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The chromate resistance phenotype of some yeast mutants correlates with a lower level of Cr(V)-species generated in the extra-cellular medium

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Abstract The paper describes the selection of chromate-resistant mutants of the yeast *Pichia guilliermondii* with a higher chromate-reducing activity and reports the EPR-study of Cr(V)-generation in the extracellular medium during the reduction of chromate by the yeast culture. It is shown that the reduction of chromate to Cr(III) species runs through the extracellular generation of Cr(V)-intermediate(s), thus supporting the assumption about the existence of an extra-cellular pathway of Cr(VI)-reduction. Furthermore, it is demonstrated that the chromate-resistance phenotype of tested mutants correlates with a lower stationary level of Cr(V)-species in the medium. It is thus suggested that isolated mutants can be used as sources of Cr(III)-biocomplexes due to their ability to effectively reduce chromate to Cr(III)-chelates with potential pharmacological applications.

Keywords Chromate reduction · Yeast · Chromium(V) · Chromate-resistant mutants · Chromium(III)-biocomplexes

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Introduction

Chromium, one of the most common elements on the Earth, forms a wide range of stable compounds in trivalent (Cr^{+3}) and hexavalent (Cr^{+6}) states, being unstable in the forms of the intermediate valences (Cr^{+5} and Cr^{+4}). Cr(VI) species are extremely toxic, whereas Cr(III)-compounds are much safer; furthermore, they can be found in many kinds of food and are widely used as pharmaceuticals and dietary supplements (e.g., chromium picolinate, chromium polynicotinate, chromium chloride) to prevent diabetes, lipoprotein abnormalities, and cardiovascular diseases (Porter et al. 1999; Vincent 1999; Cefalu and Hu 2004). While the molecular mechanisms of chromium(III) as a microelement are still unclear, some experimental evidence has been obtained in support of the important role of this metal for the normal signal transduction pathway through the insulin-stimulated insulin receptor (Yamamoto et al. 1989; Vincent 1999, 2000).

Although chromium(III) supplements are widely used (e.g., products containing chromium picolinate $Cr(pyc)_3$ have annual sales of $500 million), they possess some serious disadvantages: (1) poor solubility of chromium picolinate and chromium polynicotinate limits their low absorption up to 2–5%, and chelation of chromium chloride with large biomolecules restricts its consumption (Vincent 2003); (2) the above-mentioned salts and complexes have been shown to possess some deleterious effects, e.g., they cause the damage of chromosomes in the Chinese hamster ovary cells (Stearns et al. 1995), induction of sterility and lethal mutations in *Drosophila melanogaster* (Hepburn et al. 2003), and pathological ocular changes in rats (Amany et al. 2006).

The synthesis of novel chromium(III)-containing complexes, as well as microbial production of such compounds, is one of the possible ways of overcoming this problem. Taking into account significant kinetic inertness of chromium(III) salts in the formation of even thermodynamically stable complexes, it is promising to produce such complexes by using microbial cells capable of reducing chromium(VI) (chromate, dichromate) to Cr(III)-biocomplexes (Puzon et al. 2005; Ksheminska et al. 2006, 2008). Although the genetic and biochemical aspects of this process have been insufficiently studied, it is known that chromate anions are transported into the cells through sulfate-specific permease(s) coded, in the case of baker's yeast, by the genes *SUL1* and *SUL2* (Cherest et al. 1997) and can be reduced, as a powerful oxidative agent, to Cr(III) by cellular reducing systems which can include enzymatic and non-enzymatic pathways. Glutathione and cysteine can be regarded as the most powerful non-enzymatic chromate reductants for microbial cells, and ascorbate—for higher organisms. The bacterial reduction of chromate is well-established. It runs through the different enzymatic pathways which function under aerobic and anaerobic conditions with the use of hydrogenase, cytochrome *c*-dependent electron transfer chains, nitrate reductase, flavin reductase, ferrireductase, some flavoproteins, and NADH and NAD(P)H-dependent reductases (Ksheminska et al. 2006). For eukaryotic microbial cells and, primarily, yeasts, the data on the chromate-reducing systems are more ambiguous. It is generally unknown which system—enzymatic or non-enzymatic, intracellular or extra-cellular one—plays a leading role in the chromate detoxification process.

In our previous papers (Ksheminska et al. 2006, 2008) we have demonstrated the crucial role of the extra-cellular reduction of chromate in its detoxification by yeast cells. It was shown that Cr(III)-chelates, produced in the extra-cellular medium, are of different chemical nature and can be separated by ion-exchange chromatography at least into two species.

In this paper, the isolation of chromate-resistant mutants of the yeast *Pichia guilliermondii* with a higher chromate-reducing activity is described. Using EPR spectroscopy, we have studied Cr(V)-generation in the extra-cellular medium during the reduction of chromate by yeast cultures. It has been shown that the reduction of chromate to Cr(III) species runs through the extra-cellular generation of Cr(V)-intermediate(s), thus supporting our assumption about the existence of an extra-cellular pathway of Cr(VI)-reduction. This report demonstrates that the chromate-resistance phenotype of tested mutants correlates with a lower stationary level of extremely toxic Cr(V)-species in the extra-cellular medium.

Materials and methods

Chemicals

All used chemicals were obtained from Sigma-Aldrich and Fluka unless mentioned otherwise.

Strain

Non-conventional flavinogenic yeast *Pichia guilliermondii* ATCC 201911 (L2 *his*$^-$) was used as a parental strain for the selection of chromate-resistant (Crt^r) mutants.

Selection of the yeast mutants

The cell suspension of the yeast *P. guilliermondii* ATCC 201911 (L2 *his*$^-$) was treated by UV-radiation in the dose resulting in approximately 10% survival of the cells and then plated on the agar mineral medium supplemented with 2% sucrose, 0.1% yeast extract, histidine (40 mg/l), and 0.5 mM potassium chromate with urea (1 g/l) used as a nitrogen source. After the 8 day-incubation of the plates at 30°C, discrete colonies were picked up, cloned on the

selective medium, and tested by their growth ability in liquid media supplemented with 0.5 mM chromate.

Cultivation

The cultivation of the parental and mutant Crt^r strains of *P. guilliermondii* was carried out in Erlenmeyer's flasks under aeration in a shaker (200 rpm) at 30°C in Burkholder's mineral medium (Burkholder et al. 1944) supplemented with 2% sucrose, 0.1% yeast extract, and histidine (40 mg/l). As a nitrogen source, ammonium sulfate (3 g/l) was used. After reaching the exponential growth phase, the cells were collected by centrifugation and transferred (to the biomass 0.3 mg/ml) into the fresh growth medium of the same composition as the above-mentioned one, but supplemented with chromate in different concentrations (depending on the aim of experiments). The cultivation was continued with the periodic measuring of the biomass level and Cr(III)/Cr(VI) contents in the culture. The biomass was assayed by optical density at 540 nm (OD_{540}). The absolute dry mass, expressed in mg/ml, was calculated by using the gravimetrically obtained calibration curve.

Chemical assays

The residual chromate concentration in the extracellular liquid of yeast cultures was measured by the colorimetric diphenylcarbazide method (Marchart 1964).

Cr(III) content in the cultural liquid was determined by the colorimetric method in reaction with chromazurol S in our modification (Honchar et al. 2008). Two pools of chromium(III) were revealed: the so-called "available" (weak chromium biocomplexes) and "chelated" (strong biocomplexes not reacting with chromazurol S) ones. The latter was calculated as a difference between the total chromium content, determined after mineralization of the sample, and the "available" Cr(III) pool, measured directly in reaction with chromazurol S for samples with fully reduced chromate (zero content of residual Cr(VI) in the extra-cellular medium).

To correctly determine Cr(III)-species, produced by the yeast culture during chromate reduction, we tested a possible contribution of growth medium components to this process for the period of cultivation as well as during the analytical procedures. For this purpose and in line with the experimental variant, chromate was incubated at the same concentration with a growth medium but without inoculation of the cells. For the standard medium used in the experiments, the correction value, based on such control, was not higher than 5–7% of the initial chromate level.

In order to determine total chromium in the cells, aliquots of the washed cells were mineralized by hydrogen peroxide under acidic conditions (Honchar et al. 2008) and tested for the chromium content (in the form of Cr(III) by the reaction with chromazurol S.

All analytical data were collected from 3 replicated experiments, and at least two duplicates were used for each assay.

EPR spectroscopy

EPR measurements were performed on the X-band spectrometer Radiopan SE/X2544 with the liquid nitrogen cooling system under the following conditions: microwave frequency, 9.156 GHz; microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude 2.5 Gauss; temperature 77 K. Experimental data were collected in ASC II format. EPR spectrometer AE4700, produced by MICRODEVICE (Lviv, Ukraine) was also used. Its parameters were as follows: microwave frequency, 9.4 ± 0.05 GHz; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude 1.6 Gauss; temperature 77 K. Experimental data were printed by two-dimension recorder N307/1. As a standard for the calculation of chromium species g-factor, stable radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was used ($g = 2.0037 \pm 0.0002$) (Weil et al. 1994).

Preparation of extra-cellular samples for EPR measurements

The one-day cultures of the parental strain and two chromate-resistant mutants were concentrated by centrifugation and resuspended in the fresh growth medium supplemented with 1 or 8 mM chromate. 2 ml aliquots of the cultures were withdrawn at different time incubation (1 min, 1 and 2 h) and centrifugated (+2°C, 14,000g, 2 min). The aliquots of the supernatants (70 ± 1 µl), were placed into

microcylinders, frozen at 77 K, and stored in liquid nitrogen before EPR measurements.

Calculations

The experimental data collected from EPR spectrometer were presented in the form of function $dI/dH = f(H)$, where I is intensity of the resonance signal; dI/dH is its first derivative; H is magnetic field. For comparative quantitative analysis of resonance intensities for Cr(V)-species, generated in different yeast cultures, automatic integration of the Cr(V)-peak arias was performed by Origin 7.5 Program. For simpler evaluation of these characteristics, the heights of resonance peaks (in coordinates $dI/dH = f(H)$) were also measured. The double integration of the peak's arias was also carried out to estimate relative quantities of Cr(V)-species in the tested samples. g-Factors were calculated for the observed resonance signals with DPPH as a standard (for AE4700) or klystron with a precisely fixed frequency 9,156,780 ± 25 kHz (for Radiopan SE/X2544).

Results

Selection and description of chromate-resistant mutants of the yeast *P. guilliermondii*

Tolerance of the yeast cells to chromate strongly depends on the chemical composition of the growth medium (content of carbon and nitrogen sources, sulphate—a structural analogue of chromate), presence of reducing and chelating compounds, as well as on cultivation conditions. To isolate the chromate-resistant mutants of the yeast *P. guilliermondii*, we used selective 0.5 mM chromate-containing urea medium, since cells, grown on this nitrogen source, are more sensitive to chromate, as was previously shown by us (Ksheminska et al. 2008). As a source of sulfur, potassium sulfate was used to eliminate the prevalence of isolation of the trivial chromate-resistant mutants, impaired in sulfate/chromate transport (they often are organic sulfur-auxotrophs, unable to effectively utilize sulfate as a sulfur-nutrient). Using UV-radiation, a broad collection of chromate-resistant (Crt^r) colonies was isolated, and for 28 mutants the desired phenotype was confirmed by the growth-test in liquid cultures.

To study chromate-reducing ability of the selective chromate-resistant mutants in detail, two genetically stable representatives were chosen—Crt^r-9 and Crt^r-11. The qualitative viability test was carried out on the agar medium containing relatively high concentration of chromate—8 mM. The small aliquots (1 mU of optical density) of the tested mutants cell suspension and parental strain L2 were putted on plates (Fig. 1a–d). As shown, mutant cells survived even after 7 and 15 h of incubation. The semi-quantitative viability test, carried out with the higher aliquots of cell suspension (1 U of optical density) confirmed the chromate-resistant phenotype of the mutants. As shown for mutant L2-9 (Fig. 1e), viability of the parental strain was 2×10^{-5} after the 24-h incubation on 8 mM chromate-containing medium, whereas mutant cells formed almost a loan.

The chromate-resistant mutants L2-9 and L2-11 were studied in respect of their ability to reduce chromate in liquid media. The dynamics of changes in chromate residual content in cultures during incubation of the yeast cells (8 mg/ml) in liquid media supplemented by 1 mM and 8 mM Cr(VI) is shown in Fig. 2. At a lower initial concentration of chromate (1 mM), all tested strains, parental and mutant, almost completely reduce chromate after the 6-h incubation, and no difference was observed in the kinetics of chromate decrease in the cultures (Fig. 2a). Contrary to that, at a higher initial level of chromate (8 mM), the period of full reduction of Cr(VI) is longer, and a clear difference in the chromate-reducing activity of the strains is observed: both Crt^r mutants are more effective in the chromate detoxifying activity as compared to the parental strain (Fig. 2b).

In more detail, the growth pattern and levels of Cr(VI)- and Cr(III)-species in the cultures for chromate-resistant mutant Crt^r L2-11, compared with wild-type strain L2 of the yeast *P. guilliermondii* L2 at different concentrations of chromate in the medium, are presented in Fig. 3. During the growth delay phase with duration depending on the yeast's tolerance to chromate, the decrease of Cr(VI) due to its reduction and the appearance of the "available" Cr(III) were observed. Although the level of the "available" extra-cellular Cr(III)-species drastically decreases at the point of full chromate reduction, the total chromium content, estimated after mineralization of the cultural liquid, is very close to the initial

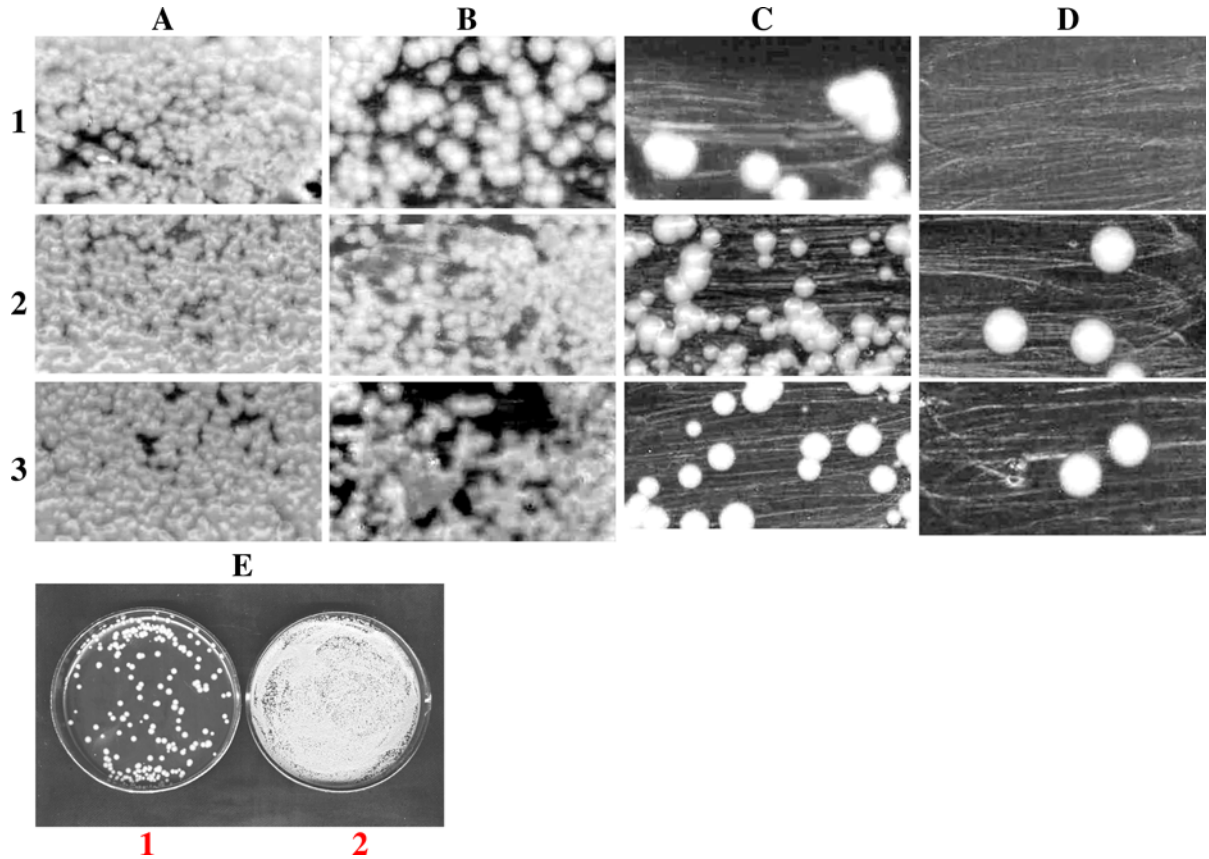

Fig. 1 The growth of chromate-resistant mutants and parental ("wild-type") strain of the yeast *P. guilliermondii* (*1*—L2; *2*—L2-9; *3*—L2-11) on agar plates during incubation with 8 mM chromate: **a** 1 min; **b** 7 h; **c** 15 h; **d** 24 h. Aliquots of the cells putted on the plates: **a**–**d** 0.01 ml of 0.1 opt.U/ml or 0.001 opt.U; **e** 0.1 ml of 10 opt.U/ml or 1 opt.U, 24 h

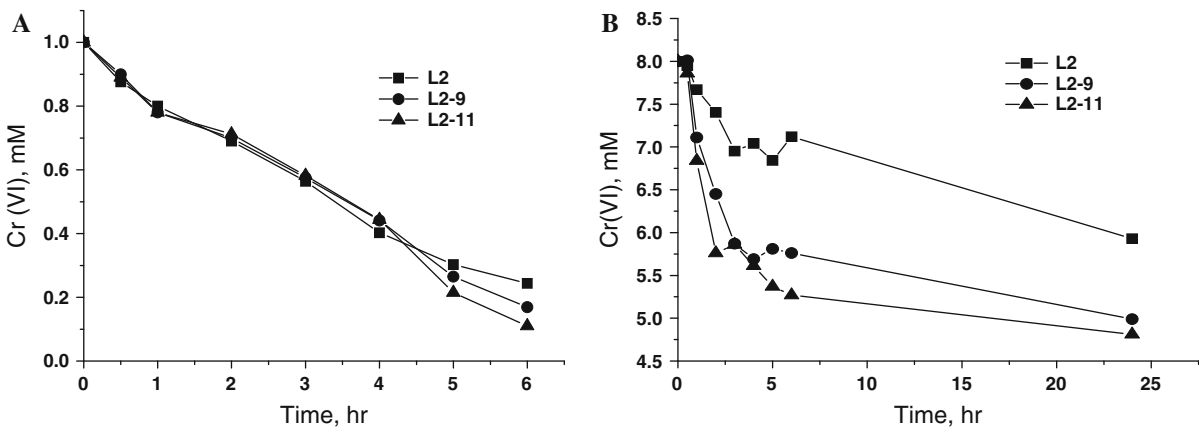

Fig. 2 Dynamics of changes of residual chromate content in the extra-cellular media during incubation with chromate of the yeast *P. guilliermondii* (parental strain L2 and Crt^r mutants L2-9, L2-11). **a** 1 mM Cr(VI); **b** 8 mM Cr(VI)

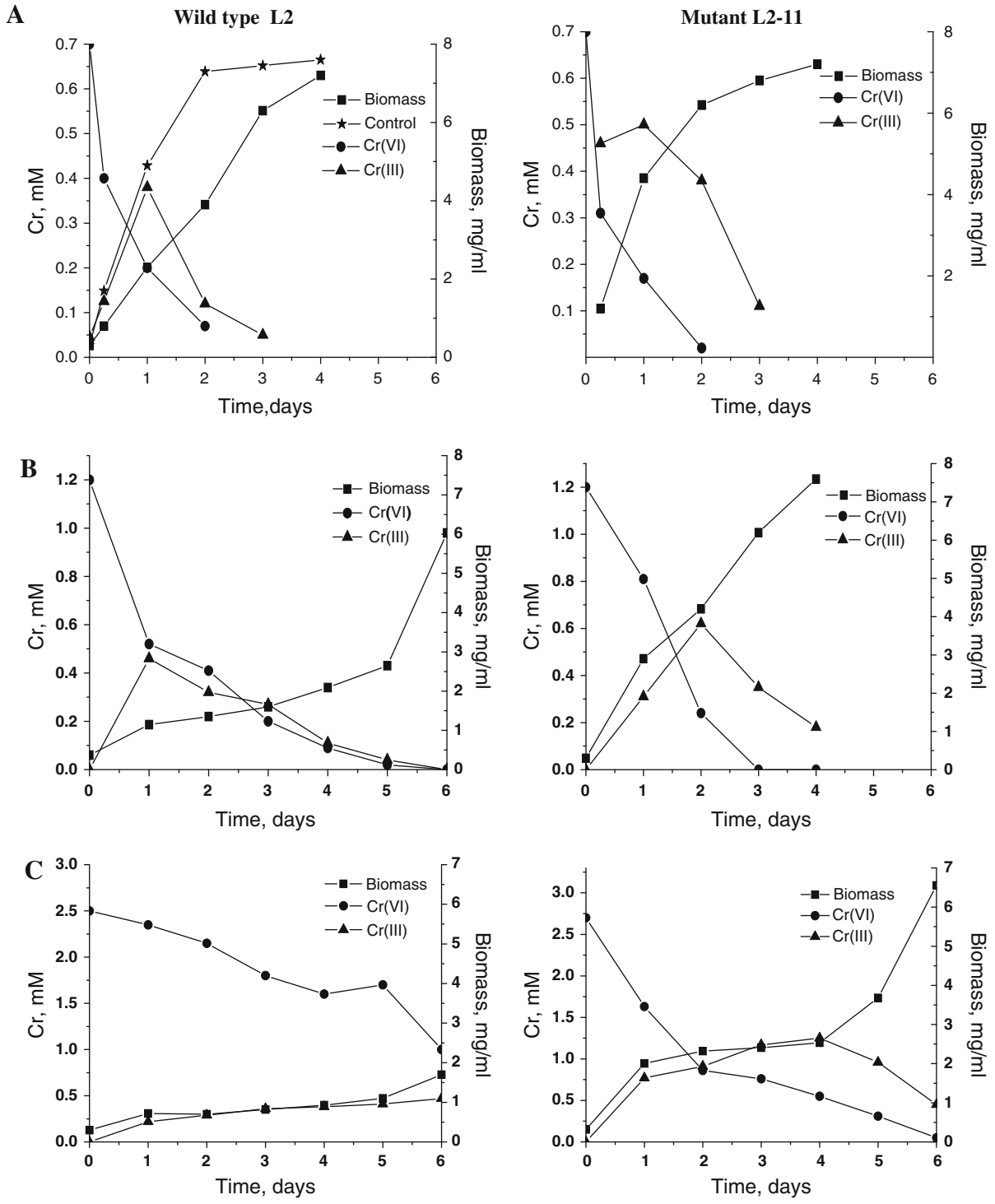

Fig. 3 Kinetics of growth and extra-cellular Cr(VI)-reduction of the parental L2 strain and chromate-resistant mutant Crt^r L2-11 of the yeast *P. guilliermondii* at different chromate concentrations: **a** 0.7 mM; **b** 1.2 mM; **c** 2.7 mM

level of chromate (data not shown). It was demonstrated that this phenomenon is related to the formation of stable Cr(III)-biocomplexes with metabolites, secreted into the cultural liquid by cells (Ksheminska et al. 2006, 2008).

Evaluation of Cr(V)-species in the extra-cellular medium during chromate reduction by the yeast cultures

Using EPR measurements, two chromate-resistant mutants (Crt^r L2-9 and Crt^r L2-11) were investigated in comparison with the parental strain L2 in regard to their ability to generate Cr(V)-radical in the extra-cellular medium. The yeast cells in growth medium were incubated at high and middle concentration of chromate, 8 and 1 mM, respectively. As shown in Fig. 4, Cr(V)-intermediate is found in the extra-cellular liquid at the earliest time of incubation (1 min) for all tested strains. During further cultivation of the cells in the presence of 8 mM chromate, an essential difference in EPR profiles of the tested strains was observed. The wild type cells L2 show a gradually increasing Cr(V)-related resonance peak (1 and 2 h), while both chromate-resistant mutants L2-9 and L2-11 are characterized by the drastically decreased level of Cr(V)-species: the first mutant—after the second hour of cultivation and the second one—even after the first hour of incubation with chromate. These experimental data corroborate our assumption about the functioning of extra-cellular chromate reduction and can be regarded as an explanation of the biochemical base of chromate-resistance of at least part of the isolated mutants. It could be thus assumed that such mutants have been adapted to chromate by decreasing the stationary level of extremely toxic Cr(V)-intermediate in the extra-cellular medium.

We investigated the Cr(V) formation in the extra-cellular medium during cultivation of different yeast strains at relatively low concentration of chromate—1 mM. It should be noted that after 5-day cultivation of the wild-type yeast L2, when the residual chromate content in the extra-cellular medium was negligible, a small Cr(V)-related resonance peak was observed (data not shown). This fact allows us to assume that a relatively stable Cr(V)-biocomplexes are generated by the cells.

Quantitative EPR-monitoring of Cr(V)-generation in the extra-cellular medium during 1 mM chromate reduction is shown in Table 1. The EPR intensities were characterized by peak area squares (S) for function $I = f(H)$, by double integration of the function $dI/dH = f(H)$, as well as by amplitude (A) for function dI/dH in relative units (as the ratio to the smallest signal value). As seen from Table 2, the Cr(V) level is low for all cultures at the earliest period of incubation (approx. 1 min), and no clear difference in the signals for wild type and mutant cells is observed. After 1 h, the Cr(V) EPR signal for wild type strain L2 drastically increases (six to seven fold). However, it does not increase for chromate-resistant mutants, particularly, for L2-11, which forms a much lower level of Cr(V)-species than the parental strain (14–80-fold, depending on the time period). Thus, the experiments on the incubation of yeast cells with less toxic chromate level (1 mM) principally support the following conclusion made for cultures supplemented with higher chromate concentration (8 mM): chromate-resistant mutants possess an ability to maintain a lower level of extremely toxic Cr(V)-intermediate in the extra-cellular medium during chromate reduction.

It is worth emphasizing (the data were extracted from the spectra shown in Fig. 4 and Table 2) that, in addition to the main resonance peak (right one, $H_{res} = 331.09 \pm 0.06$ mT), common for all tested cultures at different time of incubation, a smaller "satellite" signal is observed (left one, $H_{res} = 327.08 \pm 0.12$ mT), which is more visible during the earlier period of time (approx. 1 min) and is gradually disappearing in the later period. The g-factor calculated for the main peak is between 1.975 and 1.976 (with error $\pm$ 0.0015), and for the left one—between 1.999 and 2.000 (with the same error), that is, it appears to be similar to the published data (Table 2).

The noticeable difference in the g-factor values for two peaks can be regarded as an evidence of the formation of at least two Cr(V)-species in the extra-cellular medium of yeast cultures, assumingly being more stable (the right one and less stable—the left one). They can reflect the independent formation of structurally different Cr(V)-intermediates which are transformed into stable Cr(III)-biocomplexes or represent two intermediates sequentially converting from one to another (probably, with an earlier generation of the form 1 (left peak) to the form 2 (right peak). The difference in the g-factor values of these

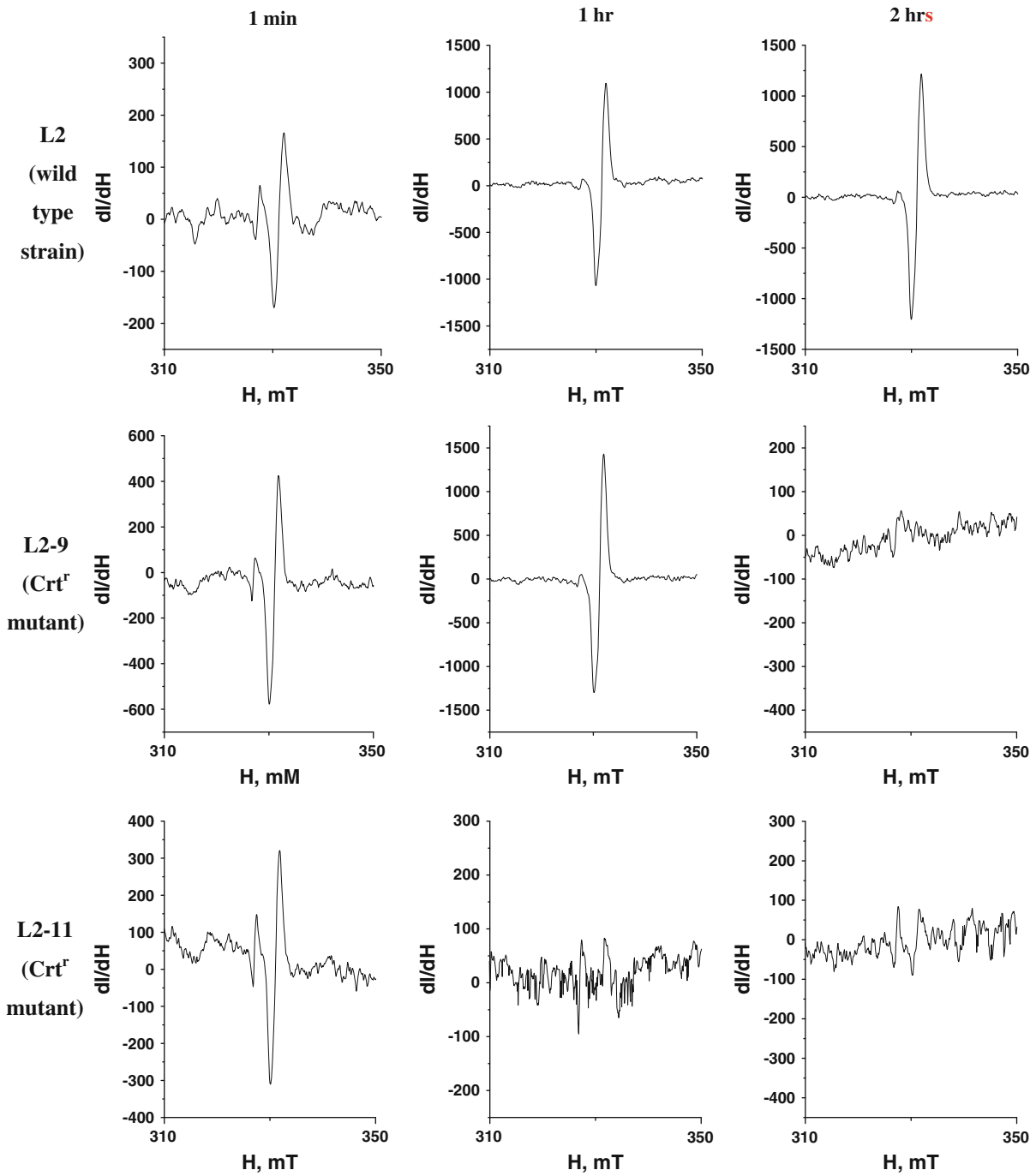

Fig. 4 EPR spectra of Cr(V) in the extra-cellular medium of the yeast *P. guilliermondii* cultures: parental ("wild type") strain L2 and Crt^r-mutants during cells' incubation with 8 mM chromate (1 min, 1 and 2 h). EPR-spectrometer: AE4700 (MICRODEVICE, Lviv, Ukraine)

intermediates can possibly be explained by different Cr(V)-center surrounding which finally resulted in the formation of different Cr(III) species, as previously demonstrated by us (Ksheminska et al. 2006).

In the experiments, presented in this paper, we observed a paramagnetic signal for Cr(III) in the extra-cellular medium of all tested cultures, which was formed as a final product of chromate reduction.

Table 1 EPR signals of Cr(V)-intermediate formed in the extra-cellular medium of the yeast *P. guilliermondii* cultures during incubation with 1 mM chromate (1 min, 1 and 2 h)

Culture		Square of peak area (S) for function $I(H)$ (double integration for dI/dH), relative units	Relative units S/S_{min}	Amplitude (A) for function dI/dH, relative units	Relative units A/A_{min}
Time	Strain				
1 min	L2	1604 ± 55	14.5	1411	4.33
	Crt^r L2-9	4151 ± 49	37.5	3814	11.7
	Crt^r L2-11	2550 ± 23	23.0	2631	8.07
1 h	L2	9340 ± 146	84.2	7291	22.4
	Crt^r L2-9	11,720 ± 72	106	9195	28.2
	Crt^r L2-11	111 ± 6.9	**= 1**	525.7	1.61
2 h	L2	10,423 ± 50	94.0	8140	25.0
	Crt^r L2-9	251 ± 10.7	2.27	326	**= 1**
	Crt^r L2-11	747 ± 25	6.74	1032	3.17

Strains: parental ("wild type") L2 and Crt^r-mutants L2-9 and L2-11. EPR spectrometer: Radiopan SE/X2544

Table 2 The comparison of g-factors for Cr(V)-species described in this paper with the data available in literature

Reference	Substance	g-Factor
Krumpolc and Rocek (1979)	Cr(V)–EHBA complex (EHBA = 2-ethyl-2-hydroxybutyric acid)	$g = 1.980$
Shi and Dalal (1994)	Product of reaction of Cr(VI) with vitamin B_2	$g = 1.9795$
Meyer et al. (1998)	*trans*-[Cr^V(N)(cyclam)($NCCH_3$)](ClO_4)$_2$ (cyclam = 1,4,8,11-tetraazacyclotetradecane)	$g_\perp = 1.997$ $g_\parallel = 1.965$
Codd and Lay (1999)	K[Cr(O)-(qaH_3)$_2$]·H_2O (qaH_5 = 1R,3R,4R,5R-1,3,4,5-tetrahydroxycyclohexanecarboxylic acid, **I**) isomers of [Cr(O)(O^3,O^4-saH)$_2$]$^{3-}$) (saH_4—shikimic acid)	$g = 1.9787$ and $g = 1.9791$ $g = 1.9800$ and $g = 1.9801$
Headlam and Lay (2001)	Cr(V)-peptide complexes	$g = 1.979$–1.986
This paper	Cr(V)-species in the extra-cellular liquid of the yeast *Pichia guilliermondii* cultures:	
	L2 (wild type strain)	g(M) = 1.975–1.976
		g(m) = 1.999
	L2-9 (Crt^r mutant)	g(M) = 1.975
		g(m) = 1.999–2.000
	L2-11 (Crt^r mutant)	g(M) = 1.976
		g(m) = 1.999–2.000

M major peak, *m* minor peak

The resonance peak of Cr(III) appeared in the field range from 151.75 to 153.02 mT, and g-factor was shown to be in the range of 4.311–4.275 (standard deviation being 0.005).

Conclusions

We have shown that the cells of the flavinogenic yeast *Pichia guilliermondii* are able to effectively reduce chromate, the main part of the final product, Cr(III)-species, being located in the extra-cellular medium. Typical representatives of chromate-resistant *P. guillirmondii* mutants can tolerate higher concentrations of chromate in the growth medium and have a better Cr(VI)-reducing activity compared to the wild type strain. The formation of paramagnetic Cr(V)-species in the extra-cellular medium was discovered for all tested cultures that supports our hypothesis about the crucial role of extra-cellular

reduction in chromate detoxification. It has been demonstrated for the first time that the chromate-resistant phenotype of mutants is related to a lower stationary lever of the extremely toxic intermediate, Cr(V), in the extra-cellular medium. At least two species of this intermediate are observed in the medium with *g*-factors 1.975–1.976 (the dominant peak) and 1.999–2.000 (the minor "satellite" peak is found only at the earlier time of the cells' incubation with chromate). We may thus conclude that the chromate-resistance phenotype of Crt^r-mutants can be explained by the biochemical adaptation of chromate homeostasis of the cells to achieve a lower production of Cr(V)-species. Further experiments are required to identify the chromate-reducing cell components/metabolites, as well as the chemical nature of organic ligands, which interact with Cr(V) and Cr(III) species. Recent publications on stable chemically synthesized Cr(V)-complexes with organic ligands (e.g., hydroxycarboxylates and model peptides) suggest that relatively stable Cr(V)-complexes an be formed in microbial cultures which are less reactive and, therefore, less toxic.

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