Mechanism of Arsenate Resistance in the Ericoid Mycorrhizal Fungus *Hymenoscyphus ericae*

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Arsenate resistance is exhibited by the ericoid mycorrhizal fungus *Hymenoscyphus ericae* collected from As-contaminated mine soils. To investigate the mechanism of arsenate resistance, uptake kinetics for arsenate ($H_2AsO_4^-$), arsenite (H_3AsO_3), and phosphate ($H_2PO_4^-$) were determined in both arsenate-resistant and -non-resistant *H. ericae*. The uptake kinetics of $H_2AsO_4^-$, H_3AsO_3, and $H_2PO_4^-$ in both resistant and non-resistant isolates were similar. The presence of 5.0 μM $H_2PO_4^-$ repressed uptake of $H_2AsO_4^-$ and exposure to 0.75 mM $H_2AsO_4^-$ repressed $H_2PO_4^-$ uptake in both *H. ericae*. Mine site *H. ericae* demonstrated an enhanced As efflux mechanism in comparison with non-resistant *H. ericae* and lost approximately 90% of preloaded cellular As (1-h uptake of 0.22 μmol g^{-1} dry weight h^{-1} $H_2AsO_4^-$) over a 5-h period in comparison with non-resistant *H. ericae*, which lost 40% of their total absorbed $H_2AsO_4^-$. As lost from the fungal tissue was in the form of H_3AsO_3. The results of the present study demonstrate an enhanced H_3AsO_3 efflux system operating in mine site *H. ericae* as a mechanism for $H_2AsO_4^-$ resistance. The ecological significance of this mechanism of arsenate resistance is discussed.

As is ubiquitous in nature with As levels being elevated by mining, industrial, and agricultural activities (Meharg et al., 1994). In the southwest of England, the mining and processing of As ore has led to highly contaminated mine spoil soils. As may be present in these spoil soils at 35 mmol kg^{-1} with arsenate being the dominant form of available soil As (Colbourn et al., 1975). Within the pH range 2.0 to 6.5 arsenate exists predominantly as $H_2AsO_4^-$ (Fergusson, 1990). The dominant vegetative cover on these mines is *Calluna vulgaris*, which is present in ericoid mycorrhizal association with the ascomycete fungus *Hymenoscyphus ericae* (Sharples et al., 2000). Ericoid mycorrhizal symbiosis is considered to be critical to the survival of plants in the order Ericales on natural heathland sites and sites contaminated with toxic metals. The principle benefit conferred upon plants by ericoid mycorrhizal association is fungus-mediated access to otherwise unavailable sources of organic nitrogen and phosphorus, whereas the fungus may also alleviate toxic metal stress under some circumstances (Smith and Read, 1997).

$H_2AsO_4^-$ resistance is exhibited by the ericoid mycorrhizal fungus, *H. ericae* (Sharples et al., 1999, 2000) and selected angiosperms, including *Holcus lanatus* (Meharg and Macnair, 1990), *Agrostis capillaris*, and *Deschampsia cespitosa* (Meharg and Macnair, 1991, 1992), collected from As-contaminated mine soils. $H_2AsO_4^-$ is a $H_2PO_4^-$ analog and competes with $H_2PO_4^-$ as a substrate for the $H_2PO_4^-$ uptake system in angiosperms (Asher and Reay, 1979; Ullrich-Eberius et al., 1989), fungi (Rothstein and Donovan, 1963; Jung and Rothstein, 1965; Beever and Burns, 1980), mosses (Wells and Richardson, 1985), lichens (Nieboer et al., 1984), and bacteria (Silver and Misra, 1988). Resistance mechanisms to $H_2AsO_4^-$ in the bacteria, *Staphylococcus aureous* and *Eschericha coli*, involve reducing cellular concentrations of As and rapidly effluxing them via a plasmid-encoded arsenical pump (Rosen, 1986; Silver and Misra, 1988). Meharg and Macnair (1990) demonstrated that $H_2AsO_4^-$ resistance in the grass *H. lanatus* was due to suppression of the high affinity $H_2PO_4^-$uptake system in $H_2AsO_4^-$ tolerant plants, which led to reduced uptake of both $H_2PO_4^-$ and $H_2AsO_4^-$.

Sharples et al. (2000) recently isolated populations of arsenate-resistant *H. ericae* from roots of *C. vulgaris* on As-contaminated mine spoil soils in southwestern England. Populations of the mycorrhizal fungus have evolved resistance to arsenate contamination in parallel with the host plant, and it seems likely that the presence of the mycorrhizal fungus in roots of *C. vulgaris* is essential its establishment and persistence on As-contaminated sites (Sharples et al., 2000). The present study investigated the mechanism of $H_2AsO_4^-$ resistance in an isolate of *H. ericae* from an As-contaminated mine site.

RESULTS

Effect of $H_2AsO_4^-$ and H_3AsO_3 on Biomass Production

The heathland *H. ericae* isolate demonstrated significantly greater sensitivity to $H_2AsO_4^-$ and H_3AsO_3

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than the mine site isolate (Fig. 1). Growth of the heathland isolate was almost completely inhibited at 1.33 mM $H_2AsO_4^-$ and above, whereas growth of the mine site isolate was inhibited by only 40% at the highest concentration tested (13.3 mM) (Fig. 1A). Biomass yield of the heathland isolate was 15 μg h^{-1} in the presence of 13.3 mM H_3AsO_3, whereas the mine isolate produced a mean biomass yield of approximately 45 μg h^{-1} at the same H3AsO3 concentration (Fig. 1B). Growth of both isolates was more severely affected by the presence of $H_2AsO_4^-$ than H_3AsO_3.

Kinetics of High Affinity $H_2AsO_4^-$ and $H_2PO_4^-$ Uptake

In $H_2PO_4^-$-deficient tissue the rate of $H_2AsO_4^-$ and $H_2PO_4^-$ uptake was dependent on concentration, and the uptake of both ions displayed saturation kinetics (Figs. 2 and 3). Single Michaelis-Menten functions were fitted to the data, representing the high affinity uptake carrier, which would predominate at low-substrate concentrations used here (Meharg and Macnair, 1990). Kinetics of both $H_2AsO_4^-$ and $H_2PO_4^-$ uptake were similar for the two *H. ericae* isolates (Table I). There was no significant difference

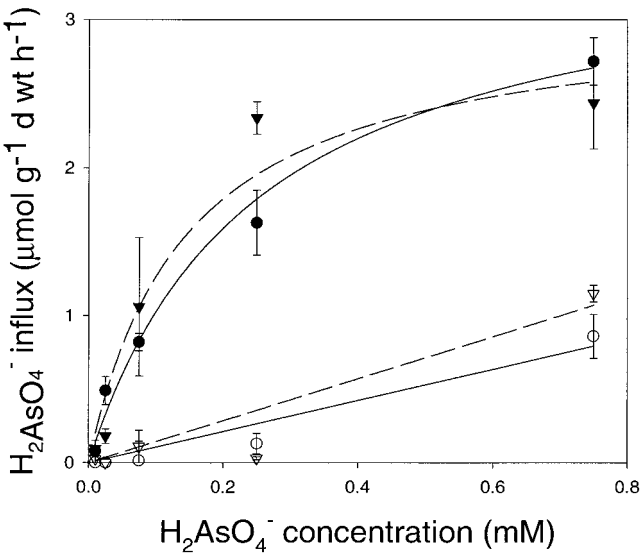

Figure 2. $H_2AsO_4^-$ influx in the absence of $H_2PO_4^-$ for mine site (▼, dashed line) and heathland (●, solid line) *H. ericae*. $H_2AsO_4^-$ influx after growth in the presence of 5 $mmol^{-3}$ $H_2PO_4^-$ for mine site *H. ericae* (▽, dashed line) and heathland *H. ericae* (○, solid line). Bars = mean ± SE (n = 3).

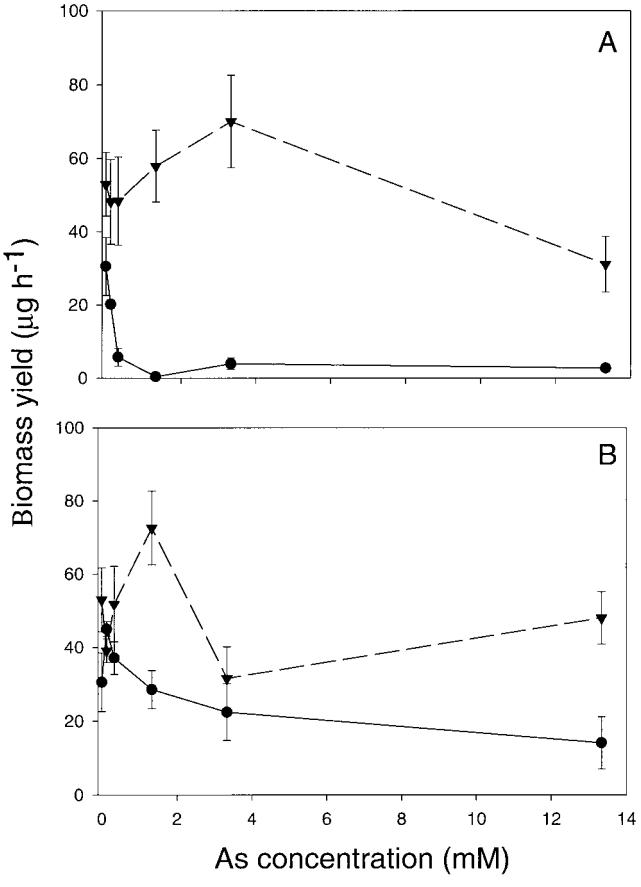

Figure 1. A, Growth of mine site (▼) and heathland (●) *H. ericae* over a range of $H_2AsO_4^-$ concentrations. Bars = mean ± SE (n = 3). B, Growth of mine site (▼) and heathland (●) *H. ericae* over a range of H_3AsO_3 concentrations. Bars = mean ± SE (n = 3).

in the uptake of $H_2AsO_4^-$ between the heathland and mine site *H. ericae* isolates, as determined by ANOVA (data not shown). Growth in the presence of 5 μM $H_2PO_4^-$ prior to uptake suppressed $H_2AsO_4^-$ uptake in both the mine site and heathland isolates (Fig. 2). At 0.75 mM $H_2AsO_4^-$ in inorganic phosphate-sufficient tissue, the rate of $H_2AsO_4^-$ uptake was approximately 2 to 3 times lower than the rate of uptake in the absence of $H_2PO_4^-$ (Fig. 2). Michaelis-Menten kinetic parameters could not be determined for isolates precultured on high concentration $H_2PO_4^-$ media, and a linear regression was fitted to the data (Fig. 2).

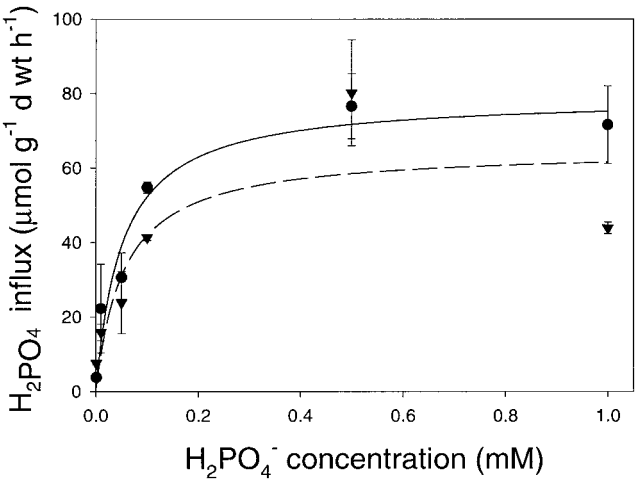

Figure 3. $H_2PO_4^-$ influx for mine site (▼, dashed line) and heathland (●, solid line) *H. ericae*. Bars = mean ± SE (n = 3).

Table I. *Kinetic parameters for $H_2AsO_4^-$, $H_3AsO_3^-$, and $H_2PO_4^-$ influx in mine site and heathland H. ericae*

Figures represent kinetic parameters ± SE (n = 3). P is the probability of the source term not being significant.

H. ericae Isolate	Species	Regression	Y0	A	K_m	V_{max}	r^2	P
					mol m^{-3}	μ*mol* g^{-1} *dry wt* h^{-1}		
Mine	H_3AsO_3	Hyperbola			3.5 ± 4.6	0.86 ± 0.91	0.9736	<0.0001
Mine	H_3AsO_3	Linear	0.0068 ± 0.0068	0.19 ± 0.014			0.9729	<0.0001
Mine	H_2AsO_4	Hyperbola			0.14 ± 0.06	3.1 ± 0.5	0.9432	0.0058
Mine	$H_2PO_4^-$	Hyperbola			0.055 ± 0.048	64.9 ± 13.9	0.7734	0.0209
Heathland	H_3AsO_3	Hyperbola			0.72 ± 0.60	0.40 ± 0.17	0.9297	0.0005
Heathland	H_3AsO_3	Linear	0.024 ± 0.016	0.22 ± 0.033			0.9014	0.0011
Heathland	H_2AsO_4	Hyperbola			0.28 ± 0.07	3.67 ± 0.37	0.9887	0.0005
Heathland	$H_2PO_4^-$	Hyperbola			0.051 ± 0.017	79.12 ± 6.51	0.9571	0.0007

Increasing $H_2PO_4^-$ concentrations up to 0.1 mM resulted in an increase in the rate of $H_2PO_4^-$ uptake in both isolates (Fig. 3). At concentrations above 0.1 mM, $H_2PO_4^-$ uptake was not further enhanced. Apparent kinetic parameters for both isolates were similar (Table I). There was no significant difference between the rates of $H_2PO_4^-$ uptake in the heathland or mine site isolate as determined by ANOVA (Minitab, SPSS, Chicago).

In comparison with the rate of $H_2AsO_4^-$ uptake, $H_2PO_4^-$ uptake was much greater in both *H. ericae* isolates at all concentrations. Both isolates demonstrated a much higher V_{max} and a lower K_m value for $H_2PO_4^-$ in comparison with $H_2AsO_4^-$ (Table I), indicating a much higher affinity for $H_2PO_4^-$ uptake.

Repression of $H_2AsO_4^-$ and $H_2PO_4^-$ Uptake

Repression of $H_2AsO_4^-$ uptake by $H_2PO_4^-$ and $H_2PO_4^-$ uptake by $H_2AsO_4^-$ was investigated at fixed concentrations. Uptake of 0.75 mM $H_2AsO_4^-$ from solution in heathland and mine site *H. ericae* isolates was reduced by pre-exposure of fungal mycelium to $H_2PO_4^-$ (5.0 μM $H_2PO_4^-$). The initial rate of $H_2AsO_4^-$ uptake in the absence of $H_2PO_4^-$ in mine site *H. ericae* was 0.22 μmol g^{-1} dry weight h^{-1}, which was lower than the initial rate of uptake in the heathland isolate (Fig. 4A). After 20 min of exposure to $H_2PO_4^-$, a rapid decrease in $H_2AsO_4^-$ uptake was observed for both isolates with mine site and heathland isolates demonstrating uptake rates of 0.14 and 0.29 μmol g^{-1} dry weight h^{-1}, respectively. After 2 h of $H_2PO_4^-$ uptake, $H_2AsO_4^-$ uptake by both isolates was almost completely suppressed (Fig. 4A).

Uptake of 0.1 mM $H_2PO_4^-$ was repressed by pre-exposure to 0.75 mM $H_2AsO_4^-$ (Fig. 4B). Initially, the rate of $H_2PO_4^-$ exposure in the heathland isolate was greater than the mine site isolate, however on exposure to $H_2AsO_4^-$, rates of $H_2PO_4^-$ uptake decreased in both isolates (Fig. 4B). After 20 min of exposure to $H_2AsO_4^-$, the rate of $H_2PO_4^-$ uptake was suppressed considerably, however, after 24 h of exposure to $H_2AsO_4^-$, $H_2PO_4^-$ uptake in both isolates was completely inhibited (Fig. 4B). There was no difference between the effects of $H_2AsO_4^-$ on $H_2PO_4^-$ uptake and $H_2PO_4^-$ on $H_2AsO_4^-$ uptake between the heathland and mine site isolate.

Efflux of As from Fungal Cells

In the methylation experiment, As was not present in the $HgCl_2$ traps for either mine site or heathland isolates. Because $HgCl_2$ complexes all volatile methylated arsines as well as AsH_3, this indicates that

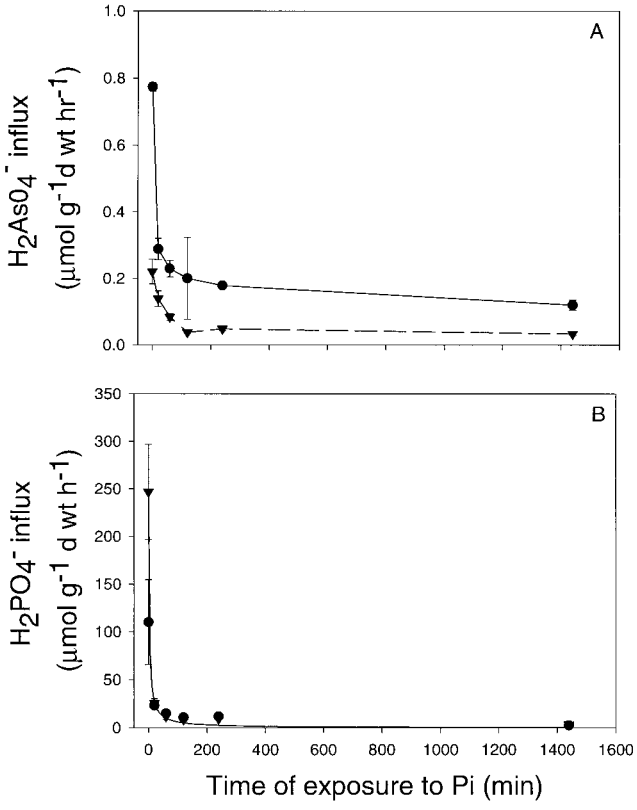

Figure 4. A, Uptake of 0.75 mM $H_2AsO_4^-$ in mine site (▼) and heathland (●) *H. ericae* after periods of exposure to 5.0 μM $H_2PO_4^-$. Bars = mean ± SE (n = 3). Second order decay curves were fitted to the data (Sigma Plot, Jandel Scientific). B, Uptake of 0.1 mM $H_2PO_4^-$ in mine site (▼) and heathland (●) *H. ericae* after periods of exposure to 0.75 mM $H_2AsO_4^-$. Bars = mean ± SE (n = 3). Second order decay curves were fitted to the data (Sigma Plot, Jandel Scientific).

neither isolate methylated $H_2AsO_4^-$ (data not shown).

Efflux of As from mine site *H. ericae* mycelia was more rapid than for the heathland isolate (Fig. 5). After 1 h of incubation in 0.75 mM $H_2AsO_4^-$ (to load cells with As) and transfer to $H_2AsO_4^-$-free media for time periods of up to 24 h, As concentration in the mine site isolate tissue decreased significantly ($P < 0.001$) (Fig. 5). After 5 h in $H_2AsO_4^-$-free media, the mine site isolate lost 83% of its initial As concentration in comparison with the heathland isolate, which lost 13%, showing enhanced As cell efflux in the mine site *H. ericae* isolate. Similar trends were found after loading for 10 min, 20 min, and 4 h exposure to 0.75 mM $H_2AsO_4^-$ (data not shown). Of the total As effluxed from cells, 71.6% was H_3AsO_3 with the remainder of As being lost as $H_2AsO_4^-$. The majority of As lost from the heathland isolate similarly was in the form of H_3AsO_3 (71.3%). These results indicate an enhanced H_3AsO_3 efflux mechanism in the mine site *H. ericae* isolate.

Uptake of $H_2AsO_4^-$ over Time

The long-term uptake of $H_2AsO_4^-$ was much greater in the heathland *H. ericae* isolate than the mine site isolate (Fig. 6). Accumulation of $H_2AsO_4^-$ by the heathland isolate did not differ significantly from linearity with respect to time ($r^2 = 0.973$) over 2 h, however, after 2 h of exposure, decreased accumulation was observed (Fig. 6). This decrease in As accumulation after 2 h seems likely to reflect a response to $H_2AsO_4^-$ toxicity. In contrast, while the mine site isolate demonstrated increased accumulation of As over 20 min there was no increase in accumulation after 2 h and this was sustained for up to 24 h.

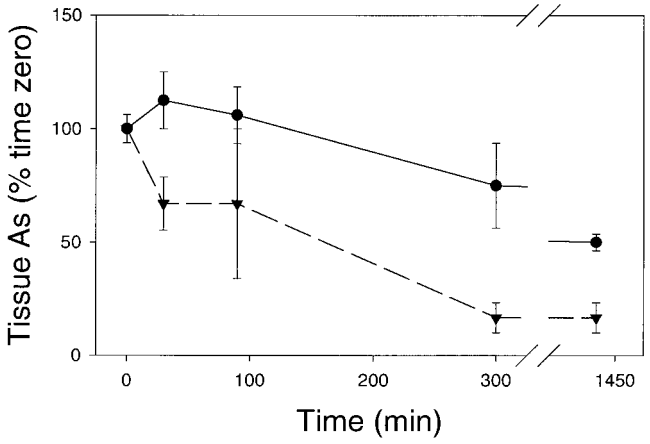

Figure 5. As content of mine site (▼) and heathland (●) *H. ericae* tissue over time as a percentage of total As content after incubation in 0.75 mM $H_2AsO_4^-$. Bars = mean ± SE ($n = 3$).

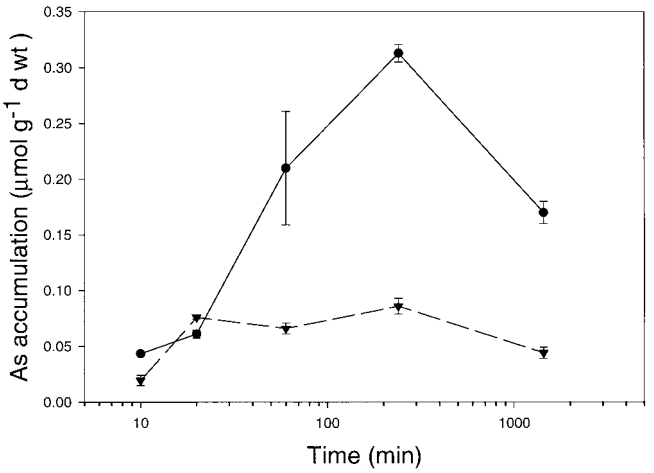

Figure 6. Long-term accumulation of 0.75 mM $H_2AsO_4^-$ in mine site (▼) and heathland (●) *H. ericae*. Bars = mean ± SE ($n = 3$).

Uptake of H_3AsO_3

The rates of H_3AsO_3 uptake by heathland and mine site *H. ericae* isolates were similar with uptake in both isolates increasing linearly in response to increasing H_3AsO_3 concentrations (Fig. 7). Although Michaelis-Menten functions could be fitted to the data, linear models demonstrated the best fits (Table I). Uptake of H_3AsO_3 in mine site and heathland *H. ericae* isolates at low H_3AsO_3 concentrations (0.01 mM H_3AsO_3) was 3- to 4-fold less than the rate of $H_2AsO_4^-$ uptake at the same concentration (Figs. 2 and 6). At 0.75 mM H_3AsO_3, the rate of H_3AsO_3 uptake was 15 times less than the rate of $H_2AsO_4^-$ uptake with both isolates have a lower affinity for H_3AsO_3 than $H_2AsO_4^-$.

DISCUSSION

Populations of $H_2AsO_4^-$-resistant *H. ericae* have been isolated from As/Cu mine soils (Sharples et al.,

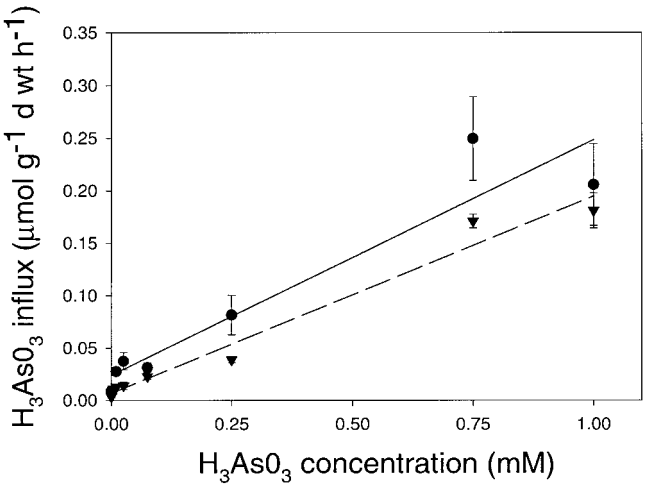

Figure 7. H_3AsO_3 influx for mine site (▼) and heathland (●) *H. ericae*. Bars = mean ± SE ($n = 3$). Linear regressions were fitted to the data (Sigma Plot, Jandel Scientific).

2000), where soil solution $H_2AsO_4^-$ content is 20 to 75 times higher than natural heathland soils that have not been contaminated by mining and other industrial processes. The present study demonstrates increased $H_2AsO_4^-$ and H_3AsO_3 resistance in an isolate of *H. ericae* obtained from an As/Cu mine site in comparison with an isolate from an uncontaminated heathland site. Growth of the heathland isolate was completely inhibited at 1.33 mM $H_2AsO_4^-$, whereas the mine site *H. ericae* isolate continued growth at the highest concentration tested (13.3 mM) (Fig. 1A). H_3AsO_3 is usually considered to be the most toxic form of As available to plants and fungi, however both isolates of *H. ericae* demonstrated greater sensitivity to $H_2AsO_4^-$ (Fig. 1), although rates of growth of both isolates were similar in the absence of As.

$H_2AsO_4^-$ behaves as a $H_2PO_4^-$ analog (Meharg and Macnair, 1990) and is accumulated by the $H_2PO_4^-$ transport system in a wide range of organisms (Meharg and Macnair, 1992). Inorganic phosphate transport across membranes is carrier-mediated and described by Michaelis-Menten kinetics (Beever and Burns, 1980). Adaptation of the $H_2PO_4^-$ uptake system is a mechanism of $H_2AsO_4^-$ resistance in the grasses *H. lanatus*, *D. cespitosa*, and *A. capillaris* (Meharg and Macnair, 1990, 1991, 1992, 1994). In *H. lanatus*, $H_2AsO_4^-$ resistance is achieved by constitutive suppression of the high-affinity uptake system by carrier synthesis inhibition and is independent of plant phosphorus status (Meharg and Macnair, 1992). The present study demonstrates similar apparent K_m and V_{max} parameters for $H_2AsO_4^-$ transport and $H_2PO_4^-$ transport in $H_2AsO_4^-$-resistant and -nonresistant *H. ericae* (Table I). Mine site and heathland *H. ericae* apparent K_m values (0.14 ± 0.06 mM and 0.28 ± 0.07 mM, respectively) were higher than values previously reported for $H_2AsO_4^-$-resistant *H. lanatus* (0.074 mM) (Meharg and Macnair, 1992) and other fungi (Sharples et al., 1999). For example, *Saccharomycetes cerevisiae* has a K_m for $H_2AsO_4^-$ of 0.004 mM (Jung and Rothstein, 1965), and *Candida tropicalis* has a K_m value of 0.005 mM (Beever and Burns, 1980). The apparent V_{max} value for $H_2AsO_4^-$ uptake obtained for *H. ericae* (Table I) was similar to those reported for *S. cerevisiae* (10.2 μmol g^{-1} dry weight h^{-1}), lower than the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Sharples et al., 1999), yet higher than those found in $H_2AsO_4^-$-resistant *H. lanatus* (Meharg and Macnair, 1990).

Both isolates demonstrated similar H_3AsO_3 transport kinetics, however influx was markedly lower than when compared with the rate of $H_2AsO_4^-$ uptake (Figs. 2 and 7). This decrease was expected because H_3AsO_3 is not a $H_2PO_4^-$ analog and is therefore not transported by $H_2PO_4^-$ carriers. It is not known how H_3AsO_3 enters cells, however the present study seems to indicate passive diffusion (Fig. 7).

Pregrowth of *H. ericae* in the presence of 5 mM $H_2PO_4^-$ significantly suppressed the uptake of $H_2AsO_4^-$ for both mine site and heathland isolates (Fig. 2). Suppression of $H_2AsO_4^-$ uptake by long-term exposure to $H_2PO_4^-$ has also been demonstrated in the plant *Hordum vulgare* and *Silene vulgaris* (Lee, 1982; Paliouris and Hutchinson, 1991). In the case of *H. vulgare*, plants grown in the presence and absence of 0.5 mM $H_2PO_4^-$, demonstrated $H_2AsO_4^-$ uptake rates of 27.7 and 81.6 nmol g^{-1} fresh weight h^{-1}, respectively (Lee, 1982). Meharg and Macnair (1991) suggest that $H_2AsO_4^-$ resistance in *H. lanatus* is due to a decrease in the concentration of protein carriers in the plasma membrane rather than a change in the carrying capacity of the protein.

$H_2AsO_4^-$ uptake was rapidly repressed on exposure to $H_2PO_4^-$ in both mine site and heathland *H. ericae* (Fig. 4). Similarly, the presence of $H_2AsO_4^-$ rapidly repressed $H_2PO_4^-$ uptake (Fig. 4). Rapid repression of the high affinity $H_2PO_4^-$ uptake system in plants under high plant $H_2PO_4^-$ status has long been reported (Meharg and Macnair, 1992). The nature of this repression differs for different species. Repression may occur by a decrease in V_{max} with little or no change in the K_m, which occurs in algae (McPharlin and Bieleski, 1987), bacteria (Beever and Burns, 1980), and selected plants (Anghinoni and Barber, 1980; Lee, 1982; Cogliatti and Santa Maria, 1990; Jungk et al., 1990). Repression of the high affinity uptake system in the vesicular arbuscular mycorrhizal fungus *Gigaspora margarita* is achieved by increase in the apparent K_m with little change in the apparent V_{max} (Thompson et al., 1990), whereas repression in the plant, *Solanum tuberosm,* is achieved by both an increase in apparent K_m and a decrease in apparent V_{max} (Cogliatti and Clarkson, 1983). Changes in $H_2PO_4^-$ uptake with changing $H_2PO_4^-$ status may be under allosteric control (Levebvre and Glass, 1982; Schorring and Jensen, 1984) and by the synthesis and breakdown of transport sites (Jeanjean, 1973; Drew et al., 1984). Because the speed of repression is too rapid to be explained by protein turnover, under short exposure times it is likely that for *H. ericae* both $H_2PO_4^-$ and $H_2AsO_4^-$ act allosterically.

Short-term uptake of $H_2AsO_4^-$ was similar between isolates, however in the longer term, accumulation of As by the heathland isolate decreased significantly in 24 h (Fig. 6). Such a decrease in the rate of As accumulation in the heathland isolate may reflect cell death in response to $H_2AsO_4^-$ toxicity. Arsenate causes toxicity in fungi and plants by interfering with aerobic phosphorylation, following intracellular reduction of $H_2AsO_4^-$ to H_3AsO_3, which then breaks down protein sulfydryl groups (Ullrich-Eberius et al., 1989).

The present study demonstrates the ability of an isolate of *H. ericae* from a mine site to efflux H_3AsO_3 from its hyphae (Fig. 5), which may provide $H_2AsO_4^-$ resistance to this isolate. H_3AsO_3 efflux has

been reported as a mechanism of $H_2AsO_4^-$ resistance in the bacterium *S. aureous* (Broer et al., 1993) and the yeast *S. cerevisiae* (Wysocki et al., 1997) and contrasts to the mechanism of resistance reported in higher plants (Meharg and Macnair, 1990). Arsenate resistance in *S. cerevisiae* is mediated by an H_3AsO_3 transporter (Wysocki et al., 1997), and in the case of *S. aureous*, intracellular $H_2AsO_4^-$ is reduced to H_3AsO_3 before being actively exported from the cells (Broer et al., 1993). The present study suggests a similar mechanism of As resistance in *H. ericae* at As-contaminated mine sites. The steady state of As accumulation observed in the mine site isolate after 20 min is probably not due to a suppression of the high affinity uptake system but rapid internal reduction of $H_2AsO_4^-$ to H_3AsO_3, which then initiates efflux of H_3AsO_3 from the hyphae. The ability of the mine site *H. ericae* isolate to efflux $H_3AsO_3$from cells into the surrounding soil indicates a need for enhanced resistance to H_3AsO_3. Arsenate was much more toxic to *H. ericae* than H_3AsO_3 (Fig. 1), which supports efflux of H_3AsO_3 as the mechanism of $H_2AsO_4^-$ resistance in *H. ericae*.

The mechanism of $H_2AsO_4^-$ resistance we have described in *H. ericae* is likely to be of ecological importance for its host plant (*C. vulgaris*) on contaminated mine sites. Arsenite efflux enables the fungus to retain its ability to transport inorganic phosphate from the soil (much of which will, in turn, is transferred to the host plant), whereas effluxing absorbed $H_2AsO_4^-$. The fungus may thus act as a filter to maintain low plant As levels, while maintaining an adequate supply of phosphorus to the host (Sharples et al., 2000). Efflux of H_3AsO_3 from the fungal cells into the soil also ensures that re-absorption of As from the soil is limited.

MATERIALS AND METHODS

Fungal Material

The arsenate-resistant *Hymenoscyphus ericae* genotype was isolated from roots of *Calluna vulgaris* obtained from the Gawton United mine (Devon, S.W. UK) whereas the non-resistant genotype was obtained from *C. vulgaris* roots from an uncontaminated natural heathland site at Aylesbeare Common (Devon, S.W. UK). These fungal isolates were randomly selected from approximately 25 isolates from each site and were previously identified by PCR-RFLP analysis (Sharples et al., 2000). Although the characteristics of arsenate absorption and arsenite efflux have only been studied in detail for single isolates from each population, preliminary experiments for absorption and efflux by further isolates indicate consistent patterns for absorption/efflux within the mine site or heathland populations (data not shown). The fungi were maintained on modified Melin Norkrans agar medium (MMN) containing: 3.79 mM $(NH_4)_2HPO_4$; 2.21 mM KH_2PO_4; 0.57 mM $MgSO_4 \cdot 7H_2O$; 0.23 mM $CaCl_2 \cdot 6H_2O$; 0.43 mM NaCl; 0.034 mM FeEDTA; 55.5 mM D-Glc; and 0.3 μM thiamine, adjusted to pH 5.5, and incubated at 25°C. In $H_2AsO_4^-$ and H_3AsO_3 uptake experiments, mycelia were grown in liquid MMN for 17 d and transferred to $H_2PO_4^-$-free MMN for 48 h prior to uptake analysis. To determine the effects of $H_2PO_4^-$ on $H_2AsO_4^-$ uptake, mycelia were grown in liquid MMN containing 5 μM $H_2PO_4^-$ for 17 d before $H_2AsO_4^-$ uptake. Mycelia for $H_2PO_4^-$ uptake studies were grown on liquid MMN containing 0.01 mM $H_2PO_4^-$ for 17 d at 25°C.

Effect of $H_2AsO_4^-$ and H_3AsO_3 on Biomass Production

Two plugs (6-mm diameter) of each *H. ericae* isolate were cut from the edge of actively growing mycelia on MMN and inoculated into 9-cm-diameter Petri dishes containing 25 mL of liquid MMN. After 11 d of incubation at 25°C, fungal plugs were transferred to 25 mL of liquid MMN supplemented with either $H_2AsO_4^-$ or H_3AsO_3, supplied as Na_2HAsO_4 and $NaAsO_2$, respectively, at concentrations of 0, 0.67, 1.33, and 4.67 mM. For all treatments, $H_2PO_4^-$ concentration in the medium was adjusted to 0.01 mM. After 7 d of incubation, mycelial mats were removed from basal medium, dried overnight (80°C), and the biomass increase determined gravimetrically. All treatments were replicated three times.

Kinetics of $H_2AsO_4^-$ and $H_2PO_4^-$ Uptake

To determine $H_2AsO_4^-$, H_3AsO_3, and $H_2PO_4^-$ uptake, three replicate mycelial mats of each isolate were incubated in 25 mL of aerated test solution for 20 min (except when uptake was determined with respect to time). Test solutions contained 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.5 mM $Ca(NO_3)_2$, and different concentrations of either $H_2AsO_4^-$, H_3AsO_3, or $H_2PO_4^-$ in the form of $Na_2HAsO_4 \cdot 7H_20$, $NaAsO_2$, and Na_2HPO_4, respectively. In the experiments to determine the rate of $H_2PO_4^-$ uptake, [^{32}P] (as NaH_2PO_4, supplied by Amersham) was added to give an activity of 37 kBq mL^{-1}. Using the methodology of Meharg and Macnair (1990), uptake was terminated by rinsing mycelia in 25 mL of an ice-cold solution containing 1 mM Na_2HPO_4, 10 mM MES, and 0.5 mM $Ca(NO_3)_2$. Mycelia were transferred to 25 mL of an aerated ice-cold solution of the same composition for 10 min to ensure desorption of $H_2AsO_4^-$, H_3AsO_3, or [^{32}P] from the cell-free space. Mycelia were dried (80°C, 24 h) and biomass determined gravimetrically before analysis.

Repression of $H_2PO_4^-$ and $H_2AsO_4^-$ Uptake

To investigate the effects of $H_2PO_4^-$ on $H_2AsO_4^-$ uptake, three replicate mycelial mats of each isolate were pre-incubated in liquid MMN containing 5 μM $H_2PO_4^-$ for 0, 20, 60, 120, 240, or 1,440 min prior to $H_2AsO_4^-$ uptake (as described above). The effects of $H_2AsO_4^-$ on $H_2PO_4^-$ uptake were determined by pre-incubating three replicate mycelia in 0.75 μM $H_2AsO_4^-$ for 0, 20, 60, 120, 240, or 1,440 min before exposure to [^{32}P]-uptake solution.

Methylation and Efflux of As by Fungi

Methylation of $H_2AsO_4^-$ was investigated using a modified method of Gates et al. (1997), involving the chemofo-

cussing of volatile As species on mercuric chloride. Conical flasks were inoculated with 20 mL of a solution containing 10 mM MES, 0.5 mM $Ca(NO_3)_2$, and 0.67 mM $H_2AsO_4^-$ for mine site *H. ericae* mycelia and 0.27 mM $H_2AsO_4^-$ for heathland *H. ericae*. These $H_2AsO_4^-$ concentrations were the approximate $H_2AsO_4^-$ EC_{50} values of the mine and heathland population (preliminary data by Sharples et al., 2000) (data not shown). Polyurethane plugs were soaked in 0.1 M $HgCl_2$ and oven dried at 50°C for 12 h before being placed inside a glass condenser fitted to the conical flasks. Three replicate flasks were set up for each isolate. The presence of As was indicated by brown discoloration of the $HgCl_2$ plugs and after 24 h of incubation, $HgCl_2$ plugs were removed and analyzed for As by atomic absorption spectrometry.

To investigate the mechanism of $H_2AsO_4^-$ resistance, mycelia of each isolate ($n = 3$) were exposed to $H_2AsO_4^-$ uptake solution for 10 min, 20 min, 1 h, 4 h, or 24 h. After termination of uptake, mycelia were transferred to 25 mL of liquid MMN containing no $H_2PO_4^-$ for 0, 30 min, 90 min, 5 h, or 24 h. Mycelia were then dried (80°C, 24 h) and biomass determined gravimetrically before As analysis.

Speciation of As

Three replicate mycelia of each isolate were incubated in 0.75 mM $H_2AsO_4^-$ uptake solution for 1 h. Uptake was terminated and mycelia transferred to a 2-mL test tube containing 1 mL of $H_2PO_4^-$-free liquid MMN. Fresh liquid MMN was continually pumped into and out of the test tube at a flow rate of 0.7 mL min^{-1} and removed from the tube at the same rate for 5 h. After 5 h, fungal material was dried, digested, and analyzed for As. Two milliliters of MMN pumped from the test tube was also analyzed for $H_2AsO_4^-$ and H_3AsO_3 using atomic absorption spectrometry (Glaubig and Goldberg, 1988).

Analysis

To determine $[^{32}P]H_2PO_4^-$ uptake, dried fungal mycelia were placed in 20-mL glass scintillation vials, to which 10 mL of deionized water was added. $[^{32}P]$-activity was determined by Cherenkov counting using a Tri Carb 2100TR liquid scintillation counter (Packard Bell, Sacramento, CA) with data corrected for quenching.

As was determined by digesting mycelia in 2 mL of concentrated nitric acid (Aristar grade) using a block digester, for 1 h at 120°C followed by 1 h at 180°C, to evaporate the samples to dryness. The As residue was redissolved in 20 mL of a solution containing 5% (v/v) HCl (Analar grade) containing 20 mM potassium iodide. The amount of As present in the digests was determined using hydride generation interfaced with an atomic absorption spectrometer (ThermoUnicam Solaar 929, Cambridge, UK). As species were determined using pH selectivity, $H_2AsO_4^{3-}$ reduced to arsine (AsH_3) by $NaBH_4$ at pH < 6, whereas H_3AsO_3 reduced to AsH_3 at pH 7.

Statistical Analysis

Data were analyzed by ANOVA using the computer package Minitab v. 11 (Minitab, State College, PA). Curve fitting was carried out using the fitting regimes within the computer package Sigma Plot (Jandel Scientific, Erkrath, Germany), which uses the Marquardt non-linear curve fitting algorithm (Marquardt, 1963).

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