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The Role of Thiol Species in the Hypertolerance of *Aspergillus* sp. P37 to Arsenic*

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***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 with that of the arsenic-sensitive *A. nidulans* TS1 strain. Increasing levels of arsenic in the medium did not diminish the intracellular pool of reduced glutathione in *Aspergillus* sp. P37, in sharp contrast with the decline of glutathione in *A. nidulans* under the same conditions. Furthermore, concentrations of arsenic that were inhibitory for the sensitive *A. nidulans* strain (*e.g.* 50 mM and above) provoked a massive formation of vacuoles filled with thiol species. Because the major fraction of the cellular arsenic was present as the glutathione conjugate $As(GS)_3$, it is plausible that the arsenic-hypertolerant phenotype of *Aspergillus* sp. P37 is in part due to an enhanced capacity to maintain a large intracellular glutathione pool under conditions of arsenic exposure and to sequester $As(GS)_3$ in vacuoles. High pressure liquid chromatography analysis of cell extracts revealed that the contact of *Aspergillus* sp. P37 (but not *A. nidulans*) with high arsenic concentrations (≥150 mM) induced the 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 we advocate the role of As(GS)3 complexes in arsenic detoxification.**

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 B. C. (1). Such long-term arsenic-rich niches have select microbial populations that thrive among concentrations of the metalloid that are altogether lethal for all other life forms. The strain *Aspergillus* sp. P37, isolated from Rio Tinto, exhibits a hypertolerant 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 to date. This strain exceeds by at least 20-fold the endurance to arsenic forms exhibited by the akin species *Aspergillus nidulans* (2).

Because arsenate, the main chemical form of arsenic 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 demonstrated that the arsenic-hypertolerant phenotype of *Aspergillus* sp. P37 is not related to the transport (influx/efflux) of the oxyanion (4). This finding was evidenced by experiments showing that both arsenate and phosphate uptakes in *Aspergillus* sp. P37 were similar to those shown by a reference, the arsenic-sensitive *A. nidulans* strain. In contrast, *Aspergillus* sp. P37 reduced As(V) to As(III) more readily than did the control *A. nidulans*. On this basis, it was proposed that arsenic reduction contributed decisively to the hypertolerance phenomenon (4). Still, the reduction of arsenate originates arsenite, a more toxic derivative that requires further detoxification. Consequently, there should be additional features of this strain contributing to arsenic resistance.

Because *Aspergillus* sp. P37 does not methylate arsenic to any significant extent,[1] the one plausible mechanism for such a detoxification is conjugation with a thiol species, as is the case in plants. In this case, reduced glutathione and poly-γ-glutamylcysteinylglycines, called phytochelatins (PCs),[2] bind metal ions, including arsenite, with high affinity, thereby counteracting the toxic effect of these ions. PCs have the general structure $(\gamma\text{-EC})_n S$, in which n varies between 2 and 11 (5). The synthesis of PCs from GSH is catalyzed by phytochelatin synthase, a constitutive enzyme requiring activation by heavy metals or metalloids (6). The treatment of plants with a γ-glutamylcysteine synthetase inhibitor, L-buthionine sulfoximine, produces arsenic hypersensitivity both in normal and arsenate-hypertolerant plants (7), whereas the GSH-overproducing *Arabidopsis thaliana* presents only a moderately higher arsenic tolerance (8). Interestingly, PC-deficient *A. thaliana* with normal GSH levels appeared to be hypersensitive to various heavy

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[1] D. Cánovas and V. de Lorenzo, unpublished observations.
[2] The abbreviations used are: PC, phytochelatin; GST, glutathione *S*-transferase; HPLC, high pressure liquid chromatography; mBrB, monobromobimane.

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metals, including arsenic (9), revealing that PCs contribute decisively to arsenic tolerance. From this background, the question is whether PCs or other thiol compounds could also account for the tolerance of *Aspergillus* sp. P37 to arsenic. 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 its arsenic tolerance depends on the efflux of arsenic across the plasma membrane and on the vacuolar sequestration of $As(GS)_3$ (14). On the contrary, *Schizosaccharomyces 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 arsenic resistance phenotype *Aspergillus* sp. P37. As shown below, we have found similarities and differences between the mechanisms that operate in this fungus and those driving arsenic tolerance in plants. Our data show that although intracellular pools of glutathione remain basically unaltered in *Aspergillus* sp. P37 upon exposure to arsenate for short periods, their intracellular location changes dramatically in the presence of the oxyanion. Furthermore, our results suggest that arsenic-glutathione complexes are accumulated into vacuoles, thereby contributing to the high tolerance *in vivo* to the heavy ion.

EXPERIMENTAL PROCEDURES

Fungal Strains and Media—The arsenic-hypertolerant strain *Aspergillus* sp. P37 was isolated from the Rio Tinto in southwestern Spain and has been described in detail previously (2). The reference strain *A. nidulans* TS1 (*biA1 methG1 argB2*) was kindly provided by T. Suárez (15, 16). Isogenic *gstA*$^+$ *A. nidulans* MH3408 (*bia1 niiA4 amds-lacZ*) and Δ*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 (1% yeast extract, 2% peptone, and 2% glucose) 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 ranging 0–15 mM. The gradient of arsenic concentration was made as follow. First, a bottom layer of medium was poured in a Petri dish on a surface sloping 5°. After hardening of the medium, the plate was transferred from the slant to a 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 2-μl drops of a suspension of fungal conidiospores (~10^8 spores/ml) were placed along the gradient and allowed to dry. Plates were incubated for 3 days at 37 °C to allow fungal growth. Images on plates were recorded by using a Chemidoc device (Bio-Rad).

Identification of Acid-soluble Thiols by HPLC—Fungi were grown for 5, 12, or 24 h in YPD medium containing increasing concentrations of arsenate at 37 °C. The biomass was filtered and washed with distilled water. Extraction of acid soluble compounds was carried out as described previously (20). Briefly, ~5–15 mg of freeze-dried fungal material was ground in a mortar in 0.1% trifluoroacetic acid with 6 mM diethylenetriaminepentaacetic acid at pH ≤ 1. *N*-Acetyl-cysteine was added during grinding as an internal standard. Samples were taken from a minimum of two separate experiments. The specimens under analysis were filter centrifuged at 10,000 × *g* for 15 min. Freshly extracted samples were immediately derivatized according to Sneller *et al.* (21). To this end, 250 μl of each specimen was treated with monobromobimane (mBrB; Molecular Probes, Inc.) in 4-(2-hydroxyethyl)-piperazine-1-propanesulphonic acid and diethylenetriaminepentaacetic acid buffer for 30 min at 45 °C in the dark. 300 μl of methanesulfonic acid was then added to stop the reaction. Derivatized samples were stored in the dark at 4 °C until analysis. At that point, derivatized thiols were separated in a Nova Pack C_{18} column (Waters, Milford, MA) and eluted with a slightly concave methanol gradient of 12–25% (v/v) for 15 min and then with 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—Whereas 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-glutathione complexes, samples of the fungal strains under scrutiny, grown in the presence or absence of arsenate, were extracted in trifluoroacetic acid at pH 3, separated by HPLC, and detected by the post-column derivatization method with 5,5′-dithiobis(2-nitrobenzoic acid) as described previously (21). The $As(GS)_3$ standard for this procedure was made by the 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 h. After this period, the thiol-specific fluorescent reagent mBrB was added at a final concentration of 100 μM, 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—The 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 (PerkinElmer 2100; PerkinElmer Life Sciences) with the method of hydride generation as described previously (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 arsenic-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 arsenic-exposed *Aspergillus* sp. P37 had any connection with the mechanism(s) that accounts for the hypertolerant phenotype or whether it was part of a nonspecific response to stress. To address this question, we considered a number of alternatives including the possibility that, similarly to plants, arsenic could end up in vacuoles following conjugation with thiol compounds (see "Discussion"). To either regard or discard this possibility, we ran 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 arsenic-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 and/or a more even distribution of the -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—To provide a profile of the different solutes containing thiol groups that form or accumulate upon the 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 arse-

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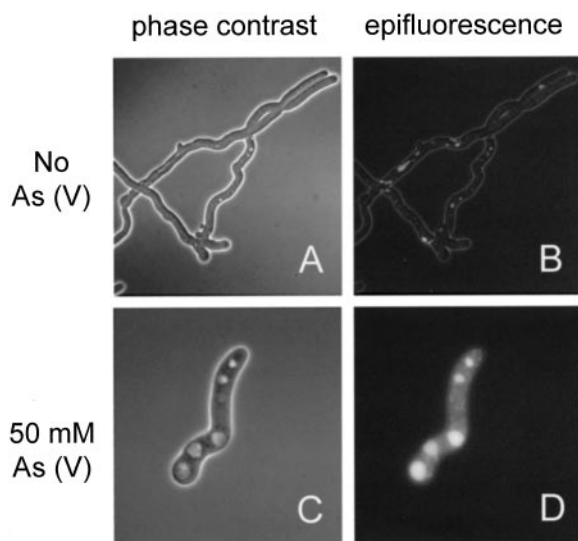

FIG. 1. **Thiols are accumulated in the vacuoles in response to arsenic.** *Aspergillus* sp. P37 was grown without (*A* and *B*) or with 50 mM (*C* and *D*) sodium arsenate for 3 h and then stained with mBrB for an additional hour. Cells were visualized by phase-contrast (*A* and *C*) or fluorescence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

nate. Acid-soluble compounds were then extracted from the biomass, derivatized with mBrB, and submitted to HPLC analysis as explained under "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 arsenic, 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 for 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 γ-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 the free thiol species accumulated in vacuoles consist of GSH.

Fig. 3*A* shows the evolution of GSH buildup in *Aspergillus sp*. P37 or *A. nidulans* TS1 through time under growing concentrations of arsenate. It is noteworthy that the 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 h). Only after 12 h of incubation and with superior arsenic levels in the medium did *Aspergillus* sp. P37 cells accumulate significantly higher amounts of glutathione, and a peak of the intracellular GSH pool was reached after 24 h of incubation with the highest arsenic concentration (Fig. 3*A*). 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 ($\leq$17%) than the GSH levels of the uninduced cells of *A. nidulans* TS1. Further increases in the level of the metalloid in the medium resulted in a reduction of the glutathione pool of *A. nidulans* TS1, surely due to the toxic effect exerted by arsenic on sensitive cells (Fig. 3*A*). Yet, the most informative outcome of the results shown in Fig. 3*A* is that GSH pools of *Aspergillus* sp. P37 treated with 50 mM arsenic for 5 h (a concentration that sufficed for a considerable vacuolation of thiol-containing compounds as detected with epifluorescence microscopy; Fig. 1) were not that different ($\leq$ 10%) from the levels measured in non-treated control cells. This result suggested that, within a certain arsenic 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 (labeled 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. 3*B*). Purification of the compound Y by HPLC and further analysis by matrix-assisted laser desorption ionization time-of-flight mass spectroscopy showed three major peaks (698, 714, and 730). One of these peaks 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 to elevated concentrations of arsenate, as it was not detected in cultures with <100 mM of the oxyanion (Fig. 3*B*). Cultures amended with subinhibitory concentrations of other heavy ions such as Cd^{2+} (0.1 mM), As^{3+} (25 mM), Cr^{3+} (50 mM), or Cu^{2+} (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 arsenic-resistant microorganisms (9). Therefore, unlike in 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 arsenic detoxification system.

Formation of Arsenic-Glutathione Complexes in Arsenic-stressed Aspergillus sp. P37 Cells—In view of the above data, 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 bound to arsenic when cells grow in the presence of the oxyanion. To address this issue, we first tackled the question of whether both As(III) and As(V) could form stable conjugates with reduced GSH under our analytical conditions. To this end, because 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. 4*A*). 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 that appears at the $As(GS)_3$ retention time point is likely to be a side reaction of some of the As(III) produced upon the 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 task, the fungi were grown in the presence or absence of 50 mM for 24 h, its biomass was collected and extracted in trifluoroacetic acid, and the soluble fraction was separated by HPLC and detected by the post-column derivatization method mentioned previously (21). Fig. 4 shows that although the non-treated sample produced only the peak of arsenic-free

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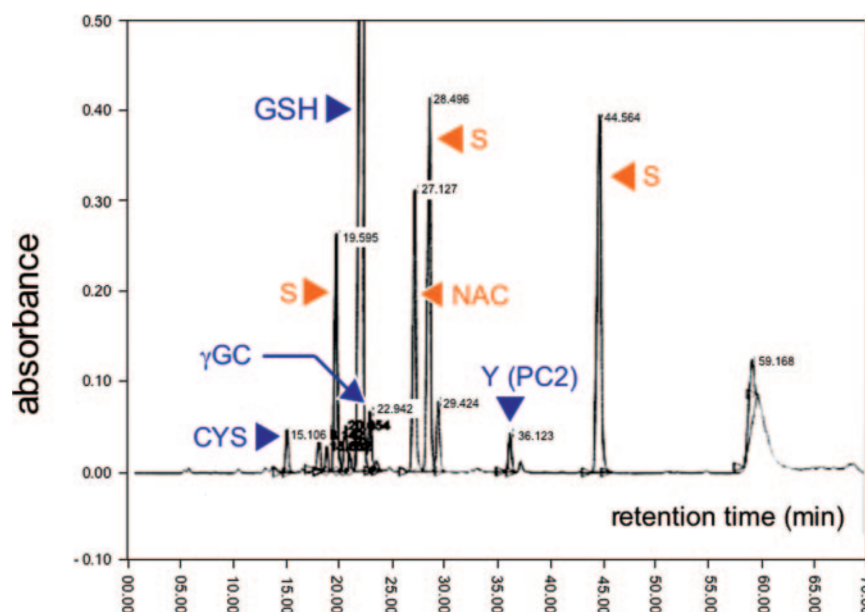

FIG. 2. **Fractionation of the intracellular thiol content of fungal samples.** Compounds containing thiol groups were extracted from fungal biomass as explained under "Experimental Procedures" and identified and quantified by HPLC. The sample shown corresponds to *Aspergillus* sp. P37 grown for 24 h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

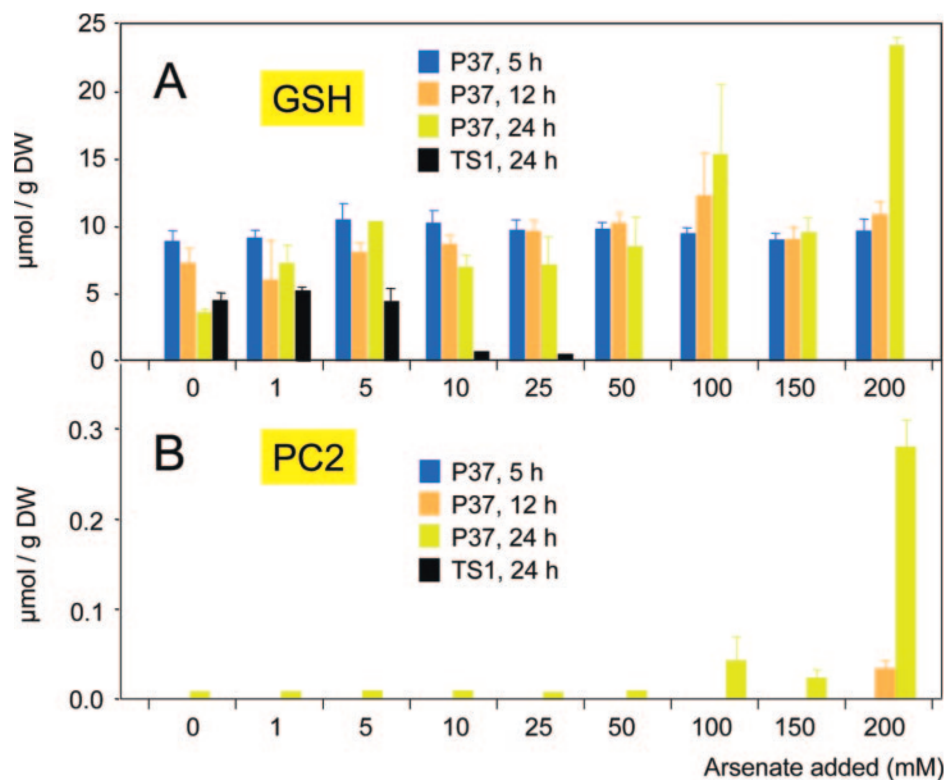

FIG. 3. **Accumulation of thiol compounds in *Aspergillus* sp. P37 and *A. nidulans* TS1.** *A*, buildup of glutathione in As(V)-exposed cells. The plot shows the GSH contents of *Aspergillus* sp. P37 and *A. nidulans* TS1 under the concentrations and time of exposure indicated in each case. Note the 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 phytochelatin-like compound PC2. The peak labeled *Y* in Fig. 2 is an alleged phytochelatin-2 which 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 analyzed once. *Bars* indicate S.D. *DW*, dry weight.

GSH, the specimen exposed to arsenate yielded two major chemical species. As displayed in Fig. 4*C*, one peak matched GSH, whereas the other corresponded to $As(GS)_3$. By judging the cognate peak areas, it would appear that the share of glutathione complexed with arsenite accounted for up to 35–40% of the whole GSH pool. Because the only form of arsenic that 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 the interpretation of the above data, *A. nidulans* TS1 was subjected to the same procedure for detecting the formation of $As(GS)_3$. Yet, because this strain starts being sensitive to arsenic at concentrations >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 h, 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 to 65%.

An Involvement of Glutathione-S-transferase(s) (GST) in the Response to Arsenic?—The above data (Fig. 4*A*) reveal 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 arsenic-resistant and arsenic-sensitive *Aspergillus* strains when exposed to high concentrations of the heavy ion. The manifest question is whether the formation of such $As(GS)_3$ compounds *in vivo* occurs also non-enzymatically or is it the result of a specific biocatalytic step. The ubiquitous enzyme GST has been conserved through the evolutionary scale, from bacteria to mammals, as a major instrument for

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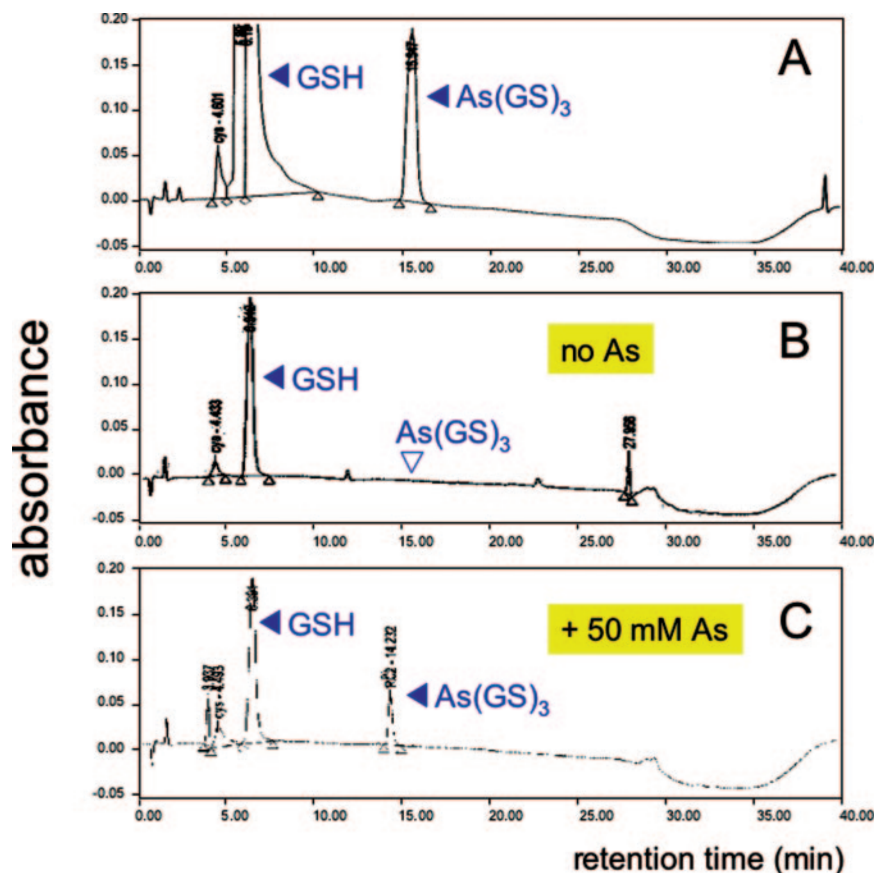
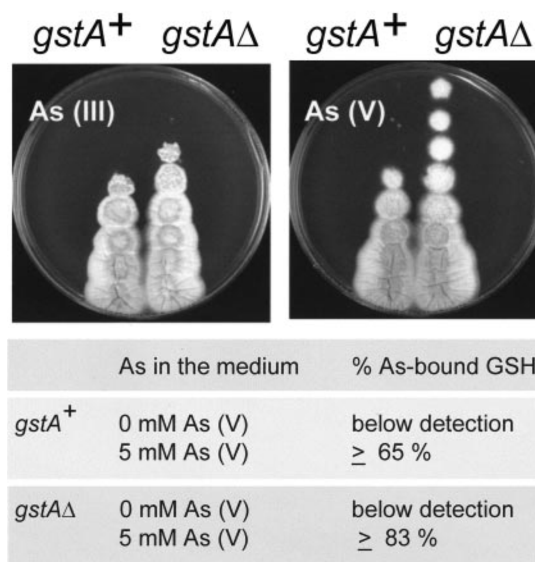


FIG. 4. **Formation of $As(GS)_3$ complexes.** *A*, generation and analysis of standards. As(III) was conjugated chemically with GSH, and the resulting $As(GS)_3$ was assigned a distinct HPLC peak (retention) time, ~15.5 min) identified with the post-column derivatization method explained under "Experimental Procedures." With the same fractionation procedure, GSH produced a peak with a reaction time of ~6 min. *B*, *Aspergillus* sp. P37 grown in the absence of arsenate lacks $As(GS)_3$. 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.

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 that are thus inactivated and, in some cases, channeled toward 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 as to 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 the formation of $As(GS)_3$ in *Aspergillus.* The experiment had to be limited to the one *A. nidulans* strain in which a well characterized Δ*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 Δ*gstA* mutant strain *A. nidulans* MH9986. The results unequivocally showed (Fig. 5) that the Δ*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 *versus* arsenic-bound glutathione in either strain, we measured these two chemical species in cells of the $gstA^+$/Δ*gstA* strains grown in the presence or absence 5 mM arsenate. Fig. 5 shows that cells lacking GstA had an even higher share of arsenic-bound glutathione (~83%) as compared with the wild type cells. We cannot rule out the existence of other proteins with GST activity that specialize in linking GSH to arsenite enzymatically. Another explanation for the superior tolerance to arsenic and the presence of large amounts of $As(GS)_3$ in the Δ*gstA* strain is that much of the glutathione-arsenic 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").



	As in the medium	% As-bound GSH
$gstA^+$	0 mM As (V) 5 mM As (V)	below detection ≥ 65 %
gstAΔ	0 mM As (V) 5 mM As (V)	below detection ≥ 83 %

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 arsenic-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 under "Experimental Procedures."

Arsenic Accumulation in Cells—As the role of thiol compounds in the response of *Aspergillus* sp. P37 and *A. nidulans*

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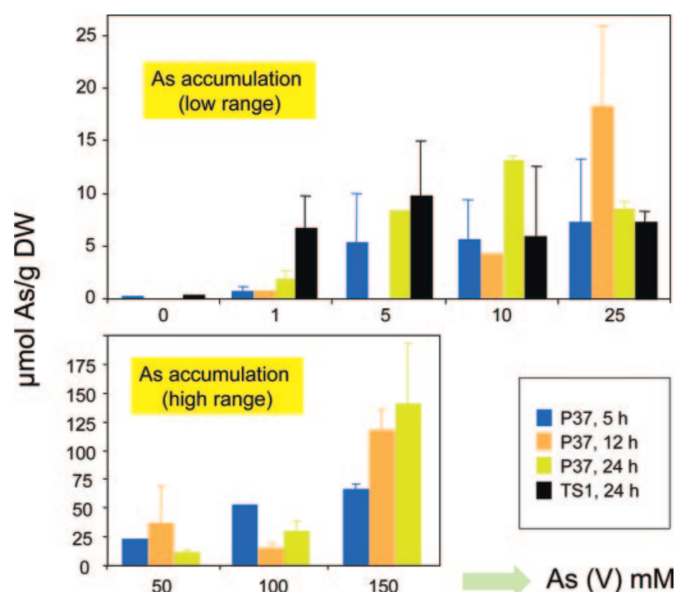

FIG. 6. **Accumulation of arsenic in *Aspergillus* sp. P37 and *A. nidulans* TS1.** The plots show the arsenic contents of *Aspergillus* sp. P37 and *A. nidulans* TS1 under the concentrations and time of exposure indicated in each case. The higher range of arsenic concentrations (50–150 mM) could be tested only in *Aspergillus* sp. P37, whereas *A. nidulans* TS1 was inspected 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 under "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 analyzed once. *Bars* indicate S.D. Note the very different scales of the lower *versus* higher ranges of arsenic concentrations. *DW*, dry weight.

TS1 to arsenic becomes exposed by the results above, the question to tackle at this point is whether or not hyper-resistant cells accumulate arsenic concomitantly with vacuolation and the formation of glutathione-arsenic conjugates. This could be inferred from the data on the buildup of $As(GS)_3$ under various conditions spelled out previously but needed to be quantified prior to assigning to arsenic 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. 3*A*), we detected only minor differences in the arsenic-accumulation capacity of the two *Aspergillus* strains at concentrations of arsenate $\leq$ 25 mM (*i.e.* below growth limit for *A. nidulans* TS1; Fig. 6*A*). However, increasing arsenic levels in the medium beyond 50 mM produced a notable increase in arsenic accumulation in *Aspergillus* sp. P37 (Fig. 6*B*), which was concomitant with the induction of high vacuolation within the hypertolerant cells (Fig. 1) (2). Because maximum contents of arsenic 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 that 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 arsenic concentrations far above the average inhibitory levels of reference microorganisms.

DISCUSSION

Different biological systems have evolved diverse strategies of enduring the toxic effects of arsenic. The fact that many (if not most) microorganisms bear at least one arsenate reductase and a membrane-bound pump complex for the 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 oxidizing atmosphere. With little variation, such an arsenate reductase/extrusion pump theme seems to account for many arsenic 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 arsenic-hypertolerant phenotype at concentrations of the oxyanion $\geq$ 200 mM. Furthermore, the patterns of the steady-state accumulation of arsenic and the arsenate uptake kinetics of *Aspergillus* sp. P37 was not that different from those of the arsenic-sensitive reference strain *A. nidulans* TS1 (4). Eukaryotes and multicellular organisms do add more mechanisms of arsenic 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 with sugars in seaweed; all of these possibilities contribute 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 arsenic related to those found in prokaryotic and multicellular strategies. Such a blend appears to be the case in the strain that is the subject of this study. From our observations it follows that other mechanisms should operate in the system and that intracellular sequestration mechanisms could be the likely means to account for the hypertolerance phenotype of *Aspergillus* sp. P37.

S. cerevisiae tolerates a certain level of arsenic exposure by the sequestration of $As(GS)_3$ in vacuoles mediated by the Ycf1 transporter (14). Ycf1p is a homologue of the multidrug resistance-associated protein, 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 behavior; during short periods of incubation (5 h) 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-catalyzed arsenate reduction in bacteria, plants, fungi, and animals, and arsenate reduction is a prerequisite for arsenic efflux from the cells (32). Second, GSH binds arsenite to form $As(GS)_3$, which allows sequestration in vacuoles mediated by an ATP binding cassette-type glutathione conjugate transporters (32). *A. thaliana* plants overexpressing the *Escherichia coli* γ-glutamylcysteine synthetase (the limiting step in the formation of GSH and PCs in plants) were only moderately resistant to arsenic compared with 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 a thiol-based hypertolerance to arsenic. Such a capacity is indeed present in *Aspergillus* sp. P37 (4), as sug-

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gested by the much higher arsenite efflux following arsenate exposure in comparison with *A. nidulans* TS1. Furthermore, *Aspergillus* P37 maintained higher GSH levels under arsenic exposure compared with *A. nidulans* TS1 and was even able to further increase the GSH level under very high arsenic conditions (Fig. 3*A*), 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 the possibility 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 arsenic, 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 the reduction of arsenate to arsenite, the latter is quickly bound by GSH. Only after exposure to arsenic is the thiol pool 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 arsenic-glutathione or arsenic-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). Because arsenite-GSH complexes are unstable at neutral pH but not under acidic conditions, we believe 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 selenium (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 arsenic. 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 such a role for other GST proteins existing in *Aspergillus*. These results also reveal a difference in the detoxification pathways of arsenic compared with those of 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 multidrug resistance-associated protein homologue (34). Although, PCs are potent arsenic detoxifiers in plants and even in some yeasts such as *S. pombe* (9, 35), *Aspergillus* sp. P37 only accumulated 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 a 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 the 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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