The Role of Thiol Species in the Hyper-Tolerance of
*Aspergillus* sp. P37 to Arsenic

by

David Cánovas†+, Riet Vooijs‡+, Henk Schat‡, and Víctor de Lorenzo†§¶

†Centro Nacional de Biotecnología-CSIC, Campus UAM-Cantoblanco, Madrid 28049, Spain, ‡Department of Ecology and Physiology of Plants, Faculty of Earth and Life Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands, and §Centro de Astrobiología (INTA-CSIC), Torrejón de Ardoz, Madrid 28850, Spain.

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†These two authors have contributed equally to this paper

¶ To whom correspondence should be addressed: Centro Nacional de Biotecnología-CSIC, Campus UAM-Cantoblanco, Madrid 28049, Spain. Tel.: 34-91 585 45 36; Fax: 34-91 585 45 06; E-mail: vdlorenzo@cnb.uam.es

* Present address: Department of Genetics, University of Melbourne, Victoria 3010, Australia

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SUMMARY

Aspergillus sp. P37 is an arsenate-hypertolerant fungus isolated from a river in Spain with a long history of contamination with metals. This strain is able to grow in the presence of 0.2 M arsenate, i.e., 20-fold higher than the reference strain, Aspergillus nidulans TS1. Although Aspergillus sp. P37 reduces As(V) to As(III), which is slowly pumped out of the cell, the measured efflux of oxyanions is insufficient to explain the high tolerance levels of this strain. To gain an insight into this paradox, the accumulation of acid-soluble thiol species in Aspergillus sp. P37 when exposed to arsenic was compared to that of the As-sensitive A. nidulans TS1 strain. Increasing levels of arsenic in the medium did not diminish the intracellular pool of reduced glutathione (GSH) in Aspergillus sp. P37, in sharp contrast with the decline of glutathione in A. nidulans under the same conditions. Furthermore, concentrations of As which were inhibitory for the sensitive A. nidulans strain (e.g. 50 mM and above) provoked a massive formation of vacuoles filled with thiol species. Since the major fraction of the cellular arsenic was present as the glutathione-conjugate As(GS)$_3$, it is plausible that the As-hypertolerant phenotype of Aspergillus sp. P37 is in part due to an enhanced capacity to maintain a large intracellular glutathione pool under conditions of As exposure, and to sequester As(GS)$_3$ in vacuoles. HPLC analysis of cell extracts revealed that contact of Aspergillus sp. P37 (but not A. nidulans) with high As concentrations (≥150 mM) induced production of small quantities of a distinct thiol species indistinguishable from plant phytochelatin-2. Yet, we argue that phytochelatins do not explain arsenic resistance in Aspergillus and advocate the role of As(GS)$_3$ complexes in arsenic detoxification.
INTRODUCTION

Arsenic is a major environmental pollutant that typically contaminates the soil and water of areas subject to intensive mining and metallurgical activities. Some of such areas (such as the Rio Tinto district in Southern Spain) have been in operation since ancient times and can be traced to the mining of pyrites by the phoenician colonizers several centuries BC (1). Such a long-term arsenic-rich niches have selected microbial populations which thrive under concentrations of the metalloid which are altogether lethal for all other life forms. The strain Aspergillus sp. P37, isolated from Rio Tinto, exhibits a hyper-tolerant phenotype for arsenate (e.g., up to 0.2 M) that seems to approach the maximum resistance to the oxyanion found in the biological world so far. This strain exceeds by at least 20-fold the endurance to As forms exhibited by the akin species Aspergillus nidulans (2).

Since arsenate, the main chemical form of As encountered under aerobic conditions, is a phosphate analogue, it is taken up into cells through the phosphate uptake system (3). Yet, in a previous study, we have demonstrated that the As hyper-tolerant phenotype of Aspergillus sp. P37 is not related to transport (influx/efflux) of the oxyanion (4). This was evidenced by experiments showing that both arsenate and phosphate uptakes in Aspergillus sp. P37 were similar to those shown by a reference, As-sensitive A. nidulans strain. In contrast, Aspergillus sp. P37, reduced As(V) to As(III) more readily than the control A. nidulans. On this basis, it was proposed that arsenic reduction contributed decisively to the hypertolerance phenomenon (4). Still, reduction of arsenate originates arsenite, a more toxic derivative, which requires further detoxification. Consequently, there should be additional features of this strain contributing to As resistance.
Since *Aspergillus* sp. P37 does not methylate As to any significant extent (Canovas et al., unpublished) the one plausible mechanism for such a detoxification is conjugation with thiol species, as is the case in plants. In this case, reduced glutathione (GSH)\(^1\) and poly-\(\gamma\)-glutamylcysteinylglycines, called phytochelatins (PCs), bind metal ions, including arsenite, with high affinity, thereby counteracting their toxic effect. PCs have the general structure \((\gamma\text{-EC})_n\text{S}\), in which \(n\) varies between 2 and 11 (5). Their synthesis from GSH is catalyzed by phytochelatin synthase (PCS), a constitutive enzyme requiring activation by heavy metals or metalloids (6). Treatment of plants with a \(\gamma\)-glutamylcysteine synthetase inhibitor, L-buthionine sulfoximine, produces arsenic hypersensitivity, both in normal and arsenate-hypertolerant plants (7), while GSH-overproducing *Arabidopsis thaliana* present only a moderately higher As tolerance (8). Interestingly, PCs-deficient *A. thaliana* with normal GSH levels appeared to be hypersensitive to various heavy metals, including As (9), revealing that PCs contribute decisively to As tolerance. On this background, the question is whether PCs or other thiol compounds could also account for *Aspergillus* sp. P37 tolerance to As. Functional PCs are present in all plants examined, but have been found only in a small number of animals and fungi (5,10-13). The archetypical yeast, *Saccharomyces cerevisiae*, lacks functional PCs and As tolerance depends on its efflux across the plasma membrane and on vacuolar sequestration of As(GS)\(_3\) (14). On the contrary, *S. pombe* (9), does produce PCs, which determine the metallo-tolerance of this yeast to a number of metal ions.

In this work, we have investigated the role of thiol compounds in the As resistance phenotype *Aspergillus* sp. P37. As shown below, we have found similarities and differences between the mechanisms which operate in this fungus and those driving arsenic tolerance in plants. Our data shows that while intracellular pools of glutathione remain basically unaltered in *Aspergillus* sp. P37 upon exposure to arsenate during short times of exposure, their intracellular location change dramatically in the presence
of the oxyanion. Furthermore, our results suggest that As-glutathione complexes are accumulated into vacuoles, thereby contributing to the high tolerance *in vivo* to the heavy ion.

**EXPERIMENTAL PROCEDURES**

*Fungal strains and media—* As-hypertolerant strain *Aspergillus* sp. P37 was isolated from the River Tinto in Southwestern Spain and previously described in detail (2). The reference strain *Aspergillus nidulans* TS1 (*bia1 methG1 argB2*) was kindly provided by T. Suárez (15,16). Isogenic *gstA*+ *A. nidulans* MH3408 (*bia1 niiA4 amds-lacZ*) and *gstA* *A. nidulans* MH9986 (*amdS-lacZ gstA::riboB2*) strains were obtained from Michael Hynes (17). The sporulation conditions for *A. nidulans* (18) were applied to produce conidiospores in *Aspergillus* sp. P37. Fungal strains were regularly cultured in complete YPD medium (19) to which glucose (1%) was added as a carbon source.

*Arsenic resistance assays—* Arsenic resistance was estimated from plates containing a gradient concentration of arsenate or arsenite raging 0-15 mM. The gradient of arsenic concentration was made as follows: a bottom layer of medium was poured in a Petri dish on a surface sloping by 5º. After hardening of the medium, the plate was transferred from the slant to flat plane. The upper layer of medium containing the desired concentration of the arsenic species to be assayed was then poured onto the plate, and the medium was allowed to solidify. Eight drops of 2 l of a suspension of fungal conidiospores (~10⁸ spores/ml) were placed along the gradient and allowed to dry. Plates were incubated for 3 days at 37 ºC to allow fungal growth. Images on plates were recorded by using a Chemidoc device (Bio-Rad, Cupertino CA).
Identification of acid-soluble thiols by HPLC— Fungi were grown for 5, 12 or 24 hr in YPD medium containing increasing concentrations of arsenate at 37 ºC. Biomass was filtered and washed with distilled water. Extraction of acid soluble compounds was carried out as previously described (20). Briefly, about 5-15 mg of freeze-dried fungal material was ground in a mortar in 0.1% trifluoroacetic acid with 6 mM diethylenetriaminepenta-acetic acid at pH ≤ 1. N-acetyl-Cysteine (NAC) was added during grinding as an internal standard. Samples were taken from a minimum of 2 separate experiments. The specimens under analysis were filter-centrifuged at 10,000 x g for 15 min. Freshly extracted samples were immediately derivatied according to Sneller et al. (21). To this end, 250 l of each specimen was treated with monobromobimane (mBrB; Molecular Probes, Inc., USA.) in 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid and diethylenetriaminepentaacetic acid buffer for 30 min at 45 ºC in the dark. 300 l of methanosulphonic acid was then added to stop the reaction. Derivatized samples were stored in the dark at 4 ºC until analysis. At that point, derivatized thiols were separated in a Nova Pack C18 column (Waters, Milford, MA) and eluted with a slightly concave methanol gradient of 12% to 25% (v/v) for 15 min and then a linear methanol gradient from 25% to 50% (v/v) for 15-50 min (20). Fluorescent molecular species were detected using a Waters 474 fluorescence detector. Compounds were identified and quantified by comparison with standards and corrected for different reactivities to mBrB as described (21).

Determination of arsenite-glutathione complexes— While arsenite does not form stable complexes with glutathione at neutral pH, As(GS)3 species are stable at pH 3. Thus, for the determination of arsenite-glutathioine complexes, samples of the fungal strains under scrutiny, grown in the presence or absence of arsenate, were extracted in trifluoroacetic acid at pH 3.0, separated by HPLC and detected by the postcolumn derivatization method with 5,5'-dithiobis(2-nitrobenzoic acid) described previously (21).
The As(GS)$_3$ standard for this procedure was made by chemical reaction of GSH with sodium arsenite at pH 3 (21).

**Microscopy** — Cells were pictured without fixation by phase-contrast microscopy using a Sensys charge-coupled camera (Photometrics) and a Zeiss Axiolab HBO 50 microscope. For the visualization of thiol groups in the vacuoles, *Aspergillus* sp. P37 cells were induced with or without 50 mM arsenate for 3 hr. After this period, the thiol-specific fluorescent reagent monobromobimane (mBrB, see above) was added at a final concentration of 100 M, still in the presence of arsenate. Incubation continued in the dark for one more hour, after which samples were washed with fresh medium and visualized with epifluorescence microscopy.

**Measurement of arsenic in fungal samples**— Total arsenic present in fungal biomass was determined in acid-soluble extracts prepared as explained above. The oxyanion was quantified on a flame atomic spectrophotometer (Perkin Elmer 2100, Perkin Elmer Nederland, Nieuwerkerk a/d/Jssel, The Netherlands) with the method of hydride generation as previously described (22).

**RESULTS**

**Exposure to arsenate triggers a massive accumulation of thiolic compounds in the vacuoles of Aspergillus sp. P37** — This work was prompted by our early observations of morphological changes in cells of *Aspergillus* sp. P37 exposed to growing concentrations of arsenate (2). A simple perusal of cells grown in the presence of 50 mM As(V) under a phase contrast microscope revealed the formation of large vacuoles to the point of bulging out the hyphal filaments and causing a deformation in cell shape (2). Yet, this observation could not be interpreted rigorously *per se*, as a negative control.
was not feasible: the As-sensitive *A. nidulans* reference strain could not grow at such high concentrations of the oxyanion. Furthermore, many generic stress conditions trigger formation of vacuoles in *Aspergillus* species (23,24). It was therefore unclear whether the buildup of vacuoles in As-exposed *Aspergillus* sp. P37 had any connection with the mechanism(s) which account for the hyper-tolerant phenotype or it was part of a non specific response to stress. To address this, we considered a number of alternatives including that, similarly to plants, As could end up in vacuoles following conjugation with thiol compounds (see Discussion below). In order to either regard or discard this possibility we run a simple test in which *Aspergillus* sp. P37 cells incubated in 50 mM arsenate were treated with the fluorescent thiol-specific reagent mBrB. This compound permeates freely cell membranes and reacts with -SH groups producing a strong fluorescence. Fig. 1 shows that the bulk of mBrB-reactive material accumulates in the large vacuoles of As-treated *Aspergillus* sp. P37. This outcome indicated that vacuoles served as sites of storage for thiol-containing compounds. In the absence of arsenate in the culture medium, cells did not show any significant vacuolation nor did they emit bright fluorescence in the presence of mBrB (Fig. 1). This could be due to a lower content of thiol species o and/or a more even distribution of -SH compound through the different cell compartments. The two outstanding questions originated by these results deal with the nature of the thiol compounds detected and whether or not they are conjugated with arsenic. The following sections address these aspects.

**Identification and quantification of acid-soluble thiol species in Aspergillus sp. P37**— In order to have a profile of the different solutes containing thiol groups that form or accumulate upon exposure of cells to arsenic, fungi (either *Aspergillus* sp. P37 or control strain *A. nidulans* TS1) were grown at different times in media containing increasing concentrations of arsenate. Acid-soluble compounds were then extracted from the biomass, derivatized with mBrB and submitted to HLPC analysis as explained.
in Experimental Procedures. The use of standards allowed the unequivocal identification of most of the thiol compounds that reacted with mBrB. Fig. 2 shows one of such profiles, from *Aspergillus* sp. P37 treated at the highest concentration of As, 200 mM, and for the longest time, 24 h (i.e., conditions favoring formation of thiol-containing vacuoles). The major peak in all cases (accounting up to 95% of the whole of soluble -SH compounds) corresponded to GSH. This was accompanied by a number of other thiol-containing compounds (including cysteine and \(\gamma\)-glutamylcysteine; Fig. 2), which appeared in the chromatogram as minor components of the free thiol pool. It is thus reasonable to presume that the bulk of free thiol species accumulated in vacuoles consist of GSH.

Fig. 3A shows the evolution of GSH buildup in *Aspergillus* sp. P37 or *A. nidulans* TS1 along time under growing concentrations of arsenate. It is noteworthy that accumulation of glutathione in *Aspergillus* sp. P37 did not change significantly when the cultures were induced with moderate concentrations of the oxyanion for relatively short periods of time (up to 5 hours). Only after 12 h of incubation and superior As levels in the medium, *Aspergillus* sp. P37 cells accumulated significantly higher amounts of glutathione, a peak of intracellular GSH pool being reached after 24 hours of incubation with the highest As concentration (Fig. 3A). In contrast to *Aspergillus* sp. P37, *A. nidulans* TS1 accumulated the highest concentration of glutathione at a concentration of arsenate as low as 1 mM. This concentration was, in any case, only slightly higher (<17%) than the GSH levels of uninduced cells of *A. nidulans* TS1. Further increases in the level of the metalloid in the medium resulted in a reduction of the glutathione pool of *A. nidulans* TS1, surely due to the toxic effect exerted by arsenic on sensitive cells (Fig. 3A). Yet, the most informative outcome of the results shown in Fig. 3A is that GSH pools of *Aspergillus* sp. P37 treated with 50 mM As for 5 hours (a concentration which sufficed for a considerable vacuolation of thiol-containing compounds as detected with
epifluorescence microscopy; Fig. 1), were not that different (≤ 10%) from levels measured in non-treated control cells. This result suggested that, within a certain As concentration range and exposure time, the effect of the oxyanion in the hypertolerant fungus is not so much to enhance GSH levels as to provoke a reallocation of the pre-existing cellular pool into the new vacuoles.

A role for phytochelatin-2 in the detoxification of heavy metals in Aspergillus strains?—The type of analysis shown in Fig. 2 revealed also that a minor peak (labelled initially as Y) was recurrently present in samples of Aspergillus sp. P37 grown in the highest concentrations of arsenic for the longest period of time (Fig. 3B). Purification of the compound Y by HPLC and further analysis by MALDI-TOFF-MS showed 3 major peaks (698, 714 and 730). One of them corresponded to the molecular weight of plant phytochelatin-2 (PC2, Fig. 2) conjugated to one molecule of mBrB (730; data not shown), suggesting that the peak Y of Fig. 2 did correspond to PC2. The minor contribution of this peak to the pool of free thiols argues against any relevant role in tolerance to arsenic. Yet, its presence was specific of elevated concentrations of arsenate, as it was not detected in cultures with less than 100 mM of the oxyanion (Fig. 3B). Cultures amended with subinhibitory concentrations of other heavy ions such as Cd²⁺ (0.1 mM), As³⁺ (25 mM), Cr³⁺ (50 mM) or Cu²⁺ (50 mM) also failed to induce the peak at the PC2 position (data not shown). We could not detect this peak either in A. nidulans TS1 grown in the presence of arsenate, arsenite, copper, cadmium or chromium. Furthermore, the presence of phytochelatin-like molecules is rare in As-resistant microorganisms (9). Therefore, unlike plants, where PCs are major players in the response to the oxyanion and other heavy metals (20,22,25), the low level of PC2 accumulation in Aspergillus sp. P37 seems to be a marginal phenomenon rather than a constituent of the As detoxification system.
Formation of arsenic-glutathione complexes in As-stressed Aspergillus sp. P37 cells—

In view of the data above, the next logical question was whether the glutathione present at different levels and compartments in Aspergillus sp. P37 and A. nidulans TS1 was bound or not to arsenic when cells grow in the presence of the oxyanion. To address this issue we first tackled whether both As(III) and As(V) could form stable conjugates with reduced GSH under our analytical conditions. To this end, since arsenite does not make stable complexes with glutathione at neutral pH, the GSH solution was mixed with sodium arsenite at pH 3. An HPLC protocol was then employed to separate and quantify free GSH from its complex with arsenite, As(GS)₃. By exploiting a post-column derivatization procedure with 5,5'-dithiobis 2-nitrobenzoic acid (21), the two chemical species could be separated with HPLC with clear cut retention times (Fig. 4A). When As(V) was employed instead of As(III), we could not detect any significant conjugation of the oxyanion with GSH under the test conditions, as the very minor peak which appears at the As(GS)₃ retention time is likely to be a side reaction of some of the As (III) produced upon chemical reduction of As(V) with the GSH of the reaction mixture (not shown).

With this reference in hand, we set out to determine the presence of arsenite-glutathione complexes in Aspergillus sp. P37 strains under the conditions of vacuolation shown in Fig. 1. For this, the fungi were grown in the presence or absence of 50 mM for 24 hours, its biomass collected and extracted in trifluoroacetic acid, the soluble fraction separated by HPLC and detected by the postcolumn derivatization method mentioned before. Fig. 4 shows that while the non-treated sample produced only the peak of As-free GSH, the specimen exposed to arsenate yield two major chemical species. As displayed in Fig. 4C, one matched GSH, while the other corresponded to As(GS)₃. By judging the cognate peak areas, it would appear that the share of glutathion complexed with arsenite accounted for up to 35-40% of the whole GSH pool. Since the only form of
arsenic which reacts non-enzymatically with GSH is As (III), the results of Fig. 4 also indicate that reduction of the oxyanion is a prerequisite for its conjugation with glutathione.

To have a reference for interpretation of the data above, *A. nidulans* TS1 was subject to the same procedure for detecting formation of As(GS)₃. Yet, since this strain starts being sensitive to As at concentrations higher than 5 mM (2), we employed this maximum level of the oxyanion in our determinations. *A. nidulans* TS1 was thus cultured in 5 mM arsenate for 24 hours, subjected to an acid extraction and submitted to the same HPLC and post-column derivatization analysis as before. The share of As(GS)₃ found in the intracellular pool of glutathione was in the range of 60-65 %.

An involvement of glutathione-S-transferase(s) in the response to arsenic? — The data above (Fig. 4A) reveals that GSH and arsenite (but not arsenate) can react non-enzymatically to yield As(GS)₃. This compound is found in considerable levels both in As-resistant and As-sensitive *Aspergillus* strains when exposed to high concentrations of the heavy ion. The manifest question is whether formation of such As(GS)₃ in vivo occurs also non-enzymatically or it is the result of an specific biocatalytic step. The ubiquitous enzyme glutathione-S-transferase (GST) has been conserved through the evolutionary scale, from bacteria to mammals, as a major instrument for responding to chemical and environmental stressors. This is because this broad substrate enzyme facilitates the binding of the free thiols of glutathione to a variety of substrates, which are thus inactivated and, in some cases, channeled towards specific detoxification mechanisms (26). In mammals exposed to arsenic GSTs have been involved in the formation of the As(GS)₃ complex (27). Thus the question arises on whether the same is true in all cases. In *A. nidulans*, the gene *gstA*, encoding a GST, is involved in resistance to heavy metals (including selenite) and xenobiotics (17). We thus set out to
examine whether Gsta also mediated formation of As(GS)$_3$ in *Aspergillus*. The
experiment had to be limited to the one *A. nidulans* strain where a well characterized
gstA mutant is available along with its gsta$^+$ counterpart (17), although we argue that,
within limits, the setup has a common value to other *Aspergillus*. Fig. 5 shows the
experiments made in this respect. In one case, spots of the gsta$^+$ *A. nidulans* strain
MH3408 were plated along 0-15 mM gradients of either arsenite or arsenate in parallel
to the isogenic gsta mutant strain *A. nidulans* MH9986. The results unequivocally
showed (Fig. 5) that the gsta mutant was more tolerant than the wild type to As (V)
and slightly more permissive to arsenite. To check whether the lack of Gsta had an
effect on the intracellular contents of free vs. As-bound glutathione in either strain, we
measured these two chemical species in cells of the gsta$^+$/ gsta strains grown in the
presence or absence 5 mM arsenate. Fig. 5 shows that cells lacking Gsta had an even
higher share of As-bound glutathione (~83 %) as compared to the wild type cells.
Although we cannot rule out the existence of other proteins with GST activity specialized
in linking enzymatically GSH to arsenite, a plausible explanation for the superior
tolerance to As and the presence of large amounts of As(GS)$_3$ in the gsta strain is that
much of the glutathione-As conjugate may form non-enzymatically. The lack of Gsta
could thus release a fraction of the GSH otherwise committed to various enzymatic
reactions into a form available for binding arsenite chemically (see Discussion).

*Arsenic accumulation in cells* — As the role of thiol compounds in the response of
*Aspergillus* sp. P37 and *A. nidulans* TS1 to As becomes exposed by the results above,
the question to tackle at this point is whether or not hyper-resistant cells accumulate As
concomitantly to vacuolation and formation of glutathione-As conjugates. This could be
inferred from the data on the buildup of As(GS)$_3$ under various conditions spelled out
before, but needed to be quantified prior to assign As accumulation a definite share in
the resistance phenomenon. The results of Fig. 6 show the absolute levels of arsenic
found in the acid soluble extracts recovered from the biomass of *Aspergillus* sp. P37 and *A. nidulans* TS1 grown under various concentrations and exposure times to the oxyanion. Similarly to the situation of intracellular glutathione levels (Fig. 3A), we detected only minor differences in the As-accumulation capacity of the two *Aspergillus* strains at concentrations of arsenate ≤ 25 mM (i.e., below growth limit for *A. nidulans* TS1; Fig. 6A). However, increasing arsenic levels in the medium beyond 50 mM produced a notable increase in As accumulation in *Aspergillus* sp. P37 (Fig. 6B), which was concomitant with the induction of high vacuolation within the hypertolerant cells (Fig. 1; (2)). Since maximum contents of As in *Aspergillus* sp. P37 biomass occurred at the highest concentrations of the oxyanion in the medium, we concluded that accumulation of the heavy ion in a non-bioactive form contributes to the high-tolerance phenotype which is characteristic of this strain. As discussed below, this result, together with those described in previous paragraphs, allows a more complete picture of how *Aspergillus* sp. P37 endures As concentrations way above the average inhibitory levels of reference microorganisms.

**DISCUSSION**

Different biological systems have evolved diverse strategies of enduring the toxic effects of arsenic. That many (if not most) microorganisms bear at least one arsenate reductase and a membrane-bound pump complex for extrusion of arsenite back to the external medium is perhaps an indication of the early evolutionary success of such a mechanism during the rise of an oxydizing atmosphere. With little variations, such an arsenate reductase / extrusion pump theme seems to account for many As resistance systems found in the prokaryotic word (28). *Aspergillus* sp. P37 does display an enhanced reduction of arsenate and a subsequent efflux of arsenite to the external medium (4). Yet, the extent of such a reduction/export is insufficient for explaining the As
hypertolerant phenotype at concentrations of the oxyanion $\geq 200$ mM. Furthermore, the patterns of steady-state accumulation of arsenic and the arsenate uptake kinetics of *Aspergillus* sp. P37 was not that different from the As-sensitive reference strain *A. nidulans* TS1 (4). Eukaryotes and multicellular organisms do add more mechanisms of As tolerance instead of or in addition to reduction/export. Arsenic can be methylated by microorganisms and mammalian cells, converted into arsenocholine or arsenobetaine, or associated to sugars in seaweeds, all these possibilities contributing to the repertoire of biological responses to the oxyanion (29). Fungi occupy a distinct evolutionary domain in which we could expect a blend of mechanisms for resistance to As related to those found in prokaryotic and multicellular strategies. And such appears to be the case in the strain that is the subject of this study. From our observations it followed that other mechanisms should operate in the system and that intracellular sequestration mechanisms could be the likely means to account for hypertolerance phenotype of *Aspergillus* sp. P37.

*S. cerevisiae* tolerates a certain level of As exposure by sequestration of As(GS)$_3$ in vacuoles mediated by the Ycf1 transporter (14). Ycf1p is a homologue of MRP, which has been associated with arsenic resistance in human cells, (30). GSH depletion was also found to make mammalian cells very sensitive to arsenic (31). The results reported in this work reveal the key role of soluble thiol species, in particular GSH, in orchestrating the response of *Aspergillus* sp. P37 to either low or high concentrations of arsenic. In both sensitive (*A. nidulans* TS1) and resistant (*Aspergillus* sp. P37) strains, GSH was the major compound in the intracellular thiol pool. We clearly observed different patterns of GSH accumulation in response to arsenate stress. When the concentration of arsenate exceeded 5 mM, the intracellular pool of GSH was greatly reduced in *A. nidulans*. However, *Aspergillus* sp. P37 showed the opposite behaviour: during short periods of incubation (5 hours), arsenate did not affect the GSH pool.
However, 24 h of incubation with arsenate triggered an increase in the accumulation of
GSH. Such an accumulation could contribute to arsenate detoxification in different ways.
First, GSH can serve as the electron donor in enzyme-catalysed arsenate reduction in
bacteria, plants, fungi and animals, and arsenate reduction is a prerequisite for arsenic
eflux from the cells (32). Second, GSH binds arsenite to form As(GS)₃, which allows
sequestration in vacuoles, mediated by ABC-type glutathione conjugate transporters
(32). *Arabidopsis thaliana* overexpressing the *E. coli* γ-glutamylcysteine synthetase (the
limiting step in the formation of GSH and PCs in plants) were only moderately resistant
to As compared to the wild type. On the other hand, plants overexpressing the *E. coli*
arsenate reductase -ArsC- were hypersensitive to arsenate. However, combined
overexpression of γ-glutamylcysteine and ArsC produced a dramatic increase in
arsenate tolerance and arsenic accumulation (8). These results indicate that enhanced
arsenate reductase capacity is a prerequisite for thiol-based hypertolerance to As. Such
a capacity is indeed present in *Aspergillus* sp. P37 (4), as suggested by the much higher
arsenite efflux following arsenate exposure, as compared to *A. nidulans* TS1.
Furthermore, *Aspergillus* P37 maintained higher GSH levels under arsenic exposure
compared to *A. nidulans* TS1, and was even able to further increase the GSH level
under very high As conditions (Fig. 3A), which might explain its superior intracellular
detoxification capacity.

Both *A. nidulans* and *Aspergillus* sp. P37 accumulated considerable amounts of arsenic
as As(GS)₃, (Fig. 4), suggesting a pivotal role for GSH in intracellular arsenic
sequestration. Although we cannot rule out that the As(GS)₃ complexes found in cells
(see Fig 4c) may result from the chemical reaction between arsenite and GSH during
the extraction procedure, we argue that these complexes are indeed generated in vivo.
This is because GSH is the major thiol present in cells even in the absence of As and,
under our experimental conditions, arsenate did not react with GSH. In addition, there
were considerable amounts of intracellular arsenite, which has high avidity for thiol
groups. Thus, we hypothesize that upon reduction of arsenate to arsenite, the latter is
quickly bound by GSH. Only after exposure to As, the thiol pool is relocated into the
vacuoles, probably as As(GS)$_3$. This phenomenon is not devoid of precedents, as
As(III)-tris-thiolate conjugates have been found in vivo in cells of Indian mustard by X-
ray absorption spectroscopy -although the complexes could not be assigned to As-
glutathione or As-phytochelatin (33). It is likely that formation of the As(GS)$_3$ conjugate
per se is not sufficient for detoxification. The localization of the major share of thiols
within the vacuoles of *Aspergillus* sp. P37 under high arsenate exposure implies that
vacuolar sequestration of As(GS)$_3$ represents an essential component of the arsenate
hypertolerance mechanism in this strain. The fungal vacuole is an acidic compartment
(23). Since arsenite-GSH complexes are unstable at neutral pH but not under acidic
conditions, we it is plausible that the acidic conditions present in the vacuole can serve
as an arsenic trap by stabilizing As(GS)$_3$.

A side aspect of this work reveals that the heavy metal stress-responsive GST enzyme
GstA is not necessary for the conjugation of arsenite with glutathione in *A. nidulans*. The
deletion of this gene resulted in a phenotype of sensitivity to heavy metals and Se (17).
On the contrary, the loss of this gene resulted in an increased resistance to arsenic (Fig.
5) as well as to the systemic fungicide carboxin (17). It is possible that overexpression of
gstA as part of a stress-related response contributes to the decrease in the intracellular
pool of free glutathione available for reaction with As. Mutation of the *gstA* gene would
thus release the GstA-engaged pool of GSH and increase the amount of GSH for
arsenic detoxification. These results show that GstA does not mediate arsenic
resistance in *A. nidulans*, but they do not rule out a role for other GST proteins existing
in *Aspergillus*. These results also reveal a difference in the detoxification pathways of
arsenic compared to other heavy metals in *Aspergillus*. 
In general, plants use PCs to counteract the toxic effect of heavy metals (25). Metal-phytochelatin complexes are transported into the vacuoles of plant cells by an MRP homologue (34). Although, phytochelatins (PCs) are potent As detoxifiers in plants and even in some yeast such as S. pombe (9, 35), Aspergillus sp. P37 only accumulated a low amounts of an alleged PC2 at very high levels of arsenate exposure. Furthermore, other metals, among which cadmium is one of the most potent inducers of PC synthesis in plants (5), failed to induce the PC2 peak in both Aspergillus strains. Therefore, it is unlikely that this PC-like compound could play a role in the detoxification of arsenic and heavy metals in Aspergillus. Phytochelatins, unlike GSH, contain vicinal thiols, which display high affinity for arsenite and can form stable complexes. Given the lack of a definite role of phytochelatins in heavy metal resistance in Aspergillus, we believe that compartmentalization of As(GS)$_3$ in the vacuoles (where the acidic pH stabilizes these complexes), can supplant the absence of vicinal thiols in GSH. Yet, it is intriguing that small quantities of PC2 appears only with arsenic at high concentrations in Aspergillus sp. P37.

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FOOTNOTES

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1 The abbreviations used are: GSH, glutathione; GST, glutathione-S-transferase; MRP, multidrug resistance-associated protein; mBrB, monobromo bimane; PC, phyochelatin; PCS; phytochelatin synthase.

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FIGURE LEGENDS

FIG. 1. **Thiols are accumulated in the vacuoles in response to arsenic.** *Aspergillus* sp. P37 was grown without (A and B) or with 50 mM (C and D) sodium arsenate for 3 hr, and then stained with mBrB for an additional hour. Cells were visualized by phase-contrast (A and C) or fluorescence (B and D) microscopy. Note that the bulk of fluorescence corresponding to mBrB bound to thiols was localized in the vacuoles.

FIG. 2. **Fractionation of the intracellular thiol content of fungal samples.** Compounds containing thiol groups were extracted from fungal biomass as explained in Experimental Procedures and identified and quantified by HPLC. The sample shown corresponds to *Aspergillus* sp. P37 grown for 24 h at ≥ 100 mM As (V). The identified peaks correspond to glutathione (GSH), cysteine (CYS); γ-glutamynilcysteine (γGC); N-acetylcysteine (NAC), used as internal standard; S, solvent; Y, thiolic compound corresponding to phytochelatin 2 (PC2). See text for explanation.

FIG. 3. **Accumulation of thiol compounds in Aspergillus sp. P37 and A. nidulans TS1.** (A) Buildup of glutathione in As(V)-exposed cells. The plot shows the GSH contents of *Aspergillus* sp. P37 and *A. nidulans* TS1 under the concentrations and time of exposure indicated in each case. Note the increase of GSH *Aspergillus* sp. P37 under the most extreme conditions and the rapid depletion of the same compound in the sensitive *A. nidulans* TS1. (B) Quantification of the intracellular phytochelatine-like compound PC2. The peak labeled Y in Fig. 2 is an alleged phytochelatin-2 which, as shown in the figure, was only detected in cells of *Aspergillus* sp. P37 grown over 100 mM sodium arsenate for increased periods of time. Results shown are the average of 2-4 repetitions, except for *Aspergillus* sp. P37 grown at 5 mM for 24 h, which was only analysed once. Bars indicate SD.
FIG. 4. **Formation of As(GS)_3 complexes.** (A) Generation and analysis of standards. As (III) was conjugated chemically with GSH and the resulting As(GS)_3 was assigned a distinct HPLC peak (RT ~ 15.5 min) identified with the post-column derivatization method explained in Experimental Procedures. With the same fractionation procedure, GSH produced a peak with an RT ~ 6.0 min. (B) *Aspergillus* sp. P37 grown in the absence of arsenate lacks of As(GS)_3. The fungus was grown in standard conditions for 24 h without the oxyanion. No glutathione conjugate other than GSH itself was detected in the biomass. (C) *Aspergillus* sp. P37 grown at 50 mM As(V). Under conditions of exposure to sodium arsenate for 24, the peak corresponding to As(GS)_3 became readily detectable.

FIG. 5. **Role of *A. nidulans* GstA in tolerance to arsenic.** Isogenic *A. nidulans* MH3408 (gstA^+^) and *A. nidulans* MH9986 (gstA^-^) strains were assayed for arsenic resistance in YPD plates containing a gradient concentration of arsenite (1-15 mM) or arsenate (1-15 mM) on the plates as indicated. Note the higher tolerance of the GST-minus strain. The results of the lower table show the fraction of As-bound GSH found in the biomass of the same gstA^+^ and gstA^-^ strains grown in liquid YPD medium with or without 5 mM arsenate. Arsenite-glutathione complexes were detected as specified in the Experimental Procedures section.

FIG. 6. **Accumulation of arsenic in *Aspergillus* sp. P37 and *A. nidulans* TS1.** The plots show the As contents of *Aspergillus* sp. P37 and *A. nidulans* TS1 under the concentrations and time of exposure indicated in each case. The higher range of As concentrations (50-150 mM) could be tested only in *Aspergillus* sp. P37, while *A. nidulans* TS1 was inspect only through the lower 0-25 mM As(V) range. Total arsenic accumulated in the cells was measured with the hydride generation flame atomic
spectrophotometer method explained in Experimental Procedures. Results shown are the average of 2-4 repetitions, except for *Aspergillus* sp. P37 grown at 5 mM for 24 h, which was only analysed once. Bars indicate SD. Note the very different scales of the lower vs. higher ranges of As concentrations.
Fig. 1

Phase contrast epifluorescence

No As (V)

50 mM As (V)

Fig. 2

Retention time (min) vs. absorbance
As (III) As (V)

|                | As in the medium | % As-bound GSH |
|----------------|------------------|----------------|
| **gstA+**      | 0 mM As (V)      | below detection|
|                | 5 mM As (V)      | > 65 %         |
| **gstAΔ**      | 0 mM As (V)      | below detection|
|                | 5 mM As (V)      | > 83 %         |

Fig. 5

Fig. 6

As accumulation (low range)

As accumulation (high range)
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