Acta Biochimica Polonica

Vol. 49 No. 4/2002

959–968

QUARTERLY

The effect of vanadate on *Pichia pastoris* growth, protein kinase A activity and ribosomal protein phosphorylation

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Received: 02 July, 2002; revised: 10 October, 2002; accepted: 28 October, 2002

Key words: *Pichia pastoris*, vanadate, PKA, protein kinases, protein phosphorylation, ribosomal proteins

It was found that wild type yeast *Pichia pastoris* can tolerate vanadate concentration as high as 25 mM in the growth medium. Moreover, four vanadate-resistant *P. pastoris* strains designated JC100/1, JC100/3, JC100/9 and JC100/15 exhibiting tolerance up to 150 mM vanadate were selected. Growth of *P. pastoris* was correlated with vanadate to vanadyl reduction and its accumulation in the growth medium. In two selected strains, JC100/9 and JC100/15, protein kinase A activity was much higher in comparison to the wild type strain even without vanadate addition to the growth medium. Moreover, in the presence of vanadate, protein kinase A activity was significantly increased in the wild type and the vanadate-resistant JC100/1 and JC100/3 strains. It was also found that phosphorylation of a 40 kDa protein associated with ribosomes occured in all vanadate-resistant strains from the logarithmic, while in the wild type strain from the stationary growth phase. From the presented results it can be concluded that a protein kinase A signalling pathway(s) might be involved in the mechanism of *P. pastoris* vanadate resistance. The results also indicate a possible role of the 40 kDa protein in protection of *P. pastoris* against vanadate toxicity.

Vanadium is a group V transition metal, essential for growth and development of many plant and animal organisms. It exists in different oxidation states V^{III}, V^{IV} and V^{V}, two of which, vanadate (V^{V}) and the less toxic vanadyl (V^{IV}), are the most common ones under physiological conditions (Rehder, 1992). It is known that vanadium salts can mimic metabolic and growth promoting effects of insulin. They are able to normalize blood glu-

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Abbreviations: PKA, cyclic AMP dependent protein kinase; MAP kinases, mitogen activated protein kinases.

cose concentration in both type I and type II diabetes mellitus. Therefore, there is an interest in the use of vanadate derivatives for the treatment of diabetes (Morinville *et al.*, 1998).

Vanadate becomes toxic when present intracellularly at concentrations higher than micromolar. The mechanism of the vanadate toxic effect is connected with its structural similarity to phosphate. As a phosphate analog vanadate inhibits the activity of enzymes of phosphate metabolism such as: ATPases (Karlish *et al.*, 1979; Wach & Graber, 1991), RNAases (Lindquist *et al.*, 1973), adenylate kinases, phosphofructokinases (Chasteen, 1984) and phosphoprotein phosphatases (Morinville *et al.*, 1998). There are, however, organisms which tolerate high vanadium concentrations as, for example, some species of marine tunicates which can accumulate as high as 1 M concentration of this metal in specialized cells — vanadocytes (Carlson, 1975). Vanadate-resistant mutants of the filamentous fungus *Neurospora crassa* (Bowman *et al.*, 1983) and several yeast species: *Candida albicans* (Mahanty *et al.*, 1991), *Saccharomyces cerevisiae* (Kanik-Ennulat *et al.*, 1995) and *Hansenula polymorpha* (Mannazzu *et al.*, 1997) were also described. The mechanism of resistance of these organisms to vanadium is still not elucidated. It was hypothesized that in vanadate-resistant mutants of *N. crassa* (Bowman *et al.*, 1983) and *C. albicans* (Mahanty *et al.*, 1991) the resistance to vanadium is due to vanadate exclusion from the intracellular compartment as a consequence of the inactivation of the phosphate transport system. In contrast, in the yeast *S. cerevisiae* vanadate-resistant mutants, the phosphate transport system was not altered. According to Willsky *et al.* (1984) and Zoroddu *et al.* (1996) the vanadate oxyanion, after entering the cells by the phosphate transport system, is reduced to the less toxic vanadyl and then excreted outside the cell. In other report Bisconti *et al.* (1997) suggested that in the SC-1 vanadate-resistant strain of *S. cerevisiae* the vanadate to vanadyl reduction occured at the level of the cell envelope and not intracellularly. It was also shown that in the yeast *S. cerevisiae* vanadate inhibits the release of secretory vesicles (Lew & Sanford, 1991) and vanadate-resistant mutants show defects in glycosylation and in the secretory pathway (Kanik-Ennulat *et al.*, 1995). For the methyltrophic yeast *H. polymorpha* it was proposed that the detoxication mechanism is based on the accumulation of toxic metal ions complexed with polyphosphates in vacoules (Mannazzu *et al.*, 1997).

In this paper we present studies on vanadate effect on the growth of the yeast *Pichia pastoris*, protein kinase A activity and ribosomal protein phosphorylation. The studies were performed on the wild type and four vanadate-resistant strains selected in our laboratory.

MATERIALS AND METHODS

Strain and growth conditions. *Pichia pastoris*, strain JC100 (Cregg Lab., Oregon Institute of Science and Technology, Portland, U.S.A.) was grown under aerobic conditions in YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 28°C to the exponential or stationary growth phase.

Preparation and storage of sodium orthovanadate stock solutions. Stock solutions of sodium orthovanadate (Sigma) were prepared in deionized water, adjusted to pH 5.8 with 6 M HCl, filter sterilized and stored at 4°C. To ensure the presence of monomers in the solution and to eliminate polymeric species of $(V_{10}O_{28})^{6-}$ which are orange-yellow in colour, pH adjustment was performed stepwise with heating the solution to boiling until translucent after each part of 6 M HCl had been added (Goodno, 1979; Gordon, 1991).

***Selection of vanadate-resistant strains of* P. pastoris.** Approximately 10^8 cells from an overnight culture of the wild type strain *P. pastoris* JC100 were spread onto YPD agar plates containing sodium orthovanadate at

concentrations up to 30 mM and incubated for 5 days at 28°C. One-hundred vanadate-resistant colonies were selected and cultivated on solid YPD medium containing from 50 mM to 150 mM sodium orthovanadate under the same conditions. Four strains, designated JC100/1, JC100/3, JC100/9, JC100/15, resistant to 150 mM Na_3VO_4 were isolated.

For the growth kinetics assessment the vanadate-resistant strains were grown in liquid YPD medium without sodium orthovanadate or in the presence of 25 mM and 50 mM Na_3VO_4 under aerobic conditions at 28°C for 80 h. Yeast growth was measured in terms of absorbance at 600 nm. The kinetics of vanadyl production was analyzed by the absorbance measured at 767 nm in cell-free medium (Bisconti *et al.,* 1997).

Preparation of yeast cell-free extracts and protein kinase A assay. Yeast cells from the exponential growth phase were harvested by centrifugation and washed three times in distilled water. Cell-free extracts were prepared in a buffer containing 50 mM Tris/HCl, pH 7.5, 6 mM 2-mercaptoethanol, 10 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM EDTA, as described previously (Cytryńska *et al.*, 1999).

PKA activity was determined in the preparations obtained from cell-free extracts by ammonium sulfate precipitation at 70% saturation. The standard reaction mixture (50 μl) contained: 20 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO)_2$, 5 mM 2-mercaptoethanol, 15 μg protamine sulfate, 2 μM cAMP or cGMP, 0.03 mM [γ^{32}P]ATP (450 c.p.m. × pmol^{-1}) and crude PKA fraction (2–10 μg protein). After incubation at 30°C for 20 min the reaction mixture was spotted onto phosphocellulose filter (Whatman P81) and washed three times in 15% acetic acid. The radioactivity was determined in a scintillation counter (Cytryńska *et al.*, 2001).

Preparation of ribosomes and ribosomal protein phosphorylation. Membrane-free 80S ribosomes were released from the endoplasmic reticulum by 1% Triton X-100 treatment of the microsomal fraction. These ribosomal preparations were used for phosphorylation of ribosomal proteins by ribosome-bound protein kinases (Wojda *et al.*, 1999).

The standard reaction mixture in a total volume of 50 μl contained: 50 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM dithiothreitol (DTT), 150 μg of ribosomes and 0.09 mM [γ-^{32}P]ATP. The mixture was incubated at 30°C for 20 min and the reaction was stopped by the addition of 25 μl Laemmli sample buffer (1970). The phosphorylation level of ribosomal proteins and proteins associated with ribosomes was analysed by electrophoresis in SDS-containing 12% polyacrylamide slab gels (SDS/PAGE) according to Laemmli (1970) and subsequent autoradiography.

For isoelectrofocusing (IEF) analysis the samples after incubation, containing 1–1.5 mg of membrane-free ribosomes in 500 μl of reaction mixture, were diluted in 50 mM Tris/HCl, pH 7.5, 10 mM $Mg(CH_3COO)_2$, 1 mM DTT buffer. The ribosomes were sedimented by centrifugation at 100000 × ***g***. Ribosomal acidic proteins were extracted with 0.25 M NH_4Cl/50% ethanol and then suspended in 20 μl of a solution of 6 M urea and 2% ampholine pH 2.5–5.0. Proteins were resolved by isoelectrofocusing using 5% polyacrylamide (w/v) gels with 6 M urea and 2% ampholines in the 2.5 to 5.0 pH range (Juan-Vidales *et al.*, 1984). The separated proteins were silver stained and radioactive phosphoproteins were detected by autoradiography.

Determination of protein concentration. The concentration of protein was determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a standard.

Determination of ribosome concentration. The concentration of ribosomes (1 mg/ml has A_{260} = 11) was estimated according to Van der Zeijst *et al.* (1972).

RESULTS

Vanadate effect on *P. pastoris* growth

Four *P. pastoris* strains designated JC100/1, JC100/3, JC100/9, JC100/15 exhibiting tolerance up to 150 mM concentration of vanadate were selected on YPD agar plates as described in the Materials and methods section. The growth kinetics of these strains in liquid YPD medium in the absence and in the presence of 25 mM and 50 mM sodium orthovanadate were assessed. As can be seen in Fig. 1, the growth curves were similar for the control YPD medium and for the medium containing 25 mM vanadate during 80 h experiments. In the growth medium containing 50 mM vanadate intensive growth was observed after a 30 h lag phase in the case of the JC100/1, JC100/3, JC100/9 strains and to a much lower extent for the wild type strain. Strain JC100/15, although it tolerated 150 mM vanadate on agar plates, did not grow in the liquid medium containing 50 mM sodium orthovanadate.

During the growth of *P. pastoris* in the presence of vanadate, the medium turned dark green in colour. The dark green colour of the

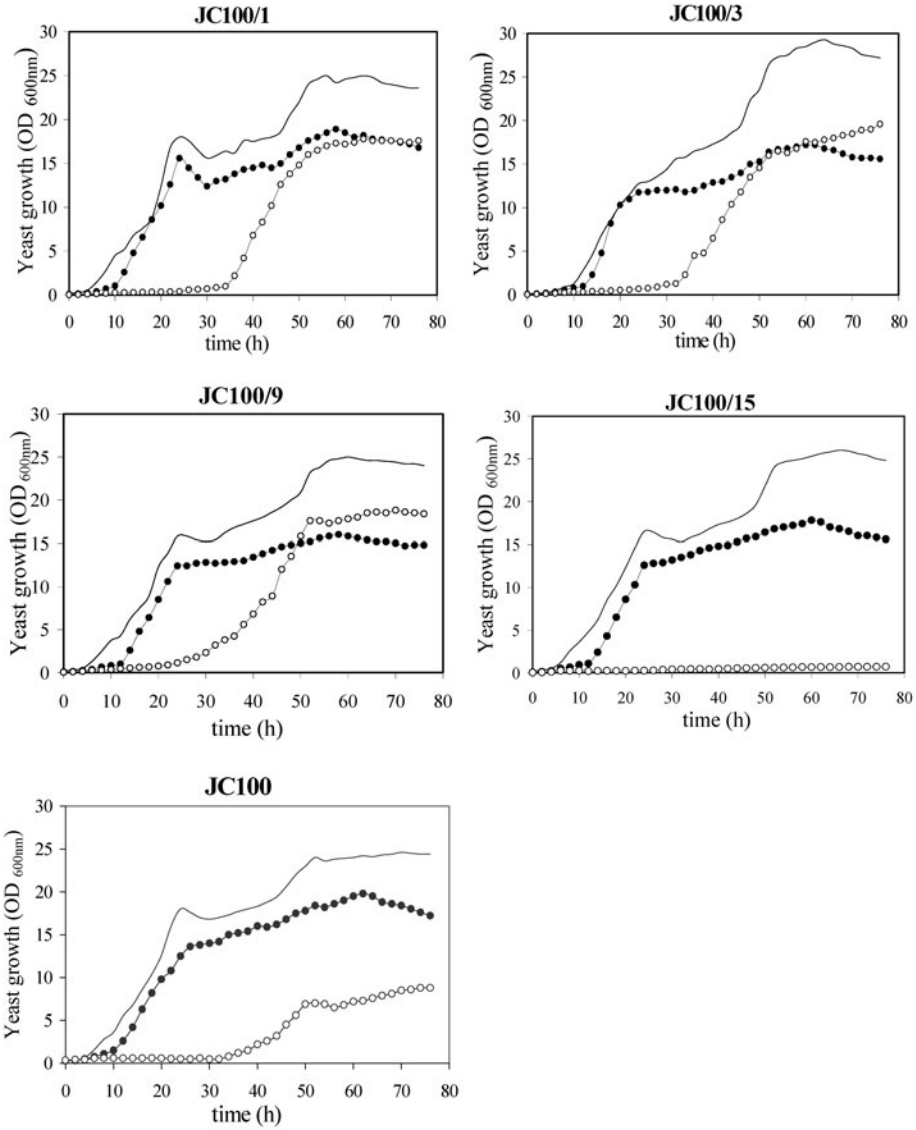

Figure 1. Growth curves of wild type and vanadate-resistant *P. pastoris* strains on YPD liquid medium.

(–) Without sodium orthovanadate; (●) with 25 mM sodium orthovanadate; (○) with 50 mM sodium orthovanadate. The presented growth curves are representative for three independent experiments.

culture was caused by the reduction of vanadate to vanadyl as was reported by Bisconti *et al.* (1997). As can be seen in Fig. 2, a significant increase in the vanadate to vanadyl reduction occured after 5 h of culturing in the medium with 25 mM vanadate, while with 50 mM vanadate significant production of vanadyl occured only after a 30 h lag phase. Without vanadate, the colour of the yeast suspension remained unchanged. In the case of strain JC100/15, which did not grow in the presence of 50 mM vanadate (Fig. 1), vanadyl was also not detected in the growth medium (Fig. 2). The obtained results indicate

Studies of PKA activity in *P. pastoris* vanadate-resistant strains

It is known that vanadate is a phosphoprotein phosphatases inhibitor *in vitro* while vanadyl *in vivo* appeares to stimulate protein phosphorylation. This indicates that vanadium can interfere with cellular regulation processes. Vanadium compounds can stimulate the activity of adenylate cyclase which controls cAMP level in the cell and as a consequence can affect PKA activity (Hackbarth *et al.*, 1980; Catalan *et al.*, 1980). Therefore, we determined PKA activity in the wild type and

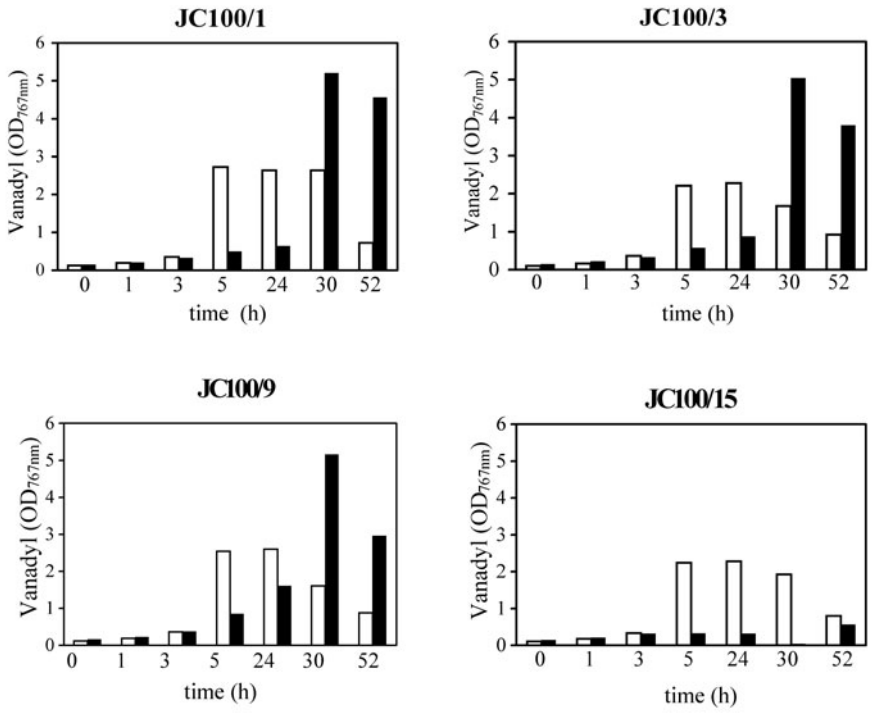

Figure 2. Vanadyl concentration in cell-free medium from cultures of vanadate-resistant *P. pastoris* strains — kinetic studies.

(❑) Absorbance at 767 nm in YPD medium in the presence of 25 mM sodium orthovanadate; (■) absorbance at 767 nm in YPD medium in the presence of 50 mM sodium orthovanadate. The presented results are representative for three independent experiments.

a correlation in the vanadate to vanadyl reduction with yeast growth kinetics. However, identification of the vanadium species present in the growth medium as well as inside *P. pastoris* cells require more thorough quantitative and qualitative analysis by using EPR and NMR spectroscopy.

vanadate-resistant strains of *P. pastoris*. For this purpose, yeast cells cultivated to the logarithmic growth phase in the absence and in the presence of 25 mM and 50 mM sodium orthovanadate were collected. Crude PKA fractions, obtained as described in the Materials and methods section, were tested for

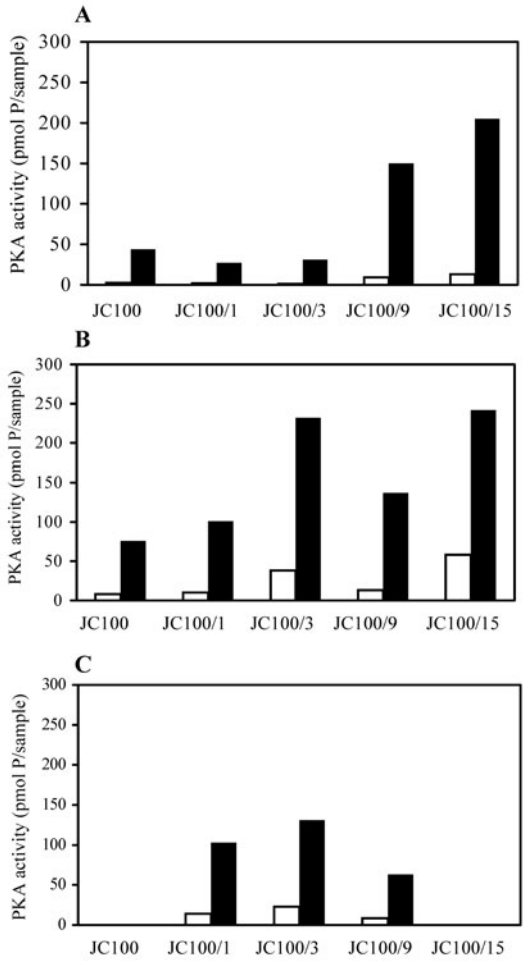

Figure 3. PKA activity in cell-free extracts of wild type and vanadate-resistant *P. pastoris* strains.

Protamine sulfate was used as a phosphorylation substrate in the absence (❑) or in the presence of cAMP (■). The studied strains were grown to the logarithmic phase in liquid YPD medium without vanadate (A), in the presence of 25 mM (B) or 50 mM sodium orthovanadate (C). PKA activity was estimated according to the Materials and Methods section. The presented results are representative for three experiments.

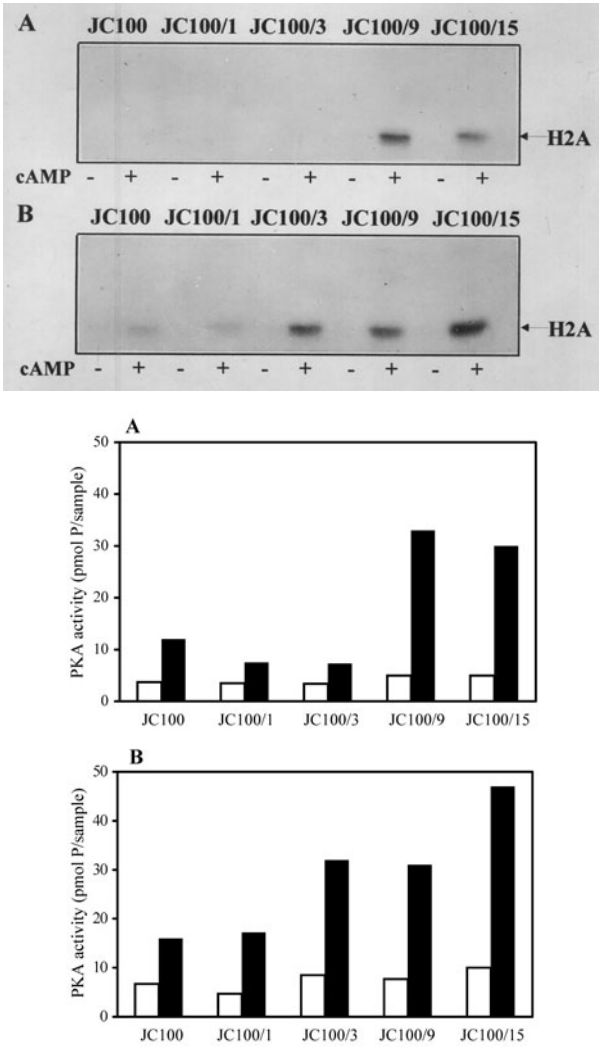

Figure 4. Phosphorylation of histone H2A by PKA from wild type and vanadate-resistant strains of *P. pastoris*.

The studied strains were grown in YPD medium without (A) and in the presence of 25 mM sodium orthovanadate (B). PKA activity was estimated as described in the Materials and Methods section, in the absence (❑) and in the presence of cAMP (■) using histone H2A as a substrate. Phosphorylation level of H2A was measured in a scintillation counter (diagram) or estimated by SDS/PAGE electrophoresis and subsequent autoradiography (photograph). The presented results are representative for three experiments.

their phosphotransferase activity in the presence of cAMP using protamine sulfate as a substrate. As can be seen in Fig. 3, in the case of cells grown in the medium without vanadate, PKA activity was detected in all the *P. pastoris* strains studied. In the vanadate-resistant strains JC100/9 and JC100/15, the PKA activity was at least 5 times higher in comparison to the wild type JC100 strain. Furthermore, in cells from cultures containing 25 mM sodium orthovanadate the PKA activity increased significantly in JC100/1 and JC100/3 while in the case of JC100/9 and JC100/15 it remained on an almost unchanged level in comparison to the results obtained for the control medium. As for cells

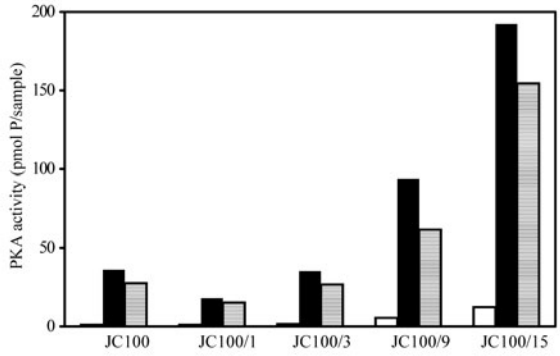

Figure 5. Comparison of PKA activity in the presence of cAMP and cGMP.

PKA activity was measured without (❑), in the presence of cAMP (■) or cGMP (▤) in cell-free extracts of the wild type and vanadate-resistant *P. pastoris* strains. Protamine sulfate was used as a phosphorylation substrate. The studied strains were grown to the logarithmic phase in liquid YPD medium without vanadate. The presented results are representative for three experiments.

which were grown on 50 mM sodium orthovanadate, in strains JC100/1, JC100/3 and JC100/9 a relatively high PKA activity was observed. Similar results were obtained using histone H2A as a phosphorylation substrate (Fig. 4). It is worth mentioning here that all the isolated fractions were stimulated by cAMP and almost to the same level by cGMP (Fig. 5). These results confirm our earlier observations obtained on *S. cerevisiae* indicating that yeast PKA can be activated by both cyclic nucleotides (Cytryńska *et al.*, 1999).

Ribosomal protein phosphorylation in *P. pastoris* vanadate-resistant strains

In parallel to the studies on PKA activity, we performed a comparative analysis of the proteins modified by kinases associated with ribosomes. For this purpose, membrane-free 80S *P. pastoris* ribosomes were incubated with [γ-^{32}P]ATP in the absence of exogenous protein kinases. SDS/PAGE and autoradiography analysis of phosphorylated proteins revealed two major radioactive bands of 13 kDa and 38 kDa in all the strains (Fig. 6). From earlier studies performed in our laboratory (not shown) we know that they correspond to ribosomal proteins P1/P2 and P0, respectively. Isoelectrofocusing analysis of the P1/P2 proteins extracted from ribosomes revealed eight forms differing in charge over the acidic pH range. Four of them were phosphorylated. However, no significant differences in the phosphorylation level between these forms in the yeast strains studied were observed (Fig. 7). In all the vanadate-resistant strains from the logarithmic growth phase an additional phosphoprotein of 40 kDa was detected. In the case of the JC100/1 and JC100/9 strains, a protein of 27 kDa was also phosphorylated (Fig. 6). The protein of 40 kDa was phosphorylated in the wild type strain from the stationary, but not logarithmic, growth phase. This protein could be washed out from ribosomes with a buffer containing 0.5 M KCl (not shown), which indicated that it is not a structural ribosomal protein but a ribosome-associated one.

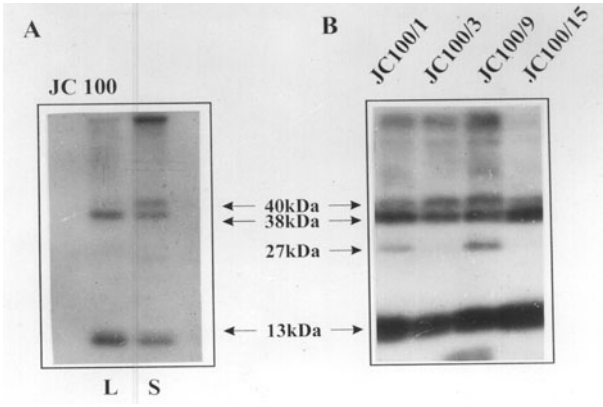

Figure 6. Endogenous protein phosphorylation in membrane-free ribosomes from wild type (A) and vanadate-resistant *P. pastoris* strains (B).

Membrane-free wild type strain ribosomes were isolated from logarithmic (L) or stationary (S) growth phase while ribosomes from the vanadate-resistant strains from logarithmic growth phase only. Yeast cells were grown in liquid YPD medium without vanadate. Ribosomal protein phosphorylation was estimated as described in the Materials and Methods section. The presented autoradiograms are representative for three experiments.

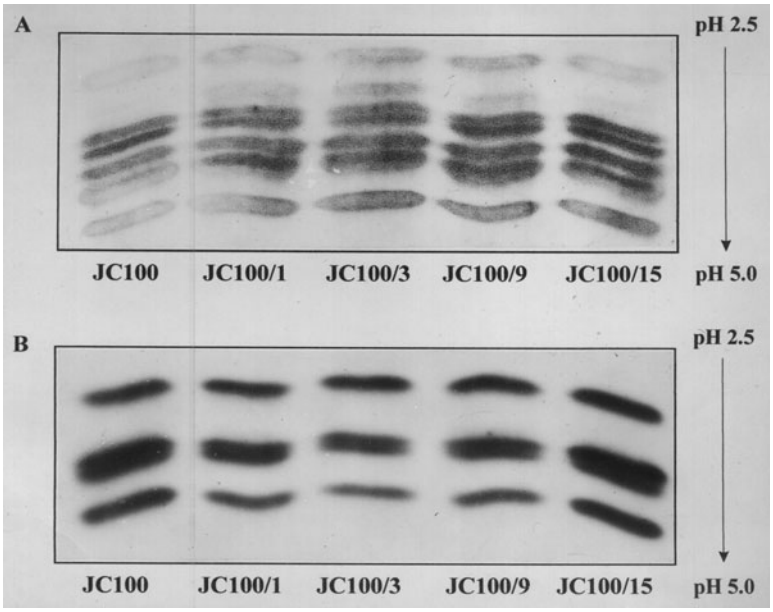

Figure 7. Isoelectrofocusing of acidic ribosomal proteins ^{32}P-labelled by ribosome-bound protein kinases.

Acidic proteins were extracted with 0.25 M NH_4Cl/50% ethanol from 1.5 mg of ^{32}P-labelled ribosomes. IEF was performed as described in the Materials and Methods section. Proteins were detected by silver staining (A) and subsequent autoradiography (B). Yeast cells were grown in liquid YPD medium without vanadate. The presented results are representative for three experiments.

DISCUSSION

We have shown that the methylotrophic yeast *Pichia pastoris* is able to grow in the presence of high, up to 25 mM, orthovanadate concentrations which are toxic to many other organisms. Furthermore, we selected four *P. pastoris* strains which can tolerate vanadate concentration as high as 150 mM. Resistance to such extremely high vanadium salt concentrations (> 96 mM) was shown for another methylotrophic, thermotolerant yeast species *Hansenula polymorpha* (Mannazzu *et al.,* 1997). Our results clearly indicate that growth of *P. pastoris* in the presence of vanadate is correlated with reduction of vanadate to vanadyl. However, it is not known if vanadate reduction occurs inside the cell or at the level of the cell envelope as it has been suggested for certain *S. cerevisiae* strains (Bisconti *et al.,* 1997).

It is known from many studies that vanadate acts as a competitor of phosphate molecules and interferes with protein phosphorylation. Moreover, according to Morinville *et al.* (1998) and Pandey *et al.* (1995), vanadate, acting as an insulin-mimetic compound, induces activation of MAP kinases and ribosomal protein S6 kinases. Our results demonstrate that in the presence of vanadate in *P. pastoris* wild type strain and two of the selected vanadate-resistant strains, JC100/1 and JC100/3, the level of protein kinase A activity was higher in comparison to the yeast growing without vanadate. It was also found that in two other selected vanadate-resistant strains, JC100/9 and JC100/15, PKA activity was much higher in comparison to the wild type strain even in the absence of vanadate. The constitutively enhanced PKA activity in the JC100/9 and JC100/15 strains can be a result of spontaneous mutation(s) which occured during strain selection in the presence of high vanadate concentration. This, however, requires further studies at the molecular level. Our data also indicate that PKA might be involved in a signalling mechanism induced by vanadate in *P. pastoris*. In addition we have found that in all four vanadate-resistant strains endogenous phosphorylation of a 40 kDa protein associated with ribosomes also occured. This protein was phosphorylated in membrane-free ribosomes from the stationary but not logarithmic growth phase of the wild type *P. pastoris* strain. The role of the 40 kDa protein is not known at present. We cannot exclude the possibility that the 40 kDa protein represents one of the molecules which participate in protecting the cells against stress conditions. This, however, needs further genetic and biochemical studies.

We would like to thank Monika Koziej for assistance in the manuscript preparation.

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