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EXPERIMENTAL ARTICLES

Chromate-Resistant Mutants of the Yeast *Pichia guilliermondii*: Selection and Properties

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Abstract—Chromate-resistant mutants of the non-conventional yeast *Pichia guilliermondii* L2 were selected by different methods. The isolated mutants were capable of better growth and higher biomass yield at toxic (1.8–2.4 mM) chromate concentrations than the parent strain. The capacity of the mutants for extracellular chromate reduction and chelation of Cr(III) in the culture liquid was demonstrated. The effectiveness of these processes was shown to correlate with the resistance of *P. guilliermondii* strains to chromate. Extracellular metabolites of the yeast cells cultivated without chromate were shown to be capable of reducing chromate and forming stable soluble Cr(III)-biocomplexes.

Keywords: *Pichia guilliermondii* yeast, chromate-resistant mutants, chromate reduction, Cr(III)-biochelation.

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Chromium and chromium compounds are widely used in many branches of industry. Due to the high toxicity and carcinogenicity of chromate [1], the development of effective methods of detoxification of chromium hydroxy anions in the environment, including methods for remediation of industrial wastes—in particular, with the use of microorganisms—is an urgent problem. It has long been considered that chromate bioremediation depends on the ability of the cells to reduce it and to sorb Cr(III), the product of this reaction [1–4].

Detailed mechanisms of the toxic action of chromate on living organisms are still imperfectly understood. They are considered to be connected with the high redox potential of chromate, its easy entrance into the cells due to the high-affinity sulfate anion transporters, and the possibility of generating intermediate reactive Cr(V) compounds in the process of cellular reduction of Cr(VI) [2, 5–6].

Intracellular reduction can be nonenzymatic or enzymatic. Under physiological conditions, ascorbic acid, glutathione, and cysteine effectively reduce Cr(VI) to Cr(III) [5, 6]. Enzymatic chromate reduction in bacteria is well studied; it can occur under both aerobic and anaerobic conditions. In bacteria capable of reducing chromate aerobically, this process is catalyzed by NADH- and NAD(P)H-dependent reductases [1, 7, 8].

In eukaryotic organisms, including yeasts, the genetic and biochemical aspects of metabolism of chromium compounds have not been studied in detail [9–11]. Thus, it is not known which system of reduction, enzymatic or nonenzymatic, intracellular or extracellular, plays the main role in the processes of chromate detoxification.

In our previous studies, it was shown that one of the mechanisms of chromate detoxification in yeasts is its extracellular reduction to less toxic Cr(III) [13–17]. It was shown that different types of yeasts, bakery and non-conventional (*Pichia guilliermondii* and *Phaffia rhodozyma*), when grown in the presence of 0.5–1 mM chromate, are capable of detoxifying it by means of extracellular metabolites accumulating in the culture liquid (CL). As the Cr(VI) level dropped, Cr(III) compounds appeared in the CL of *P. guilliermondii*, which were isolated chromatographically in the form of soluble biocomplexes of at least two types [13]. The results of the study of Cr(III) biocomplexes (by means of chromatography and denaturing electrophoresis in PAG) showed that proteins are a constituent part of some of them [14]. The method of EPR-spectroscopy revealed the presence of trace amounts of stable Cr(V) complexes in the CL of chromate-resistant *P. guilliermondii* mutants [16].

Earlier, we selected mutants of the non-conventional yeast *P. guilliermondii* resistant to 1.5 mM chromate and studied their capacity for chromium accumulation by the cells: the maximum cell level of chromium for certain strains was 8–10% of the initial chromate, which is not the main contribution to bioremediation of chromium compounds [19]. Using the model of the non-conventional yeast *P. guilliermondii* L2 and the chromate-sensitive mutant *Ph. rhodozyma*, we showed that resistance to chromate

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in yeasts was determined by their ability to withstand intracellular chromium accumulation [14]. This observation coincides with the data obtained by other investigators who studied the mechanisms of resistance of yeast [10] and bacterial cells [12] to chromate.

To study the processes of chromate reduction by yeasts, we developed approaches to quantitative determination of Cr(III) compounds in solutions: the colorimetric chromazurol method for available nonchelated Cr(III) and the method of mineralization of organic complexes containing Cr(III)/Cr(V) compounds unavailable for direct determination [18].

Studies of recent years conducted with *P. guilliermondii* showed that, in the case of complete chromate reduction, more than 90% of the reduced product remained in the CL [13]. Similar results were obtained with the mold fungus *Aspergillus* sp. [20] and plant extracts [21], which allows us to consider extracellular reduction as a very important mode of Cr(VI) detoxification and to critically reconsider the generally accepted concept of the intracellular mechanism of this process.

Thus, studying the yeast ability to reduce chromate outside the cell and to chelate the nascent Cr(III) by the products of cell metabolism with the formation of nontoxic biocomplexes is a promising direction of investigating various processes: elucidation of both the mechanisms of chromate bioremediation and the possibility of chromate biotransformation into the Cr(III) biocomplexes, which hold promise for pharmaceutical practice. In order to solve this task, strains with the highest possible resistance to chromate are required.

The goal of the present work was to obtain mutant strains of the yeast *P. guilliermondii* with an elevated level of resistance to chromate and to characterize the capacity of the mutants for extracellular chromate reduction and chelation of the reduction products. Such investigations will make it possible to study in greater detail the patterns of extracellular chromate reduction by yeasts, which will improve our understanding of the mechanisms of chromate resistance in eukaryotic cells and promote searching for optimal methods of its bioremediation.

MATERIALS AND METHODS

Yeast strains and conditions of their cultivation and incubation with chromate. The histidine-dependent strain of the flavinogenic yeast *P. guilliermondii* ATCC 90191 (L2) from the Collection of Microorganisms of the Institute of Cell Biology, National Academy of Sciences of Ukraine, was used in this work.

The yeasts were grown at 30°C on a circular shaker (200 rpm) in Burkholder basal medium containing the following (g/l): sucrose, 20; $(NH_4)_2SO_4$, 3; KH_2PO_4, 0.5; $MgSO_4$, 0.2; and $CaCl_2$, 0.15. The medium was supplemented with histidine (40 mg/l) with or without yeast extract (YE), up to 0.1%. The medium contained 0.01 mg/l of the trace elements B, Cu, Mn, and Mo, added in the form of the following compounds (mg/l): H_3BO_3, 56; $CuSO_4 \cdot 5H_2O$, 39.3; $MnSO_4 \cdot 7H_2O$, 50.4; and $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 120; as well as 2 μg/l biotin, 0.07 mg/l Zn as $ZnSO_4 \cdot 7H_2O$ (307.9 mg/l), and 0.2 mg/l Fe, which was added from freshly prepared Mohr's salt solution (1.4 mg/ml). Depending on the aim of the experiment, ammonium sulfate or urea (1 g/l) was used as a source of nitrogenous nutrition. Cells grown to the mid-exponential growth phase were transferred under sterile conditions into a fresh growth medium to be incubated with chromate. Chromate was introduced in the form of the salt K_2CrO_4 (chemically pure). The cell and chromate concentration, as well as the incubation time, varied depending on the aim of the experiment.

Measurements and analytical methods. The biomass was determined by optical density at 540 nm. The dry cell mass (mg/ml) was calculated using a calibration graph constructed on the basis of the results of gravimetric analysis of the yeast *P. guilliermondii* [16]. The residual chromate concentration in the culture liquid (CL) during incubation of cells with chromate was determined colorimetrically using the diphenylcarbazide method [23].

The concentration of available Cr(III) in the CL was determined using the chromazurol S method we developed [18]. For analysis, 1 ml of 1% SDS, 0.5 ml of 1 M acetate buffer (pH 3.5), 0.5 ml of 0.06% chromazurol S in water, and 0.1–0.5 ml of the tested sample were introduced into test tubes. The volume was adjusted to 4 ml with distilled water. The test tubes were placed in a boiling water bath for 30 min, cooled quickly, and supplemented with 1 ml of 1 M H_2SO_4. The OD_{590} of the solutions was determined using a KFK-2MP photocolorimeter. The blind sample (all the components except for chromium) and the standard chromium(III) solutions were treated in a similar way. The chromium(III) concentration was calculated with the calibration graph. The 0.05 mM Cr(III) solution in 10 mM acetate buffer, pH 3.5, was used as the standard. The store solution of 10 mM chromium(III) was prepared by dissolving the exact weight portion of metal chromium in 1 M H_2SO_4.

In order to measure the total chromium level, the samples (the cells or culture liquid) were mineralized [18] and the concentration of the resultant Cr(III) was determined with the chromazurol method.

All the experiments were made in three replicates.

Obtaining chromate-resistant mutants of *P. guilliermondii*. Group I and II mutants were obtained by UV irradiation of *P. guilliermondii* L2 suspension at a dose affording 10% survival of the cells [19]. To prepare the suspension, cells grown on solid medium for 48 h were suspended in 50 mM phosphate buffer (pH 6.7) up to 0.3 mg/ml.

Plates with agarized minimal medium containing chromate were inoculated with the mutagenized sus-

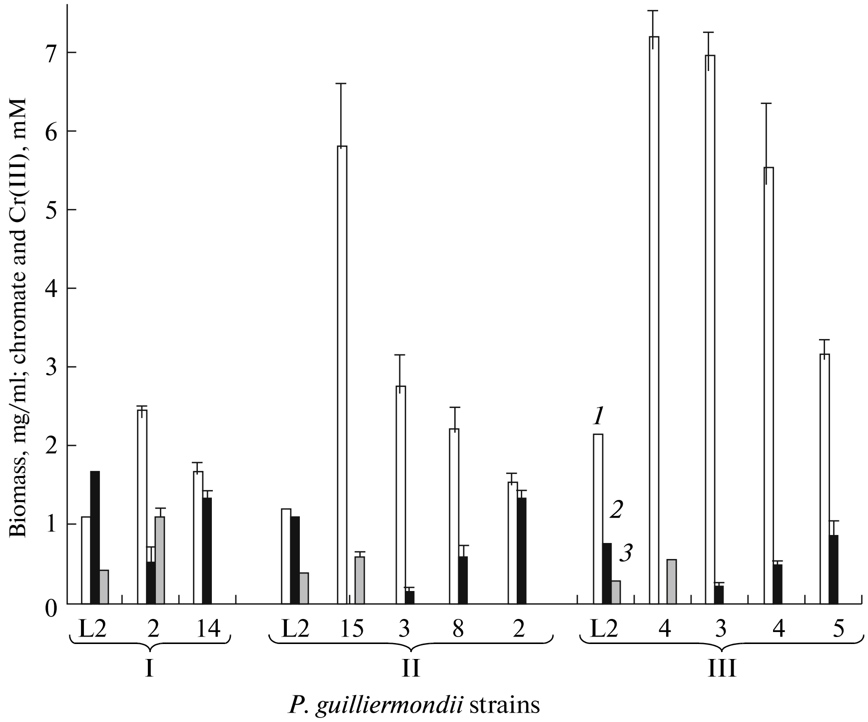

Fig. 1. Comparative characteristics of group I-III chromate-resistant mutants and the parent strain (L2) of *P. guilliermondii* by their capacity for growth and chromate reduction in medium with ammonium sulfate and YE: biomass (*1*); Cr(VI) level (*2*); Cr(III) level (*3*). Group I mutant cells (1 mg/ml) were cultivated for 2 days with 2 mM chromate; group II and III mutant cells (3 mg/ml) were cultivated for 5 days with 2.4 and 1.8 mM chromate, respectively. The digits on the X scale indicate the number of strains in each subgroup of mutants in groups I-III. The subgroup combines the strains of one group with similar characteristics.

pension: medium with 1 mM chromate and ammonium sulfate as a source of nitrogen with group I mutants and medium with 0.5 mM chromate and urea with group II mutants. Well-separated colonies that appeared 8–10 days later were subcultured onto wort agar with subsequent analysis of the capacity of group I and II mutant strains for growth and chromate reduction in liquid media in the presence of 0.1% YE.

Group III mutants were obtained using the method of long-term incubation of the initial L2 yeast culture in the presence of a high concentration of toxic chromate as a mutagenic factor [2, 3] in a liquid medium on a shaker. The cells (8 mg/ml) were incubated for 20 days with 10 mM chromate and plated on wort agar for the subsequent analysis of their resistance to chromate and the capacity for chromate reduction in liquid media.

RESULTS AND DISCUSSION

Obtaining chromate-resistant mutants of *P. guilliermondii*. Earlier, we showed that the resistance of the non-conventional yeast *P. guilliermondii* to chromate depended substantially on the composition of the nutrient medium, including the source of nitrogen (ammonium sulfate or urea) and the presence of YE, which stimulated yeast growth [24]. A good correlation was found between resistance to chromate and the capacity for its reduction by yeast cells. In the search for chromate-resistant strains, at the first stage of selection, the cell suspension after mutagenesis should be plated on minimal medium without YE. This is necessary to overcome the factors in a rich medium which stimulate cell growth and determine higher resistance of the yeast to chromate. At the second stage of selection, it is desirable to carry out screening of chromate-resistant strains in medium with YE, since the mutation that led to a changed phenotype should be expected to maximally manifest itself under conditions that are favorable for growth and chromate reduction.

Three groups of mutants were obtained with different methods as a result of selection of chromate-resistant *P. guilliermondii* L2 mutants: 16 group I strains, 28 group II strains, and 16 group III strains. Figure 1 summarizes the results of comparative study of the properties of the mutants and the initial strain L2: growth in the presence of increased chromate concentrations (1.8–2.4 mM) and the ability to reduce toxic chromate and to accumulate available Cr(III) in the CL. Figure 1 shows the averaged biomass values, as well as the levels of residual chromate and available Cr(III) in the CL of the tested subgroups of strains in each group. The Cr(III) concentrations determined

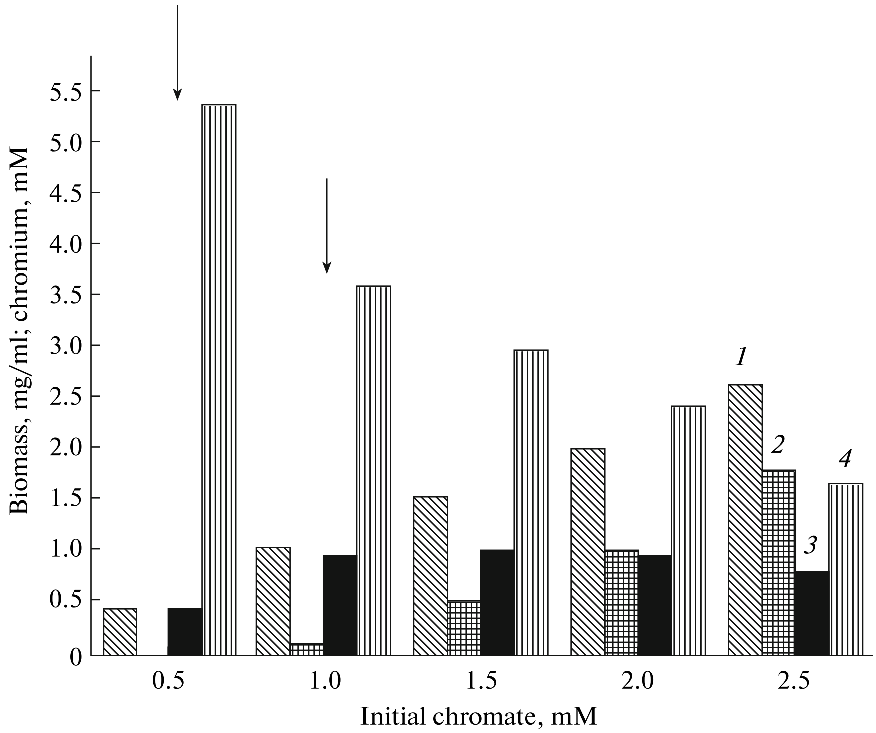

Fig. 2. Balance of different forms of chromium determined in the CL in the process of chromate reduction by *P. guilliermondii* 32-I: Cr(VI) + Cr(III) (*1*), Cr(VI) (*2*), Cr(III) (*3*), and biomass (*4*). The yeast cells (1 mg/ml) were cultivated for 24 h in the presence of different chromate concentrations. The arrows designate the initial chromate and biomass concentrations at which complete Cr(VI) reduction and the equivalent levels of available Cr(III) were observed.

by the chromazurol method are shown for the parent strain and the subgroups of mutants, which reduced chromate completely (in groups II and III) or most actively (in group I).

Properties of group I mutants. Among 40 strains grown after plating the UV-irradiated yeast cell suspension on the minimal ammonium sulfate-containing medium, 16 clones with decreased sensitivity to chromate were revealed after their incubation (1 mg/ml, 48 h) on medium with the YE and chromate (2 mM). Only two clones, strains 3-I and 32-I, differed significantly from the initial strain in the biomass level (twice as much) and the residual chromate concentration (three times lower). The remaining 14 mutants did not differ significantly from the parent strain. No substantial change in the level of chromium in the cells of the chosen mutants was noted: it varied between 0.02 and 0.05 μmol/mg cells, which corresponds to 6–8% of the initial chromate level.

Figure 2 shows the quantitative characteristics of the processes of reduction of Cr(VI) by the yeast culture of the mutant *P. guilliermondii* 32-I when the cells were incubated in the presence of different chromate concentrations, namely, the balance of different forms of chromium found in the CL. It can be seen that, at chromate concentrations from 0.5 to 2.5 mM, the sum of values of residual Cr(VI) and the Cr(III) available for determination virtually coincided with the level of the initial chromate (Fig. 2). At low chromate concentrations (0.5 and 1.0 mM), all the chromium was revealed in the form of available Cr(III). An increase in the Cr(VI) concentration was accompanied by an increase in the pool of residual chromate, but not of available Cr(III), and the total chromium, Cr(VI) and Cr(III), corresponded to the initial chromate level. Earlier, we showed that, as chromate was reduced by *P. guilliermondii* L2, the reduction product, Cr(III), was chelated by the components of the growth medium, resulting in formation of the complexes in the CL, which were not detected by the chromazurol reaction [13]. When the cells of the mutant 32-I were incubated, this phenomenon was not observed (Fig. 2). These results seemed to indicate the inability of the mutant 32-I CL to chelate Cr(III).

To test this suggestion, the dynamics of Cr(VI) reduction and Cr(III) chelation in the yeast culture was studied under conditions in which an increase in the concentration and/or range of the metabolites capable of reducing chromate and chelating Cr(III) could be expected.

Three sequential phases in the profile of the growth kinetics of the mutant 32-I were observed (Fig. 3a). During the first 24 h of cultivation, the biomass grew up, after which the cells temporarily stopped growing (the appearance of a plateau in the growth curve). During the period of cessation of growth, Cr(VI)

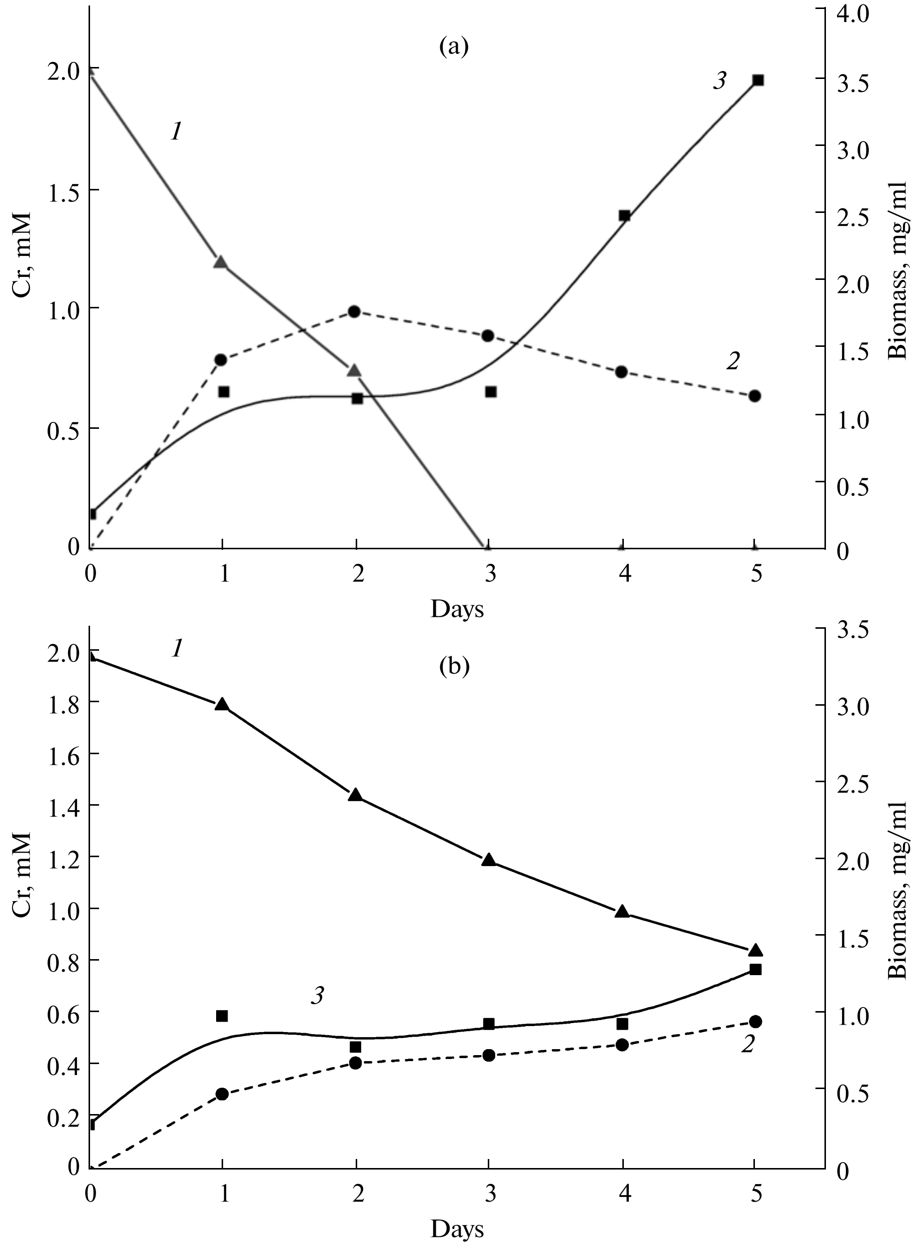

Fig. 3. Dynamics of cell growth and reduction of 2 mM chromate in the process of incubation of the yeast *P. guilliermondii*: mutant 32-I (a) and the parent strain L2 (b): Cr(VI) (*1*), Cr(III) (*2*), and biomass (*3*).

reduction continued at a sufficiently high rate, possibly resulting in accumulation of a very toxic intermediate, for example, Cr(V), to the critical level incompatible with cell growth. This suggestion was confirmed using the EPR method [16]. On the third day of cultivation, the growth resumed, probably after the concentration of the extremely toxic intermediate had decreased. Importantly, the pool of available Cr(III) decreased only after three days of cultivation and a negative balance was observed when the sum of residual chromate and the Cr(III) formed was calculated: the level of available chromium seemed to decrease, likely due to the gradual decrease in the pool of Cr(V) and to Cr(III)/Cr(V) binding by the cell metabolites. The point of resumption of growth coincided with the absence of chromate and, accordingly, an extremely toxic Cr(V) in the culture liquid; the chromium content in the cells at this stage of growth was only 4–6% of the initial chromate level. After mineralization of the CL aliquot sampled on the fifth day of cultivation, chromium was found in an amount of 89% of the initial chromate level, which confirms the hypothesis that Cr(III) binds to form complexes, where it becomes unavailable for determination in the reaction with chromazurol S. However, a sufficiently high concentration of available Cr(III), about 30% of the initial chromate, was detected in the CL of the mutant 32-I at the stage of complete chromate reduction, which may be indicative of a decreased level of the chromium-binding metabolites (Fig. 2). Nonchelated Cr(III), available in the reaction with chromazurol, was also detected in the CL of the parent strain L2 under the conditions of incomplete chromate utilization (Fig. 3b), i.e., when the cell growth curve was steadily flat. Obviously, with a low biomass in the CL, the cell metabolites were lacking for Cr(III) chelation.

The difference in the profiles of growth kinetics the mutant 32-I (Fig. 3a) and the initial strain L2 (Fig. 3b) upon incubation with 2 mM chromate is probably determined by mutation (mutations) responsible for a higher resistance of the mutant to Cr(V)/Cr(IV), the intermediate product of chromate reduction. The presence of the phase of growth delay, the plateau, the duration of this phase, or the impossibility for the growth curve to change its course and leave the plateau are most likely related to the emergence of the pool of the cell-toxic Cr(V). Earlier, we reported a similar profile of growth kinetics for strain L2 at lower concentrations (1 mM chromate) [24] and no growth delay phase was observed on incubation with 0.5 mM chromate (Fig. 4). When chromate was completely reduced, no available Cr(III) remained in the CL: it was chelated completely by the cell components. Our conclusions agree with the literature data on the untypical growth kinetics of different yeasts in the presence of chromate [4, 9]: the authors link the phenomenon of growth delay at certain chromium concentrations, i.e., the appearance of a plateau, to a toxic stress, and the resumption of cell growth, i.e., the curve taking a different course, to chromate detoxification.

These results suggest the following conclusion: the sensitivity of the yeast *P. guilliermondii* to chromate is directly associated with its reduction. The chromate-resistant mutant 32-I reduced 2 mM chromate much more actively (Fig. 3a) than the parent strain (Fig. 3b). Although Cr(III) chelation by the cell metabolites proceeded less vigorously, Cr(III) did not inhibit the growth of the mutant. It is known that Cr(III) is less toxic to yeasts (and all other living organisms) than chromate [1, 2].

Properties of group II mutants. We showed that, when *P. guilliermondii* grew in the presence of elevated chromate concentrations, a lag phase was present in the kinetics profile and its duration correlated with resistance to chromate [24]. We used this feature of the growth kinetics as a test for selecting the most chromate-resistant mutants. The screening of the strains was carried out on medium with urea on which the initial strain L2, as was shown earlier [24], was the most sensitive to chromate.

When screening the mutants in a liquid medium, we selected strains with a duration of the growth delay phase shorter than in the parent strain *P. guilliermondii* L2. Twenty-eight strains, group II mutants, were

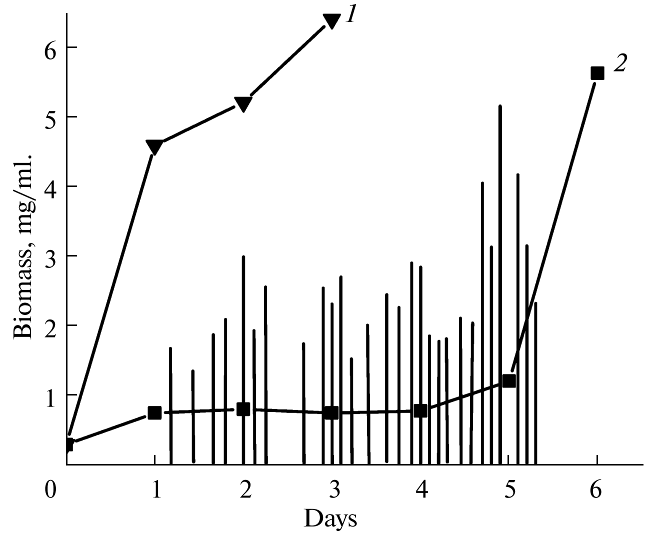

Fig. 4. Kinetics of the biomass growth of *P. guilliermondii* L2 in the presence of 0.5 mM chromate in different growth media: with $(NH_4)_2SO_4$ (*1*) and urea (*2*). Vertical lines designate the biomass values of group II chromate-resistant mutants (28 strains) in the process of incubation in the medium with urea.

selected based on this criterion (Fig. 4). The second stage of screening the chromate-resistant mutants of this group was carried out on medium with ammonium sulfate as a source of nitrogen in the presence of 2.4 mM chromate (Fig. 1, group II). The yeasts (0.3 mg/ml) were incubated for five days, controlling the biomass and the level of residual chromate as an indicator of reduction in the process of incubation. Fifteen strains were revealed that completely reduced chromate, the content of available chromium in the CL being three times lower than in the case of L2.

As can be seen from Fig. 1, almost all group II mutants grew much better in the presence of 2.4 mM chromate and utilized chromate more effectively than the parent strain L2. Fifteen mutants of this group utilized chromate completely; three strains, almost completely; and the parent strain reduced only half of the initial level of the toxic anion during this period. The mutants of the subgroup consisting of eight group II strains reduced chromate twice as effectively as L2, and only two strains did not differ substantially from L2 in this characteristics.

The fact that Cr(III) in 15 group II mutants (Fig. 1), as in strain 32-I, remained available, i.e., nonchelated, in the CL after the incubation with 2 mM chromate (Fig. 3a) under the conditions of complete chromate reduction merits attention. Available Cr(III) was revealed in the CL of L2 in the presence of residual chromate. Thus, it is difficult to compare the chelating capacity of the wild type strains and the mutants at such a high (2.4 mM) chromate concentration.

In order to assess the ability of group II mutants to reduce chromate and to chelate Cr(III), all forms of chromium were analyzed after 5 days of cultivation in the presence of 2.4 mM chromate (Fig. 5). The incubation of the parent strain in the presence of 1 mM chromate, when its complete reduction was observed, was an additional control.

At a high chromate concentration (2.4 mM), the mutants reduced chromate completely; however, the available Cr(III) determined in the CL was 17% of the total chromium. The chromium accumulated by the cells and determined after mineralization of the samples was 6% of the initial chromate (as calculated per 1 ml of the yeast culture); its bulk, 68% of the initial chromate, was reduced to Cr(III) and chelated by the CL components. Under these conditions, the parent strain L2 was inferior to the mutants of this group both in its capacity for chromate reduction and the chelating properties (only 32.5% of the initial chromate disappeared). However, under the conditions of complete reduction of 1 mM chromate, the chelating capacity of the mutants of this group was 14% lower than in the parent strain.

Properties of group III mutants. Earlier, we showed that, upon the 24-h contact of *P. guilliermondii* with 8 mM chromate, only 2×10^{-5} living cells remained in the yeast population [16]. It is also known that yeasts with chromate-reducing activity were isolated from chromium-contaminated industrial wastes. In our previous works with EPR, we showed that already at the first stage of cultivation with Cr(VI) [16, 17], Cr(V), which is known to be the most toxic form of chromium and has a mutagenic effect, was revealed in the CL of the non-conventional yeast. Considering these facts, we proposed a new approach to selecting *P. guilliermondii* mutants with increased resistance to chromate: prolonged exposure of the yeast cells to high chromate concentrations as a mutagenic factor, i.e., under incubation conditions in which only few cells survive. The cells (8 mg/ml) were incubated for 20 days in a liquid medium with ammonium sulfate, YE, and 10 mM chromate prior to plating on wort agar. Liquid medium containing 0.7 mM chromate was inoculated with individual colonies grown under such conditions; the clones that grew better were selected after 24 h of cultivation, and residual chromate was determined in their CL. Of 48 clones, we selected 16, the selection by growth of which in the presence of 1.8 mM chromate and its reduction is shown on Fig. 1. Four strains reduced chromate completely; four strains appeared to be noticeably more resistant to chromate than the parent strain, and five strains practically did not differ from L2.

In the presence of 1.8 mM chromate, all group III mutants accumulated biomass that was several times higher than that of the parent strain under the same conditions. The mutants from the subgroup consisting of four strains utilized chromate completely, and three strains utilized it almost completely.

Similarly to group I and group II mutants, group III mutants accumulated nonchelated Cr(III) under the conditions of complete chromate reduction.

Tables 1 and 2 show the characteristics of the chromate-resistant mutants from the three groups obtained by different methods. As seen from Table 1, method II, using the minimal selective medium and urea as a nitrogen source, proved to be the most successful for obtaining the mutants. Twenty-eight mutants with decreased duration of the plateau phase (Fig. 4) and possessing an increased reduction capacity constituted 51% of the total number (55) of the group II clones analyzed.

The results shown in Table 2 demonstrate that at low chromate concentration (1 mM), the parent strain and the mutant 15-III reduced all the Cr(VI) and chelated completely the Cr(III) formed over 5 days. In the same experiment (the data not shown), strains 32-I and 27-II, having quickly (for 2 days) reduced all the 1 mM chromate, proved incapable of completely chelating the Cr(III) formed after 2 or even 5 days. The concentration of the chromate reduced in the process of incubation of the yeast cells was calculated as a difference between the levels of the initial and residual Cr(VI) in the CL; chelated Cr(III) was calculated as the difference between the levels of the total Cr(III) equal to the concentration of the initial Cr(VI) and the available Cr(III). The residual Cr(VI) and available Cr(III) concentrations in the CL were determined using the diphenylcarbazide and chromazurol methods, respectively.

At higher initial chromate concentrations in the incubation medium, the superiority of the mutants of all the groups to the parent strain in their ability to both reduce chromate and chelate the Cr(III) formed was obvious. It is important to note that the chromium content in the cells for all the strains studied does not exceed 6–8%; hence, under the conditions of incomplete chromate reduction in the CL of all the strains, including the parent strain, Cr(III) remains available. Under such conditions, the yeast probably synthesize less metabolites which are capable of forming strong complexes with Cr(III) converting it to the form unavailable in the reaction with chromazurol. The chelating capacity in the CL of the parent strain L2, which is considerably inhibited at increased chromate concentrations, is significantly decreased (Figs. 1, 3b, 5). That the quantitative parameters of the processes of both reduction and chelation increase with the duration of incubation supports this suggestion; however, if these quantitative parameters are calculated for the cell biomass of the strains studied (per 1 mg of biomass), it becomes clear that the parent strain is the best in terms of this criterion. All the advantage of the mutants consists in their ability to grow more quickly and to accumulate far more biomass under conditions of increased concentrations of toxic chromate than the cells of L2. There is no doubt that the capacity for Cr(III) chelation is influenced by the difference,

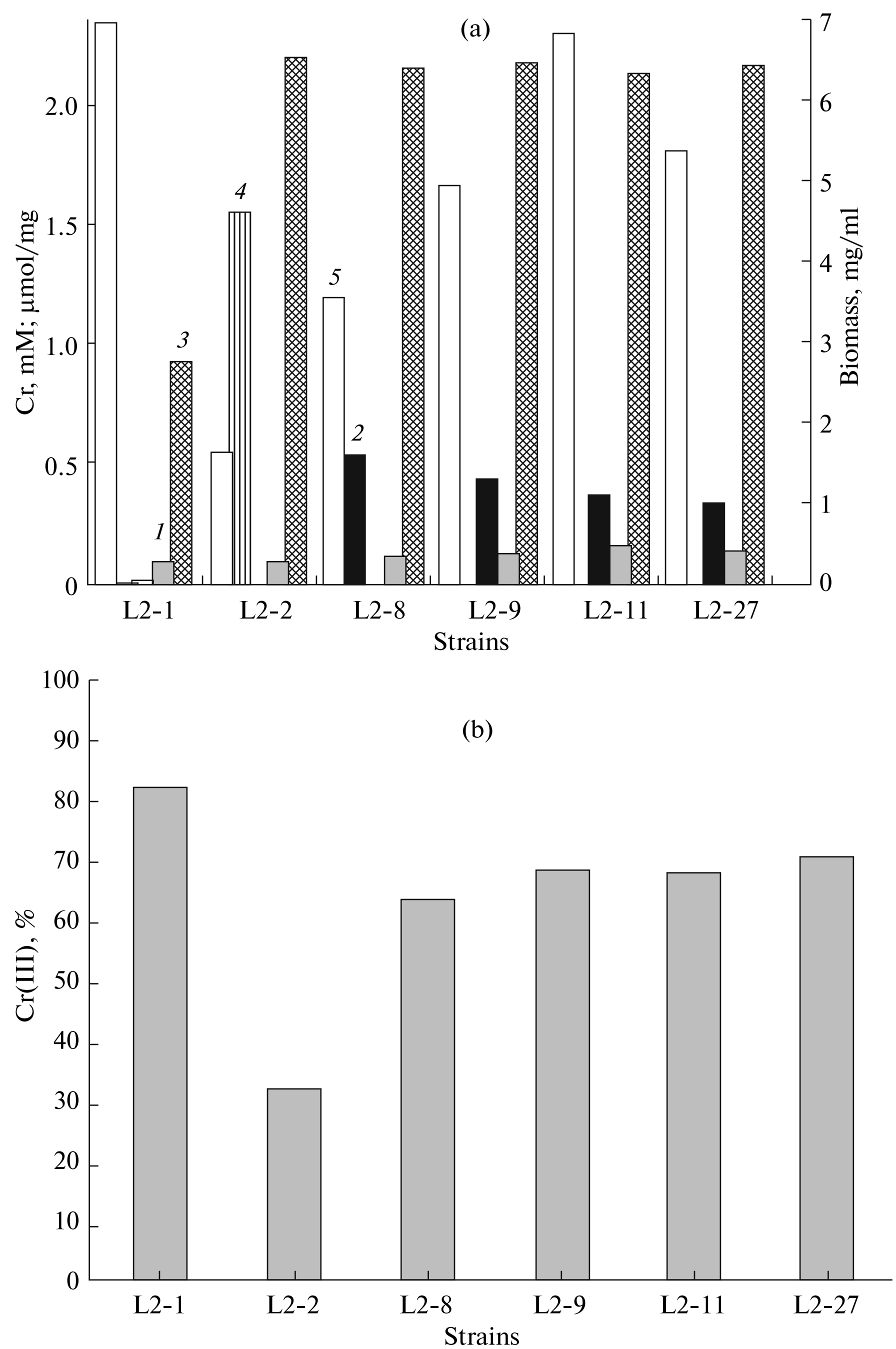

Fig. 5. The biomass and the products of Cr(VI) metabolism revealed in the culture liquid and yeast cells of the parent strain *P. guilliermondii* L2 (L2-2) and group II chromate-resistant mutants cultivated in 2.4 mM chromate (a) and the chelated Cr(III), estimated by mineralization, %, of the initial chromate concentration (b). The L2 cells were additionally incubated in 1 mM chromate (L2-1). Designations: chromium in the cells (mineralization), μmol/mg (*1*); available Cr(III) (*2*); the total chromium in the CL (mineralization) (*3*); Cr(VI) (*4*); and biomass (*5*).

though insignificant, between the biomass of the mutants and the initial strain at 1 mM chromate. Moreover, the CL of the mutants probably differs from that of L2 in the amount and range of its components.

Thus, the question remains, whether chromate reduction and Cr(III) chelation are affected by the components present inside the yeast cells and/or on the cell surface or both of these processes occur at the expense of the metabolites secreted into the CL. To investigate this problem, the processes of reduction and chealtion were studied in vitro, i.e., in the supernatant fluids of the yeast cultures that were not in contact with chromate, after removing the cells by centrifugation.

Table 1. Comparative characterization of the *Pichia guilliermondii* chromate-resistant mutants selected by different methods

Mutants from different groups; initial chromate, mM	Selection medium	Mutagenic factor	No. of strains analyzed	Effectiveness of chromate reduction in the medium with ammonium sulfate and YE								
				High			Average			Low*		
				Residual chromate, mM	No. strains selected		Residual chromate, mM	No. strains selected		Residual chromate, mM	No. strains selected	
					Number	%		Number	%		Number	%
1	2	3	4	5	6	7	8	9	10	11	12	13
32-I; 2.0	Ammonium sulfate	UV	16	0	2	12	0.1–0.6	0	0	1.4	14	88
27-II; 2.4	Urea	UV	28	0	15	54	0.11	3	11	1.35	2	7
							0.55	8	28			
12-III; 1.8	Ammonium sulfate and YE	Chromate	16	0	4	25	0.21	3	19	0.83	5	31
							0.46	4	25			

* The levels of residual chromate in the mutant and parent strain cultures did not differ significantly (Fig. 1).

Table 2. Comparative characteristics of the processes of extracellular Cr(VI) reduction and Cr(III) chelation by *P. guilliermondii* L2 mutants (the cultivation time was 5 days)

Initial chromate concentration, mM	Concentrations of reduced Cr(VI) and chelated Cr(III) in the CL, mM							
	L2		32-I		27-II		15-III	
	Cr(VI)	Cr(III)	Cr(VI)	Cr(III)	Cr(VI)	Cr(III)	Cr(VI)	Cr(III)
1.0	1.0	1.0	1.0	0.48	1.0	0.8	1.0	1.0
1.8	1.09	0.71	ND	ND	ND	ND	1.8	0.8
2.0	1.05	0.55	2.0	1.38	ND	ND	ND	ND
2.4	0.75	0.40	ND	ND	2.3	1.61	ND	ND

Note: ND stands for not determined.

Chromate reduction and Cr(III) chelation by components of the CL. In all the above experiments, the phenomena of chromate reduction and Cr(III) chelation were investigated in vivo, i.e., both the yeast cells and the cell metabolites in the CL were brought in contact with exogenous chromate. We found that not only the CL of the yeast cultures grown with chromate, but also the CL of the cells grown without chromate (CL^-), possessed chromate-reducing capacity.

In order to study the differences in the (CL^-) between L2 and the mutants obtained by different methods, the cells were incubated in the minimal medium (without chromate!) for 5 days; the cells were then removed by centrifugation and the (CL^-) was incubated with chromate. Figure 6 shows the results of the experiment in which chromate (up to 0.4 mM) was added to the (CL^-) of all the strains and its residual level in the reaction mixture was measured after certain intervals of time (2 min; 2, 4, 7, and 12 h).

When complete, down to zero (Figs. 6a, 6d), or significant (Fig. 6b) chromate reduction was recorded, new portions of chromate were added to the reaction mixture (Figs. 6a, 6b, 6d). Figure 6 shows that the (CL^-) of L2 had the maximal reducing capacity:

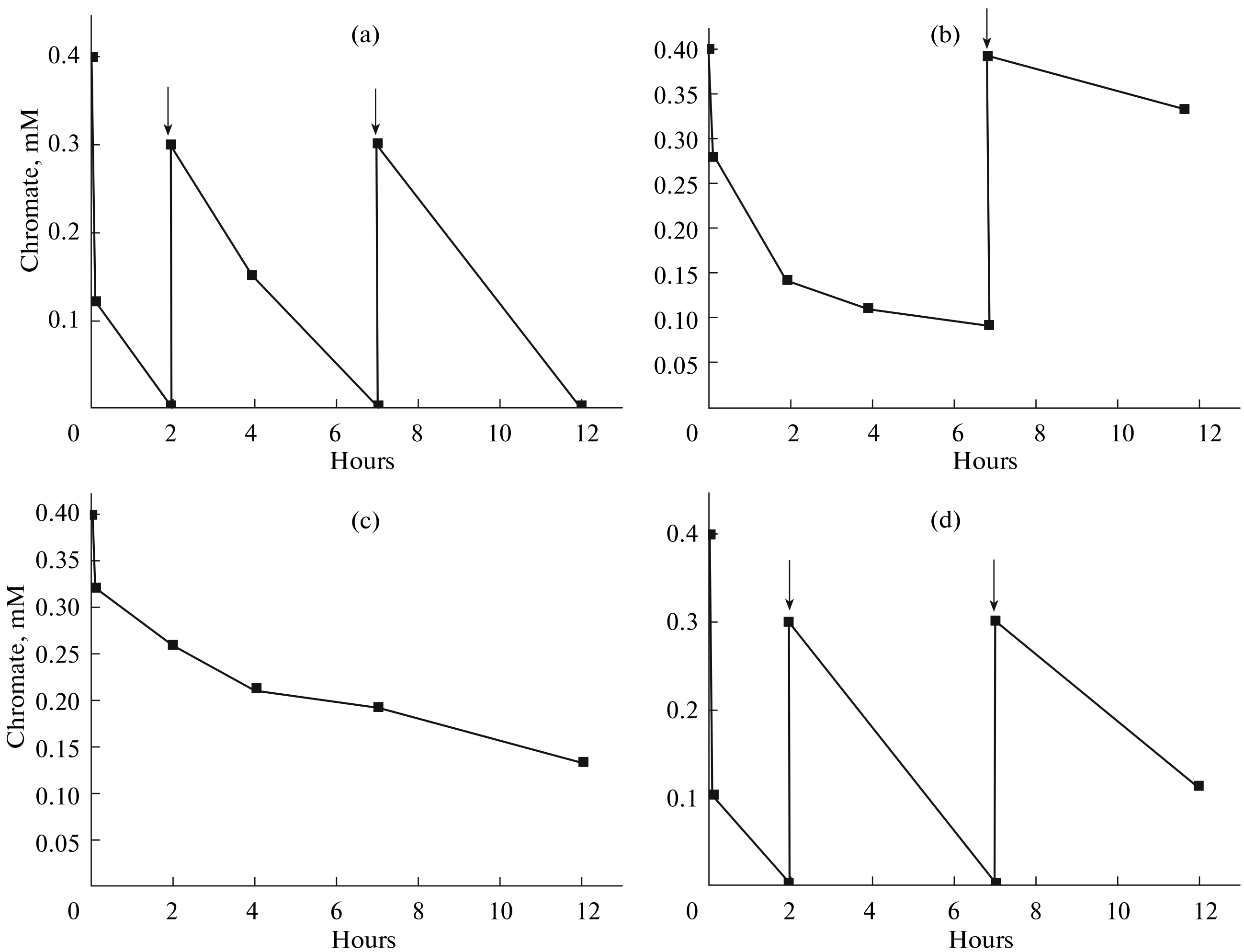

Fig. 6. Kinetics of chromate reduction by the components of the (CL^-) of *P. guilliermondii* chromate-resistant mutants: L2 (a), 32-I (b), 27-II (c), and 15-III (d). The arrows designate the moments when a new portion of Cr(VI) was added.

1 mM chromate was reduced completely in the course of 12 h. On the initial short (2 min) contact of the (CL^-) with chromate, all the strains were characterized by a high reduction rate, especially the (CL^-) of L2 and 15-III (141 and 126 nmol/min ml, respectively). Further chromate reduction during 12 h (a prolonged stage) proceeded in the (CL^-) of all the strains at a rate 140–300 times lower than the initial rate (Fig. 7). In the process, a relatively high reduction rate of about 1 nmol min^{-1} ml^{-1} was retained in the (CL^-) of strains L2 and 15-III for a longer time than in the others.

Table 3 shows the results summarizing the described experiment and its continuation: after 12 h of the incubation, a new portion of chromate, up to 0.3 mM, was added to the reaction mixture of the (CL^-) of L2 containing the forms of completely reduced 1 mM chromate. All the reaction mixtures of L2 and the mutants, each with a specified chromate concentration, were incubated at 20–25°C for an additional 12 h.

The results shown in Fig. 6 and Table 3 indicate that there exist substantial differences between the parent strain and the mutants obtained by UV irradiation (32-I and 27-II), whereas the group III mutants in which chromate was a mutagen appeared to be the closest to the initial strain L2 in terms of their capacity for extracellular reduction and chelation. It may be suggested that the (CL^-) of L2 and the mutants may differ in the number and the range of their components. This problem requires further study.

Thus, using different methods of selection, we obtained the chromate-resistant mutants of the nonconventional yeast *Pichia guilliermondii* L2, which grew better and were characterized by a higher biomass yield at toxic (1.8–2.4 mM) chromate concentrations than the parent strain. The capacity of the mutants for extracellular chromate reduction and Cr(III) biochelation in the culture liquid was characterized; it was shown that the effectiveness of these processes corre-

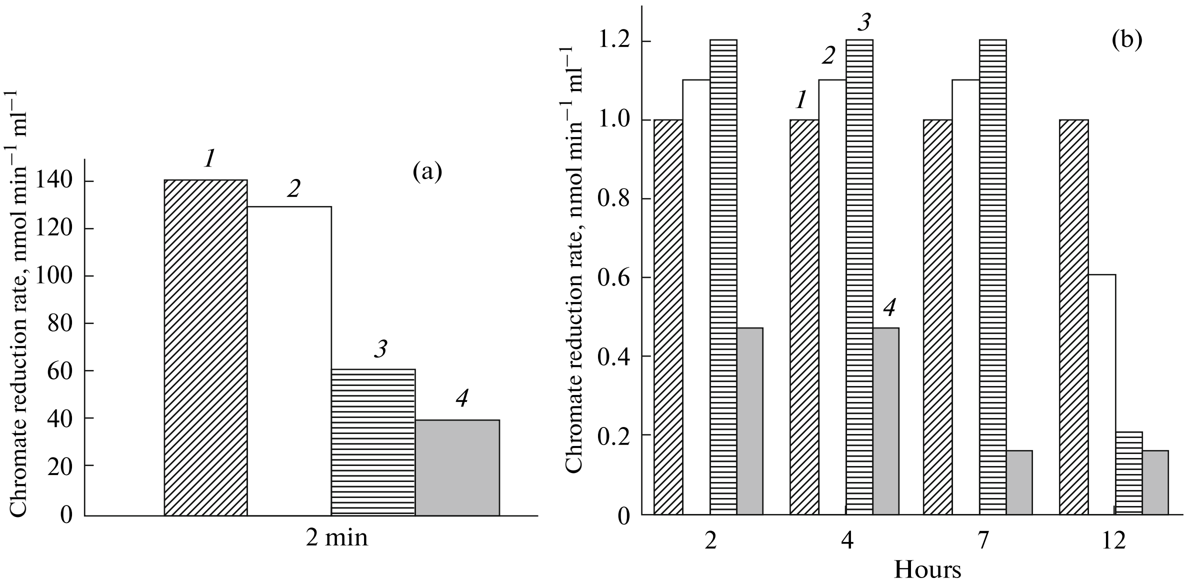

Fig. 7. Characteristics of the rates of chromate reduction (nmol min^{-1} ml^{-1}) by the components of the (CL^-) of *P. guilliermondii* chromate-resistant mutants: L2 (*1*), 15-III (*2*), 32-I (*3*), and 27-II (*4*) at the initial (a) and long-term (b) reduction stages.

lates with the resistance of *P. guilliermondii* strains to chromate. The capacity of the metabolites secreted by the yeast cells grown without chromate to reduce chromate and participate in the formation of stable soluble Cr(III)-biocomplexes has not been reported previously. The study of the particulars of extracellular chromate reduction by the non-conventional yeast *P. guilliermondii* allows us to gain a keener insight into

Table 3. Comparative characteristics of the (CL^-) of the mutant strains by the capacity for Cr(VI) reduction and Cr(III) chelation

(CL^-) of the strains; reaction time, h	Concentrations of the initial and reduced Cr(VI), available and chelated Cr(III), mM			
	Cr(VI)		Cr(III)	
	Initial	Reduced	Available	Chelated
L2;				
12 h	1.00	1.00	ND	ND
24 h	1.30	1.16	0.79	0.37
32-I;				
12 h	0.70	0.37	ND	ND
24 h	0.70	0.56	0.56	0
27-II;				
12 h	0.40	0.27	ND	ND
24 h	0.4	0.36	0.33	0.03
12-III;				
12 h	1.0	0.88	ND	ND
24 h	1.0	0.96	0.69	0.27
15-III;				
12 h	1.0	0.89	ND	ND
24 h	1.0	0.96	0.70	0.26

Note: ND stands for not determined.

understanding the mechanisms of resistance of the eukaryotic cells to stress caused, in particular, by a toxic compound.

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