ACTA MICROBIOLOGICA BULGARICA Volume 36 / 2 (2020)

Arsenate Tolerance in Saccharomyces cerevisiae is Associated with Efflux Capability

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Abstract

Arsenic is a common contaminant in soils, affecting soil microbiome and all soil organisms. The present study examined arsenate tolerance of two *Saccharomyces cerevisiae* strains isolated from contaminated soil. Their tolerance to sodium arsenate (AsV) was studied in a liquid medium for 24 hours, while the ability of the populations to reduce their intracellular arsenic content was subsequently investigated through kinetic studies of arsenate transport at 33.3, 133.2, and 266.4 μM of AsV. The dependence of tolerance on the efflux system was demonstrated by the use of amiloride hydrochloride, which is known as a suppressor of ion transport through the cell membrane. The maximum concentration tolerated by *S. cerevisiae* AS09 was found to be about 72.8% lower than that by the tolerant *S. cerevisiae* AS07. The influx and efflux of arsenate across the cell membranes of both strains were dependent on the concentration of metalloid in the medium and on the anion net balance. Both strains rapidly increased their internal As concentration to a maximum point, although only the tolerant strain was able to decrease it subsequently in all concentrations used. When the highest concentration was applied, the cells of the non-tolerant strain were found to be dead. When amiloride hydrochloride was used it was found that the tolerant cells behaved like sensitive cells, and at 266.4 μM the cells of both strains were found to be dead, because the extrusion of the toxic anion was interrupted. Kinetic differences in arsenate transport through the cell membrane explain the different degrees of tolerance of the studied strains.

Keywords: arsenate transport, yeast, influx, efflux, amiloride hydrochloride, tolerance

Резюме

Арсенът е често срещан замърсител в почвите оказвайки въздействие върху почвения микробиом и върху всички почвени организми. В настоящето проучване изследвахме толерантността към натриев арсенат (AsV) на два щама *Sacharomyces cerevisiae* изолирани от замърсени почви. Проучването се извърши в течна хранителна среда за 24 ч., докато способността на популациите да редуцират тяхното вътреклетъчно съдържание на арсен се извърши чрез изследване на транспорта на арсенат при 33.3, 133.2 и 266.4 μM AsV. Зависимостта на толерантността от извеждането извън клетката беше доказано с използването на амилорид хидрохлорид, който потиска транспорта на йони през клетъчната мембрана. Установихме, че максималната концентрация, при която все още има растеж на *S. cerevisiae* AS09 е със 72.8 % по-ниска от тази при толерантния *S. cerevisiae* AS07. Постъпването и изхвърлянето на арсенат от клетките при двата щама зависеше от концентрацията на металоида в средата и от нетния баланс на аниона. Двата щама бързо увеличиха вътрешната си концентрация на As достигайки максимум, като само толерантният щам беше способен да я намали при всички варианти. При високата концентрация се наблюдаваше загиване на клетките на нетолерантния щам. Прилагайки амилорид хидрохлорид се установи, че толерантните клетки се държат като чувствителни и при концентрация 266.4 μM клетките на двата щама загиват поради липсата на извеждане на токсичния анион навън. В заключение, различията в транспорта на арсенат през клетъчната мембрана обясняват различната толерантност на двата щама.

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Introduction

Currently, metal and metalloid pollution is one of the most important environmental problems affecting our everyday life. As a metalloid, arsenic is highly toxic, abundant in the environment, but not required for life (Rahman *et al*., 2014). In arsenic-containing habitats, soil microorganisms have developed arsenic-resistance (ars) genes. It is known that inorganic arsenate is taken up by the cells mainly via the phosphate system, while the tolerant strains are able to reduce their cell arsenate content by extrusion of arsenate out of the cell (Garbinski *et al*., 2019). Different studies have shown that some yeast strains isolated from contaminated sites possess excellent metal and metalloid scavenging capability (Nguyên-nhu and Knoops, 2002; Massoud *et al*., 2019). These cells exhibited high tolerance and may be potential candidates for the removal of metals and metalloids from wastes, wastewaters and contaminated soils. The stage has already been identified for the application of metal-resistant microbial cells in metal and metalloid harvesting. In previous publications (Shilev *et al*., 2001; Fernández *et al*., 2003; Shilev *et al*., 2007), the mechanisms of tolerance to arsenic in tolerant bacteria *Pseudomonas fluorescens,* and in non-tolerant *Bacillus* sp. were described. In those cells, arsenate tolerance was found to be strongly dependent on the transport through the cell membrane and especially on the efflux system, where different proteins are involved. In the eukaryote *Saccharomyces cerevisiae,* several phosphate transporters participate in arsenate uptake (Bun-ya *et al*., 1996; Shen *et al*., 2003). Inside the cell, the metalloid can be reduced to a form that is recognized by the extrusion system. Its presence activates the transcription of various cellular defense genes, which activate the membrane proteins for extrusion of the metalloid. Therefore, the tolerance to As depends on the presence of these proteins in the membrane. Cells remove metalloids from the cytosol either by active efflux or by sequestration in an internal organelle. Controlling the influx appears to be another way of maintaining a low intracellular metalloid content (Wang and Chen, 2006; Batista-Nascimento *et al*., 2013).

The aim of the present study was to investigate the mechanisms of sodium arsenate ($Na_2HAsO_4.7H_2O$) transport in two *S. cerevisiae* strains as a key factor for metalloid tolerance.

Materials and Methods

Previously, we isolated different strains tolerant to heavy metals and metalloids from contaminated soils (Shilev and Vancheva, 2006). Some of them were characterized and identified as *S. cerevisiae* strains and found to tolerate or not moderate to high concentrations of sodium arsenate (AsV) in the growth medium.

Cultures

In the present investigation, AsV tolerance and the kinetics of AsV transport in the cells in two strains were studied. Both strains, *S. cerevisiae* AS07 wild type (WT) and *S. cerevisiae* AS09, were pre-isolated from soil contaminated by multiple heavy metals. The former was tolerant to elevated concentrations of arsenic, while the latter was not tolerant to that metalloid.

Tolerance study

The tolerance of the above-mentioned strains was studied in a liquid medium (nutrient broth), growing them separately until optical density of approximately 0.8 at 550 nm. A portion of the yeast suspension was inoculated in a flask with a new sterile medium containing different concentrations of AsV as sodium arsenate from 0 to 5 M solution. The experiment was carried out in batch mode using 50 ml of medium per 250 ml flask. The flasks were incubated in an orbital shaker at 100 rpm and 28°C for 24 h. Growth was monitored at 550 nm. Three replicates per treatment were performed. The flask without metal was taken as a control.

Kinetic assays

In the present study, the kinetics of AsV transport in the cells of both *Saccharomyces cerevisiae* strains was studied. In the influx experiments, the cells were grown in a nutrient-broth medium (100 ml, 28ºC), in a water bath with heating and agitation. Stock solution of sodium arsenate was prepared and the relevant quantity was added when the optical density of 0.25 - 0.35 (550 nm) was reached (early exponential phase), with the purpose to obtain the final concentration of sodium arsenate in the medium (33.3, 133.2 or 266.4 μM). Samples were taken over a period of 1 h, filtered (Millipore, ⊘ 0.45 μm), washed (20 mM $MgCl_2$), and after 24-h exposure in 0.2 M HCl to break down the cells, were analyzed by atomic absorption spectrometry (Varian SpectrAA-100). Finally, the influx experiment was repeated adding Amiloride hydrochloride (Sigma Chem.) at minute 15, which affects the membrane anion transporters inhibiting the extrusion systems for metal efflux. Amiloride was dissolved in methyl sulfoxide (Fluka Chem) and added in a concentration of 2.5 mM. The experiments were repeated several times and the standard error was found to be less than 5%.

Results and Discussion

The present study is a continuation of the investigations in the field of bioremediation related to the restoration of soils contaminated with various metals and metalloids. It compares two strains isolated from such contaminated area in order to prove their tolerance or lack of tolerance to arsenic.

The detailed study of tolerance of both *S. cerevisiae* strains is presented in Fig. 1. The presence of AsV in the liquid medium led to changes in the growth rate of both strains. In small concentrations of the metalloid up to 0.5 mM, *S. cerevisiae* AS07 was able even to increase its population.

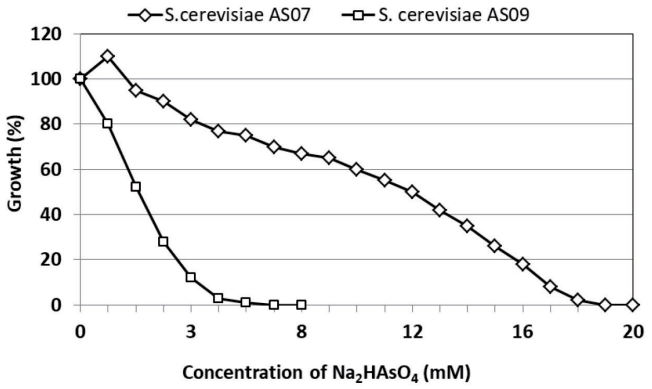

Fig. 1. Growth rate of *S. cerevisiae* AS07 and *S. cerevisiae* AS09 in presence of different concentrations of sodium arsenate expressed as a percentage of the control (growth without arsenate). Data represent the mean of three replicates, while the standard error was in the range of 5 %.

A reduction of 50% of the population after 24-h growth was observed at 11.75 mM of AsV. Moreover, the growth under such stress conditions continued until 18 mM. On the other hand, the cells of non-tolerant *S. cerevisiae* AS09 survived until 4.9 mM of AsV in the medium, while 50% growth reduction was found at 0.9 mM in the medium.

In addition, arsenate cell transport was studied in both *S. cerevisiae* strains for a period of 60 min at three concentrations of sodium arsenate in the medium – 33.3 µM, 133.2 µM, and 266.4 µM (Fig. 2).

The influx and efflux of arsenate in the cells of both strains were dependent on the concentration of the metalloid in the medium and on the net balance of the anion. Under conditions of abiotic stress produced by sodium arsenate in the medium, the internal cell concentrations also differ. The basic principle is that each microbial strain has the capability of regulating its internal ion concentration by influx and efflux operations. When the external arsenate concentration was 33.3 µM (Fig. 2 A), both cells absorbed arsenate anion at a different rate. The maximum level of internal As (0.1 µg g^{-1}) was reached by the tolerant strain after 7 min of incubation followed by efflux of As, which resulted in a 40% reduction of the initial concentration, while the non-tolerant strain continued increasing its internal values of As till the 60th minute (0.089 µg g^{-1}). Hence, for 60 min in the presence of 33.3 µM sodium arsenate the non-tolerant strain was not able to begin the efflux process.

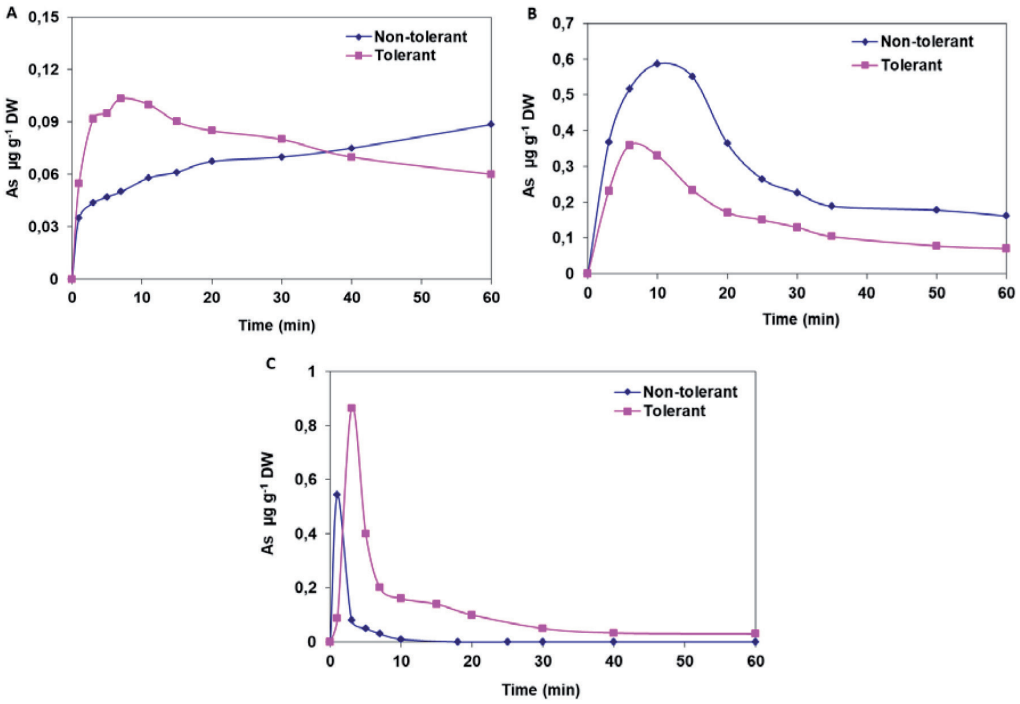

Fig. 2. Kinetic uptake of As over 60 min in tolerant and non-tolerant yeast strains, expressed per gram of dry cells, in the presence of 33.3 µM (A), 133.2 µM (B), and 266.4 µM (C) of sodium arsenate. Data represent the mean of three replicates, while the standard error was in the range of 5%.

In the case of 133.2 μM sodium arsenate, both cells reached the maximum point of internal As at the beginning of the experiment. Afterwards, the tolerant strain decreased the internal concentration by 80% by minute 60. The non-tolerant strain not only accumulated much more As (60% more), it was also able to decrease the anion concentration by 70%.

At all concentrations used at the beginning of the experiment, both strains rapidly increased their internal As concentration, reaching a maximum point, although only the tolerant strain was able to decrease it subsequently at all concentrations used. When the concentration in the medium was 266.4 μM, the non-tolerant cells died before minute 10 (evaluation of viable cells), while the tolerant cells continued extruding the anion surprisingly much more than at the lower arsenate concentration.

To demonstrate that the decrease in arsenate concentration was due to membrane transporters in the cell wall, amiloride hydrochloride, known as an inhibitor of Na^+/H^+ antiporters, was included in the experiments (Fig. 3). The result was that the efflux disappeared in all experiments and concentrations after the addition of amiloride hydrochloride. The accumulated As in the cells of the tolerant strain was found to be much lower than the As in the non-tolerant strain cells in all experiments. As a consequence, tolerant cells became sensitive. Moreover, in the case of 266.4 μM arsenate in the medium, the cells of both strains were found to be dead, because the toxic anion extrusion was interrupted.

After 60 min of incubation with 33.3 μM and 133.2 μM of sodium arsenate and amiloride hydrochloride, the cells of both strains were found to be alive, while when higher concentration was used both strain cells were dead because of higher internal concentration of anion. It seems that the low concentration did not pose a significant threat to any of the strains, as in the end the accumulated concentrations were similar and the cells were alive, while when medium concentration was used the accumulation of the non-tolerant strain was 55% more than that in the tolerant one.

In conclusion, although the maximum tolerable concentration of *S. cerevisiae* AS09 was about 72.8% lower than that of the tolerant *S. cerevisiae* AS07, 50% reduction of growth of *S. cerevisiae* AS09 represented a 92.3% decrease compared to *S. cerevisiae* AS07. On the other hand, the tolerance to arsenate is a very important quality in some habitats

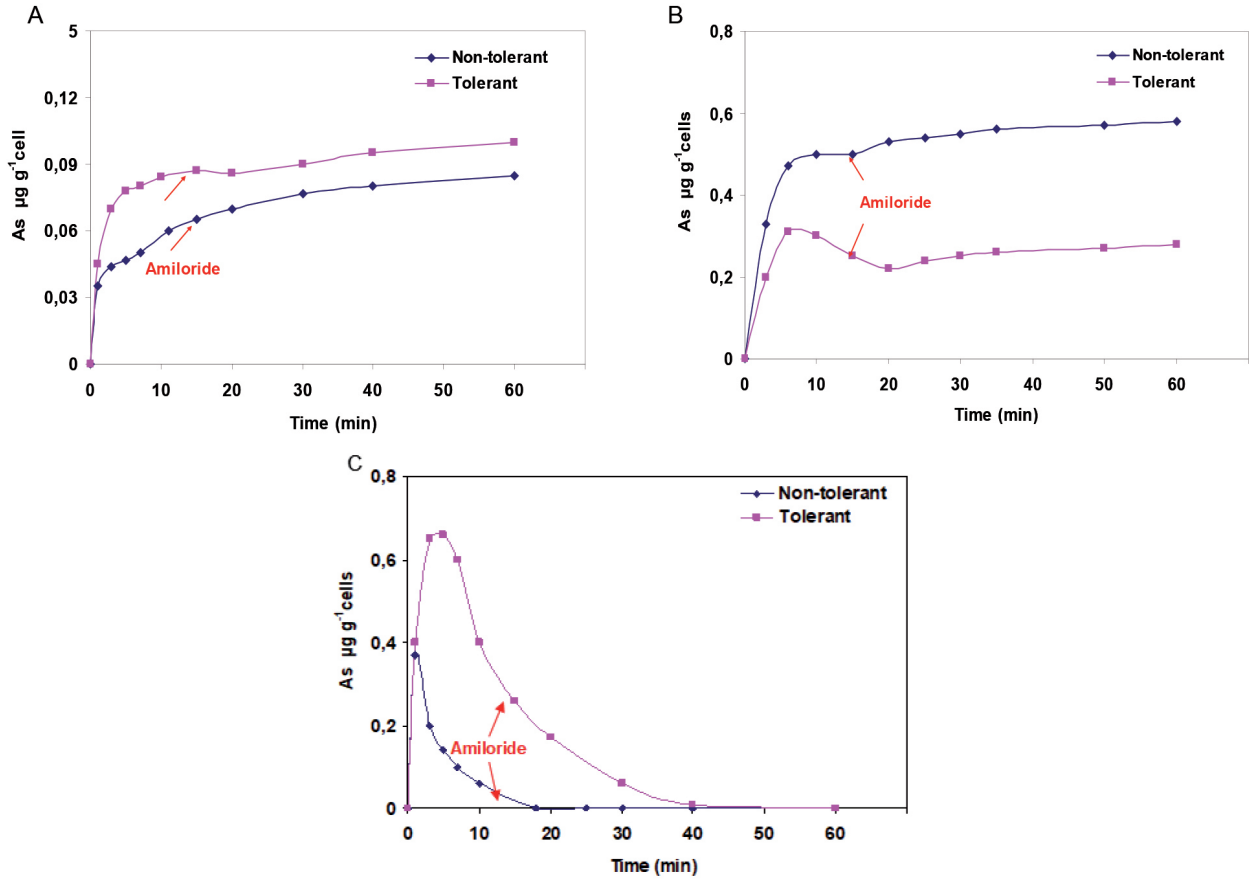

Fig. 3. Kinetic uptake of As in tolerant and non-tolerant yeast strains, expressed per gram of dry cells, in the presence of 33.3 μM (A), 133.2 μM (B) or 266.4 μM (C) sodium arsenate and 2.5 mM amiloride hydrochloride. Data represent the mean of three replicates, while the standard error was in the range of 5%

and is dependent on the possession of genes encoding production of protein transporters for arsenate efflux from the cell. In our case, the efflux of arsenate presented a typical curve for tolerant cells, but the activation times depended on the concentration in the medium. At higher arsenate concentrations, the tolerant cells response was faster – at 33.3 μM at min 10, at 133.2 μM at min 8, at 266.4 μM at min 4. The addition of an antiporter inhibitor resulted in a lack of efflux in the tolerant *S. cerevisiae* AS07 strain and it became non-tolerant, confirming the fact that tolerance to arsenate is due to an efficient efflux system in the cells.

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