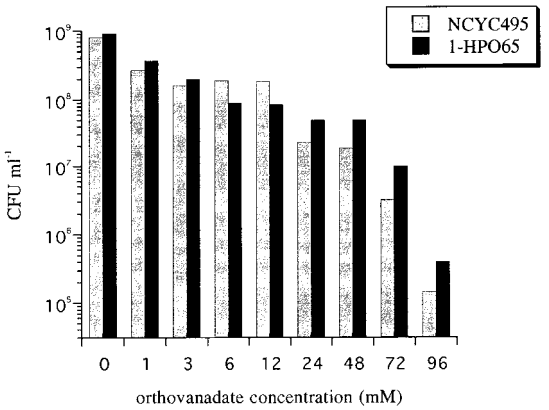

Fig. 1. The effect of vanadate on growth of two different strains of *H. polymorpha*. Viable count of cultures on GYNB or VGYNB (at different vanadate concentrations, ranging from 1 to 96 mM) of two *H. polymorpha* strains at 48 h from inoculation. The viable counts of the cultures at time 0 varied from 3.0 to 4.4×10^4 CFU ml^{-1}. The data shown are representative of two independent experiments.

tion of 1% (w/v) and stained with 4′,6-diamidino-2-phenyl-indole 2HCl (2 μg ml^{-1}; DAPI, Sigma Chemical Co.). After 20 min incubation at 4°C, cell fluorescence emission was observed through two filter combinations (UV-2A filter combination: 330–380 excitation filter/420 barrier filter for DNA detection; B-2A filter combination: 450–490 excitation filter/520 barrier filter for polyphosphate detection) assembled on a Nikon Optiphot-2 microscope equipped with an epifluorescence source (HBO 100). Fields of cells selected at random were observed and photographed with a 100× objective under visible light (as a control), and under the UV-2A and the B-2A filter combinations for detection of DNA and polyphosphate, respectively.

3. Results

3.1. Vanadate effects on cell growth

The growth of two different strains of *H. polymorpha* was assessed in the absence and presence of orthovanadate at increasing concentrations. The results are summarized in Fig. 1. After 48 h, the cell density of vanadate cultures was lower than that seen in the control, with a reduction of less than two orders of magnitude for vanadate concentrations up to 48 mM. However, both *H. polymorpha* strains were still able to grow in the presence of 96 mM vanadate.

3.2. Vanadate effects on cellular morphology and ultrastructure

The cell morphology and ultrastructure of *H. polymorpha* grown on GYNB or VGYNB were analyzed by transmission electron microscopy (Fig. 2). A significant ($P<0.001$) increase in the average vacuolar area, from 1.51 ± 1.21 μm^2 on GYNB to 4.24 ± 2.69 μm^2 on VGYNB was observed (see Fig. 2a,b). Other changes induced by vanadate were a thickening of the cell wall, an increase in cristae at the level of the plasma membrane and the formation of small vesicles in the cytoplasm (see Fig. 2c). No significant modifications in average cellular area were detected (data not shown).

3.3. Phosphate metabolism

Cellular phosphate metabolism of *H. polymorpha* grown on GYNB or VGYNB was studied by means of in vivo ^{31}P-NMR spectroscopy. As illustrated in Fig. 3 the presence of vanadium in the growth medium caused a dramatic change in peak shape, resulting in a general broadening of the P signals, together with a decrease of the Pcyt, SP and αP peaks and a simultaneous increase of the Pmp signal. The broadening and decreasing of some P signals could be due to the presence of a paramagnetic species such as vanadyl ions as already seen in *H. polymorpha* [13]. The intense signal of Pmp indicates the presence of high amounts of mobile NMR visible polyphosphates in cells grown on VGYNB.

3.4. Polyphosphate localization

In order to localise the observed polyphosphates to a cellular compartment(s), the DAPI method was used. DAPI is a well-known reagent for fluorimetric analysis of DNA [14] which has also been used for polyphosphate analysis [15]. Hence, *H. polymorpha* cells grown on GYNB or VGYNB were DAPI-stained and observed under visible light, as a control, and under the two filter combinations UV-2A and B-2A (see Section 2). When cells were examined with

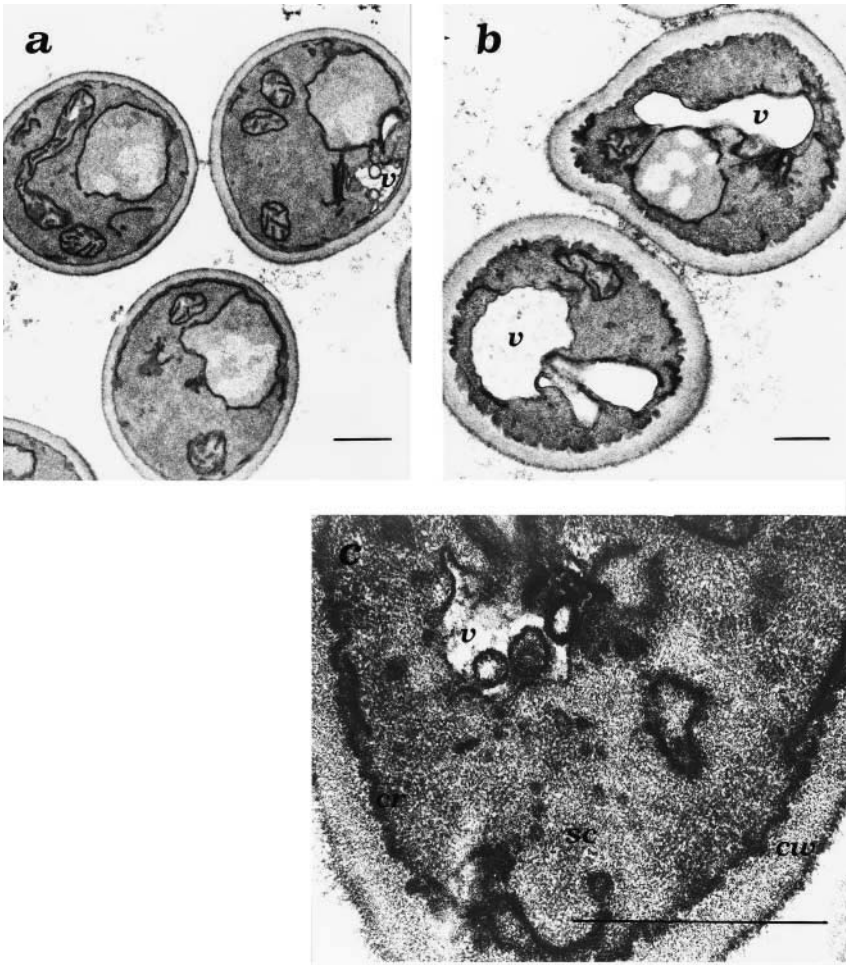

Fig. 2. Ultrastructural changes induced by vanadate in *H. polymorpha*. Cells grown to exponential phase on GYNB (a) or VGYNB (b,c) were fixed in potassium permanganate for visualization of morphological and ultrastructural modifications by transmission electron microscopy. a,b: Low-power image of whole cells (24 000×). Bar: 0.5 μm. c: High-power image of a cell portion (112 500×). Bar: 0.5 μm. v = vacuole, sc = small cytoplasmic vesicles, cw = cell wall, cr = cristae.

the B-2A filter combination, fluorescence was confined principally to spherical bodies. Comparing the images obtained with the three different wavelengths used, these fluorescent bodies were localized to the yeast vacuole (data not shown).

4. Discussion

The thermotolerant yeast *H. polymorpha* is able to grow on extremely high orthovanadate concentrations that are toxic to many other organisms. By means of ^{31}P-NMR spectroscopy, TEM analysis and fluorescence microscopy, we have shown that growth of *H. polymorpha* on vanadate-containing medium correlates with various physiological and ultrastructural modifications. These include: (i) the presence of high amounts of polyphosphates that are mainly localized in the vacuole, (ii) an increase in cell vacuolation, and (iii) the appearance of cytoplasmic vesicles and an increase in cristae at the level of the plasma membrane.

The synthesis of polyphosphates has already has been described in many microorganisms growing

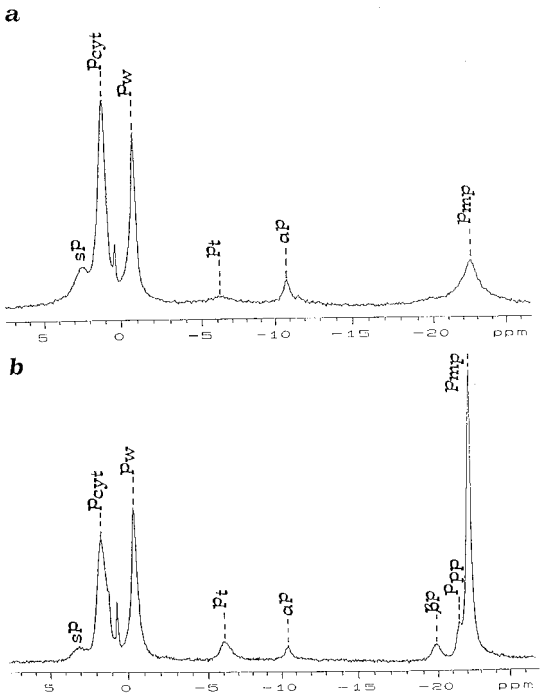

Fig. 3. ^{31}P-NMR spectra of *H. polymorpha* cells grown on (a) GYNB or (b) VGYNB. Starting from low field (on the left) the resonances are assigned as follows: the resonance at +2.5 ppm (SP) was assigned to the P of sugar phosphate; the next intense peak at about +1 ppm (P cyt) is due to cytosolic phosphate; the peak at about −1 ppm (PW) was assigned to phosphomannans of the cell wall; at −6 ppm (Pt) to the terminal phosphate of polyphosphate chains as well as the γP of ATP or the βP of ADP molecules; at −10.5 ppm (αP) to the primary phosphate of nucleoside phosphates such as the αP of the ATP and ADP molecules; at about −20 ppm (βP) to the penultimate P of different compounds such as the βP of the ATP molecule; at −21.5 ppm (Ppp) to the penultimate P of polyphosphates; the peak at −22.5 ppm (Pmp) was assigned to the middle P of polyphosphate chains. The spectra shown are from a single experiment, and are representative of two independent determinations.

under unfavourable environmental conditions [16]. They are known to act as metal sequestering agents in *Pseudomonas putida*, *Anabaena cylindrica*, a variety of eukaryotic algae, certain fungi and yeasts; studies on *Klebsiella aerogenes* have shown that accumulation of polyphosphates may correlate with heavy metal detoxification. On the other hand, in *Escherichia coli* the ability to hydrolyse polyphosphates seems to be more important for heavy metal tolerance than intracellular polyphosphate amount [17]. In yeasts and fungi a major proportion of accumulated ions is located in the vacuole where it may be in an ionic form or bound to low molecular weight polyphosphates [18]. However, the role of vacuoles in heavy metal detoxification is still controversial. While some authors hypothesized that the increase in vacuolar volume is not related to intracellular metal accumulation [19], others have suggested that vacuolation might take part in a mechanism of compartmentalization of toxic metals [20].

On the basis of our results, we can hypothesize a role for the observed modifications in *H. polymorpha*–vanadium interaction. The broadening and decreasing of the ^{31}P-NMR peak attributed to cytosolic phosphate, that is probably due to the diffuse presence of paramagnetic species such as vanadyl ions, supports the reduction of V^{V} to V^{IV} by the cells, as already observed in *H. polymorpha* by Zoroddu et al. [13]. The modification of the polyphosphate peaks indicates the presence of a high amount of ^{31}P-NMR-visible polyphosphates in cells grown on vanadate-containing medium. The observed increase in cell vacuolation, together with the high amount of polyphosphates and their vacuolar localization, suggests that once inside the cell, metal ions could be compartmentalized to the vacuole, as suggested by Davies et al. in plant cells [20], and trapped by polyphosphates. Such a compartmentalization process could be an effective detoxification mechanism which may precede the extrusion of the accumulated metals from the cell [21].

The small cytoplasmic vesicles and the plasma membrane cristae observed in *H. polymorpha* cells grown on vanadate-containing medium could be due to impaired secretion. In the yeast *S. cerevisiae* vanadate inhibits the release of secretory vesicles [22] and vanadate-resistant mutants show defects in glycosylation and in the secretory pathway [9,23]. The accumulation of the small cytoplasmic vesicles observed in *H. polymorpha* cells resembles the phenotypic modifications shown by *S. cerevisiae* vanadate-resistant mutants. While these observations would suggest that a defect in the secretory pathway could correlate to the intrinsic vanadate tolerance exhibited by *H. polymorpha*, at this stage of our investigations, it cannot be excluded that these cytoplasmic vesicles are a sign of a process leading to the extrusion of toxic vanadate molecule(s) formed intracellularly.

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