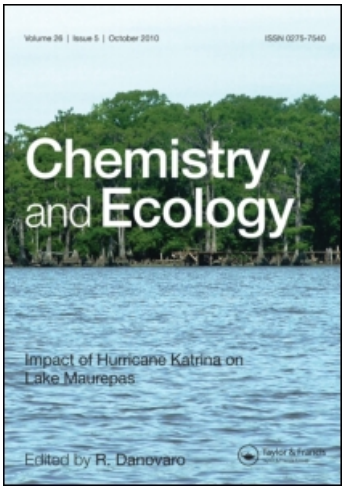

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W. James Catallo[a]; W. Henk[b]; L. Younger[b]; O. Mills[c]; D. J. Thiele[d]; S. P. Meyers[e]
[a] Laboratory for Ecological Chemistry, School of Veterinary Medicine, [b] Electron Microscopy Laboratory, Department of Veterinary Anatomy and Physiology, School of Veterinary Medicine, [c] Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA [d] Department of Metallurgical and Materials Engineering, Michigan Technological University, Houghton, MI [e] Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI

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TRACE METAL UPTAKE BY *PICHIA SPARTINAE*, AN ENDOSYMBIOTIC YEAST IN THE SALT MARSH CORD GRASS *SPARTINA ALTERNIFLORA*

W. JAMES CATALLO[a], W. HENK[b], L. YOUNGER[b], O. MILLS[c], D. J. THIELE[d] and S. P. MEYERS[e]

[a]*Laboratory for Ecological Chemistry, School of Veterinary Medicine;*
[b]*Electron Microscopy Laboratory, Department of Veterinary Anatomy and Physiology, School of Veterinary Medicine;*
[c]*Department of Oceanography and Coastal Sciences, Louisiana State University, Baton Rouge, LA 70803;*
[d]*Department of Metallurgical and Materials Engineering Michigan Technological University, Houghton MI 49931;*
[e]*Department of Biological Chemistry University of Michigan Medical School, Ann Arbor MI 48109-0606*

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The ascosporogenous marine yeast *Pichia spartinae* is a dominant endosymbiont of the marsh grass *Spartina alterniflora*. Results of previous studies suggested that *P. spartinae* is involved in iron transport processes in the grass. Of particular interest has been the mechanisms of metal uptake and metabolism by the yeast, and the ecological and plant biochemical significance of these processes. This investigation examined the uptake of iron and other metals (Zn, Cu, Cd, Ni, Pb, Cr) by *P. spartinae*, and provides data on possible mechanisms of this activity. The results suggest a) the yeast can assimilate divalent and trivalent forms of inorganic iron, as well as large organic-Fe(III) complexes, b) the uptake of inorganic trivalent iron under soluble iron-deficient conditions proceeded by a different mechanism than that of soluble Fe(II), with intracellular loadings of iron much increased under the former conditions; c) trivalent iron uptake is not mediated by hydroxamate siderophores at levels detectable by sensitive screening assays; d) the assimilation of some trace metals (Cu, Zn, Cd, Ni) is likely to be mediated by low molecular weight cysteine rich proteins, possibly metallothionein, and; e) siderophores from other fungi can provide iron for *P. spartinae*. The iron assimilation data suggested that multiple mechanisms are involved, and are influenced by the concentration and speciation of iron in the system. In general, iron assimilation mechanisms are comparable to those described for closely related yeasts, such as *Saccharomyces cerevisiae*. Among other things, these results indicated that future studies of trace metal mobilization and plant assimilation in salt marsh ecosystems must account for the activities of microbial symbionts associated with the plants.

Keywords: Pichia spartinae; metal uptake; iron; siderophores; mechanisms; salt marsh ecology

INTRODUCTION

The ascosporogenous yeast *Pichia spartinae* (Family *Saccharomycetaceae*) is found in high densities in salt marsh sediments and as an endosymbiont of the dominant macrophyte *Spartina alterniflora* (Ahearn *et al.*, 1970; Meyers *et al.*, 1970; Meyers, 1980). Viable cell densities of the yeast in the rhizosphere and vascular spaces (i.e., aerenchyma and parenchyma) of *S. alterniflora* are on the order of 10^5–10^7 cells per gram live tissue (Meyers *et al.*, 1975). Limited data suggest that this symbiosis is commensal, with the yeast able to utilize plant fluids as a carbon source without causing nutritive stress to the plant. *P. spartinae* utilizes a complex range of amines as sole nitrogen sources without prior acclimation, and produces large amounts of indole–3–acetic acid (IAA), a plant auxin (Meyers and Nicholson, 1970; Meyers *et al.*, 1975; Nakamura *et al.*, 1991). The relationship of IAA production by the yeast to growth and development in *S. alterniflora* remains undocumented.

In addition to providing biomass for detrital and aquatic food chains, *S. alterniflora* is important in salt marsh and estuarine metal cycling (Giblin and Howarth, 1984). The grass can incorporate on the order of 2 kg Fe m^{-2} yr^{-1}, with growth in dredge spoils or contaminated sediments giving rise to increased loads of zinc, copper, cadmium, and other toxic metals in tissues (Lima *et al.*, 1989; Lee *et al.*, 1981; Huiskes and Nieuwenhuiza, 1985; Alberts *et al.*, 1990). The uptake mechanisms of these metals and other cations have not been examined. The situation with iron is particularly challenging for although abundant iron is found in salt marsh sediments, its availability is extremely low (e.g., $Ksp = 10^{-38}$ to 10^{-42} for ferric hydroxides). Further, *S. alterniflora* maintains an oxidized rhizosphere where oxidization and precipitation of dissolved divalent iron occurs (Mendelssohn and Postek, 1982; Mendelssohn *et al.*, 1995). Thus, much of the plant-available iron is chemically oxidized at the rhizosphere to highly unavailable forms (e.g., oxyhydroxides), where it remains as coatings or nodules.

Active mechanisms of iron assimilation by *S. alterniflora* are not detailed, although enhanced solubility of ferric iron by metabolic and chemical acidification of the root zone has been suggested (Mendelssohn

et al., 1995). This hypothesis, however, is troublesome because pH values of the rhizosphere typically are on the order of 4–6, and the Ksp of ferric hydroxide is functionally unchanged until the pH approaches 2.0. The fungal biomass associated with *S. alterniflora* can account for up to 1% of the total biomass of the rhizosphere (Padgett and Celio, 1990), thus the relationships between metabolic activities of these microbes and plant element transport and cycling is likely to be very significant. For example, *P. spartinae* is a dominant member of a complex microbial assemblage associated with *S. alterniflora* (Meyers, 1980; Meyers *et al.*, 1975). This assemblage has different distributions and metabolic characteristics in emergent plant parts vs. the roots, rhizosphere, and detrital matter (Meyers *et al.*, 1970; Meyers *et al.*, 1975; Padgett and Celio, 1990; McClung *et al.*, 1983), where different forms and ratios of carbon and nitrogen are encountered. Several of the fungi associated with *S. alterniflora* (e.g., *Kluyveromyces drosophilarum, Rhodotorula sp., Cryptococcus sp.*) can assimilate trace iron using siderophores and other metals by various mechanisms (Meyers, 1980; Hofte, 1993). It has been suggested that *P. spartinae* also produces hydroxamate siderophores (Meyers *et al.*, 1975), but this has not been directly examined. In one study, $^{59}FeCl_2$ uptake by *S. spartinae* from solid agar occurred at almost twice the rate shown by *K. drosophilarum* (50 $nghr^{-1}$ and 28 ngh^{-1}, respectively), the latter of which produces siderophores. These data, however, suggest that metal transport systems other than (or in addition to) siderophores were used by *P. spartinae* because siderophores are specific for trivalent iron with very low affinity for divalent iron. Alternatively, it is possible that a siderophore produced by *P. spartinae* has very high affinity for Fe(II). Both possibilities rely on the assumption that chemical oxidation/precipitation of $^{59}FeCl_2$ was minor in the aerobic uptake system (i.e., divalent iron was assimilated rather than the trivalent iron from chemical oxidation).

The purpose of this work was to examine the uptake of iron and other metals by *P. spartinae*, and to provide data on the possible mechanisms of this activity. The biochemical ecological significance of metal accumulation by the fungal assemblage associated with *S. alterniflora* is discussed.

METHODS

Isolation of Cells *P. spartinae* cells were isolated from vascular spaces of *S. alterniflora* culms (Ahearn *et al.*, 1970) collected from Terrebonne

Parish, LA, in late spring. Sterile swabs were introduced to the inner root and vascular spaces after exterior parts were washed with 100% ethanol and removed with a scalpel. The swabs were streaked across glucose-amine-trace minerals (GAT) agar and the isolation medium described by Ahearn *et al.* (1970). The GAT medium contained 20 g glucose, 2 g succinic acid, 0.1% mixed amines (diethylamine:di-n-butylamine 1:1 v/v), trace inorganic nutrients (B, Mn, Cu, Zn, Cr, S, NH_4Cl, Mo, Co, Cu, Ca, Mg), 3 g deferrated peptone, and 2 g of deferrated yeast extract per litre of distilled/deionized water. Cells were examined under a light microscope and compared with a reference *P. spartinae* isolate from ATCC (Bethesda, MD). Other media used for routine growth and isolation included a reduced iron yeast extract broth (normal yeast extract deferrated using 8-hydroxyquinoline to 0.02–0.1 mg l^{-1} total iron), and normal yeast extract broth (10 gl^{-1}; 1 mg l^{-1} total iron).

Cells were incubated at 25°C on gyrorotary shakers until the stationary phase, which occurred between 24 and 96 h, depending on the medium and available iron levels.

Iron Deficient Media Glass was washed with high purity nitric acid (4 N), distilled/deionized water, and 50 mM EDTA. Media were prepared in deionized water followed by deferration using a) column chromatography over Amberlite XAD-16 and Chelex-100 resins (BioRad) or, b) multiple liquid–liquid extractions with 3% 8-hydroxyquinoline in chloroform, followed by autoclaving with basic alumina (Aldrich) and filtration/adsorption over Amberlite XAD-16. These treatments gave total iron concentrations of $< 2 \times 10^{-8}$ M in acidified extracts (i.e., $< 1 \mu g\, l^{-1}$). This amount of iron was below levels considered to be "iron limited" in media for siderophore studies (Fekete *et al.*, 1983), and was insufficient to support growth of *P. spartinae* and *Rhodotorula piminae*. Various forms of trivalent iron, including $FeCl_3$, Fe (III)-citrate, and Fe $(OH)_3$ were added to this iron deficient medium (2–200 $\mu g\, l^{-1}$) to give "iron limited" media for metal uptake and siderophore screening assays described below. Deferration also removes other essential cations (e.g., Cu, Zn, Mo), which were replaced using a trace inorganic nutrient solution prepared with ultrapure (99.99%) reagents. Siderophore-bound iron was collected by 0.22 m filtration of S-phase *R. piminae* cultures grown under iron limited conditions.

Screening for Hydroxamates. The spectrophotometric assay of Csaky (1948) was used for detection of bound and free hydroxamates in iron limited cultures. This procedure generates nitrite from hydroxylamines in solution which is detected by complexation with sulphanilic acid and α-naphthylamine followed by spectrophotometry. Preliminary screening was performed using a $HClO_4$–$Fe(ClO)_4$ colorimetric assay (Ishimaru and Roper, 1993) for free hydroxamates, but was discontinued because of relatively poor sensitivity. The universal siderophore detection assay (CAS liquid assay) of Schwyn and Neilands (1987) also was used to screen supernatants from iron limited cultures. Yeast cells were grown in a) iron deficient medium (i.e., negative control-no growth), b) the same medium augmented with small quantities of trivalent iron ($Fe(OH)_3$ or Fe (III)-citrate) and c) normal yeast extract. The iron supplements were made at several levels, with final total iron concentrations in the range of 2 to 20 $\mu g\ l^{-1}$. The yeast cells were grown for at least 96h, centrifuged (9,000 rpm/10 min), following which the supernatants were membrane filtered (0.2 μm). The resulting cell-free supernatants were used for the free hydroxamate and CAS solution assays, and wet cell pellets were used for the bound hydroxamate assays. The latter were performed on freeze-dried cell pellets. Positive controls included standard solutions of hydroxylamine hydrochloride (Aldrich), and cultures of *Rhodotorula piminae* grown under iron limited conditions.

Metal Uptake Assays. $Fe(OH)_3$ was prepared daily by mixing $FeCl_3$ in water and titrating with an excess of concentrated KOH solution. The resulting precipitate was filtered and washed with distilled water. This process was repeated until the wash was free of colour, and the pH was in the range of 8–9. The $Fe(OH)_3$ solids were air-dried, ground in a mortar and pestle, sterilized, and weighed into the deferrated media in desired amounts. Other iron sources included Fe (III) -citrate, Fe (III) -dextran and Fe (III) -complexed 1,10- phenanthroline (Sigma).

Trace metal stock solutions were prepared by mixing $CuSO_4$, $NiCl_2$, $ZnCl_2$, K_2CrO_4, $PbCl_2$, Na_2MoO_4, $CoCl_2$, $CdCl_2$ in distilled water and acidifying to pH 2 with ultrapure hydrochloric acid. This was filter sterilized (0.22 μm), and aliquots (0.25–2 ml) were added to iron limited media and normal yeast extract medium to nominal concentrations between 0.5 and 10 $\mu g\ ml^{-1}$. Controls received identical additions of the distilled water at pH 2 without metals. After 96 h growth, *P. spartinae* cells and medium were removed from the flasks

by pipette, centrifuged (8,000 rpm) and resuspended/centrifuged twice in distilled water (pH 6.0) to remove any surface-adsorbed metals and particulates. The washed cell pellets were drained, weighed, and acidified with small volumes of high purity HNO_3/H_2SO_4 (concentrated 1:1 v/v). These extracts then were analyzed using inductively coupled argon plasma emission spectrometry (ICP).

TEM and EDS Analyses. Cells for routine transmission electron microscopy (TEM) were centrifuged ($1{,}000 \times g$) and washed three times in 0.02 M s-collidine buffer (pH 7.4, in the presence of 0.2 M sucrose and 1 mM $CaCl_2$) (Schwab *et al.*, 1970). Cells were fixed for 7 h with 50% aqueous DMSO containing 2% acrolein and 2% glutaraldehyde buffered to pH 7.4 with 0.02 M s-collidine. After fixation, the cells were washed three times with s-collidine buffer and stored overnight in buffer at 4°C. For post-fixation, the cells were centrifuged from the buffer and resuspended in 3% osmium oxide in veronal acetate buffer (pH 4.0) for seven hours at 4°C. The cells then were rinsed three times in s-collidine buffer (10 minutes each), dehydrated in a gradient series of ethanol, and embedded in Spurr's resin. Ultrathin plastic sections were cut, post-stained with uranyl acetate and lead citrate, and viewed on a Philips 410 transmission electron microscope.

Cells to be analyzed for trace metals were processed as described above, except treatment with heavy metals (Os, U, and Pb) was omitted and platinum grids were not used. Analyses were carried out using an EDAX PV6100 energy dispersive X-ray spectrometer (EDS) attached to a Philips EM410 transmission electron microscope. The strong signal from copper fluorescence on metallic grids masked analyte peaks. Hence, beryllium grids and specimen holders were used to eliminate this interference (Zaluzec, 1979). Each analysis included background subtraction, and peak intensities were integrated within a window placed over the iron Kα 1 peak centered at 6.403 keV. No analytical standards were deployed and therefore only relative comparisons can be made between specimens. Analytical conditions were as follows: 60 kV accelerating voltage, 10 μA emission current, spot size 6, 300 second count time at 25–30% dead time.

Assay for Low Molecular Weight Cysteine-Rich Proteins (LMWCRPs). LMWCRPs, including metallothioneins, occur in a range of microorganisms and function as metal scavengers (Jackson and Kuske, 1993). These

polypeptides are characterized by high metal binding constants, low molecular weights (on the order of 900–6500 daltons), high levels of sulphur containing amino acids (e.g., 30% cysteine) and low levels of aromatic amino acids.

Pichia spartinae and a standard *S. cerevisiae* strain (OTY7) that produces metallothionein were grown to log phase (O.D. 640 = 1) in a synthetic complete medium lacking cysteine and methionine. These were pulse-labelled in the absence or presence of 250 μM copper sulphate in the presence of ^{35}S-labelled cysteine. Cells were harvested and lysed by vortexing in the presence of an equal volume of acid washed glass beads and Tris buffer (10 mM, pH 7.8, 10 mM PMSF, 1 mM dithioreitol) as described elsewhere (Zhou and Thiele, 1993). Extracts were treated with 500 μM copper sulphate (45 min) to reduce protein thiols, followed by electrophoresis on 20% native polyacrylamide gel. The gel was fluorographed, dried, and exposed to X-ray film for 48 h at -80°C.

RESULTS

Pichia spartinae cells demonstrated growth in iron deficient media containing low levels of either ferric or ferrous iron ($FeCl_2$, $Fe(OH)_3$, Fe (III)-citrate) as well as Fe (III)- complexed dextran and Fe (III)-1, 10-phenanthroline. Growth was inhibited in iron deficient medium alone, but occurred at substantial rates in the presence of these iron forms under both aerobic and anaerobic conditions. Aerobic cultures grew to a stationary phase within 48 h at 25°C, while anaerobic cultures reached a stationary phase only after approximately four days. Under the latter conditions, vigorous fermentation of glucose was observed, with the pH typically near 5.0 after 96 h.

Uptake of trivalent iron by *P. spartinae* was confirmed visually and analytically. Solid Fe (III)-citrate was added to the surface of iron limited agar, followed by inoculation. Cell lawns developed around the particles and within a week the red iron particulate matter was assimilated and no longer visible. ICP analysis of cells washed from these plates showed levels of iron up to 25 times higher than those grown on yeast extract agar without added iron. The mobilization of Fe (III) particles and concomitant loss of colour indicated that the iron had been reduced to Fe (II) at some point during assimilation.

Intracellular deposition of iron by the yeast was confirmed by the results of a) growth experiments in iron-deficient media containing small amounts (e.g., 10 mg l^{-1}) of $Fe(OH)_3$ followed by metal analysis of cells (Table I), TEM of thin sections of these cells showing electron dense bodies in the cytosol (Fig. 1), and EDS analyses of the cytosol and the electron dense bodies that confirmed the presence of high levels of iron vs. control cells (Fig. 2, Table I). The controls in these experiments were a) iron-deficient medium (no growth), and a 0.1% yeast extract broth containing approximately 0.1 mg l^{-1} total iron, primarily as soluble organic complexes (positive control). The 15-fold higher levels of iron in cells grown with $Fe(OH)_3$ suggested that the mechanism of uptake was different for insoluble iron than that bound to organic molecules, as in the positive controls. Thus, relative to cells grown in the iron limited medium containing $Fe(OH)_3$, positive control cells contained few electron dense bodies in the cytosol, and the iron concentration of the cytoplasm was low (as measured by ICP of cell pellets and EDS of thin sections, Table I).

Similar electron-dense bodies have been reported in *Saccharomyces vinii* exposed to iron and copper (as divalent sulphates) (Sarishvili *et al.*, 1992). These bodies were localized near the cell membrane (endocytotic sites) and within cytosolic vacuoles and were thought to be iron particles and iron complexed with biopolymers. Also reported was competition between iron and copper uptake. In the cited work, a membrane-associated redox mechanism was assumed to be involved

TABLE I Concentrations of iron detected in cell pellets and thin sections using ICP and EDS

	Treatment			
Medium[+]	*Control Cells*[±]	*iron-Limited Cells*[±]	*Control*[*] *Cells (EDS)*	*iron-Limited*[*] *Cells (EDS)*
0.114	1.34	26.58	5.84	139
0.185	1.50	21.27	6.05	151
0.110	1.69	14.55		
0.100	1.70	27.37		
$\mu = 0.13$	1.56	22.44	5.95	145
$\sigma = 0.04$	0.17	5.92	0.15	8.4

[+]ICP data in μg Fe g^{-1} cells or μg Fe ml^{-1} medium; [*]EDS integrals of α peaks with background subtraction. μ and σ are mean and standard deviation for n = 4 (ICP) and n = 2 (EDS)

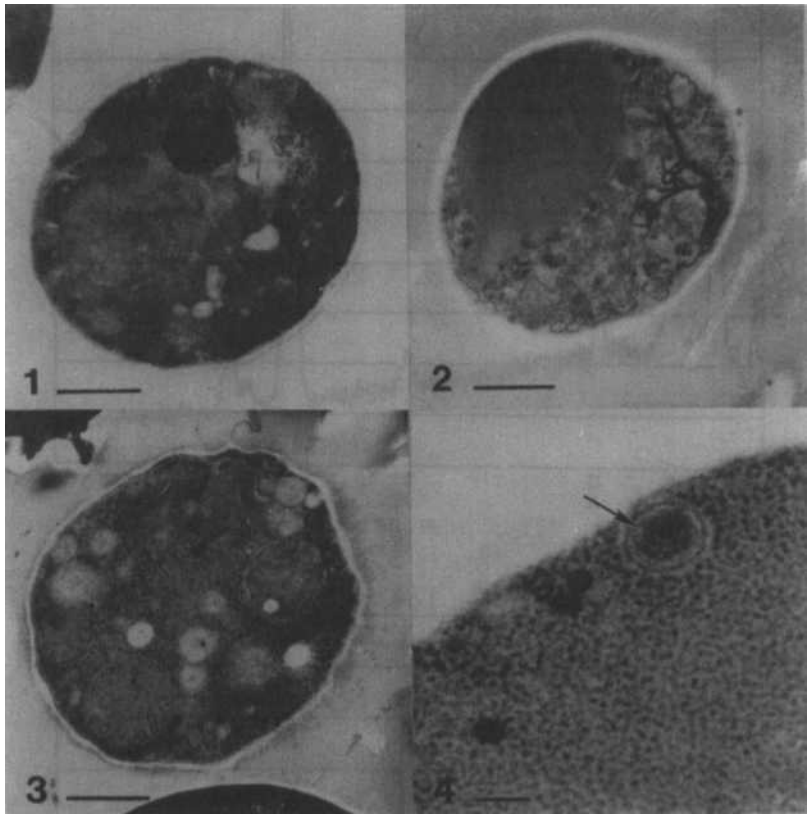


FIGURE 1 Transmission electron micrographs of *P. spartinae* cells. 1. Control cells grown in normal yeast extract medium for EDS analysis (magnification 27,000X, bar = 0.5 μm). 2. Cells grown in iron-limited media containing $Fe(OH)_3$ for EDS analysis (27,000X, bar = 0.5 μm). 3. Post-fixed sections of cells grown in iron limited media containing $Fe(OH)_3$ showing electron-dense bodies in vacuoles (27,000X, bar = 0.5 μm). 4. Post-fixed sections showing a vacuole containing electron dense bodies in ordered arrays (arrow 95,500X; bar = 0.1 μm).

in metal transport (after Lesuisse and Labbe, 1989), and the observed 75% decreases in copper uptake in the presence of iron was attributed to competition for binding sites. In the current study, significant competition between iron and copper was not observed: the intracellular levels of copper in the positive controls were approximately the same as that in the Fe $(OH)_3$ treatments, although the total amount of available iron (soluble or complexed) was much higher in the former.

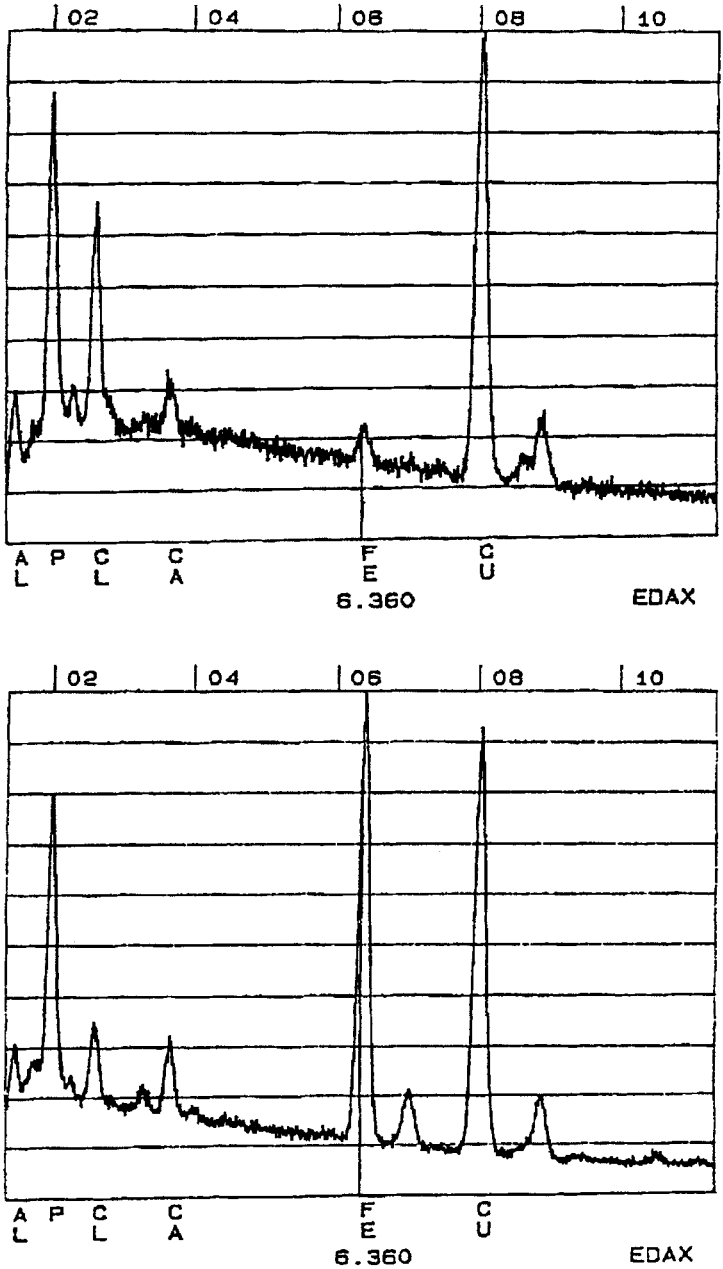


FIGURE 2 EDS spectra of thin section of *P. spartinae* cells grown in iron replete medium (top) and iron limited medium containing Fe $(OH)_3$ (bottom). The ordinate scales are identical.

Further, TEM data showed vacuoles containing electron dense bodies in *P. spartinae* cells grown under iron limited conditons (Fig. 1). The distribution of particles in the vacuoles appeared structured, as would be expected in a protein-metal complex such as ferritin. These results suggest that iron and copper were assimilated by separate mechanisms in *P. spartinae*, and that iron assimilation mechanisms were induced or promoted by low available iron.

Results of trace metal uptake assays in 1% yeast extract broth (approximately 1 $mg\,l^{-1}$ organic-complexed iron) supported the former hypothesis. It was found that *P. spartinae* accumulated zinc and copper up to 10-fold above the levels supplied in the growth medium, while nickel and cadmium showed significant enrichment (Fig. 3). In these experiments, the iron levels detected in *P. spartinae* cell pellets were constant ($3 \pm 0.2\ \mu g\ g^{-1}$) and well below those found in the iron limited cultures containing $Fe(OH)_3$.

Inducible siderophore production could account for differential levels of iron uptake observed in iron replete vs. iron limited media, as reported for other fungi (Atkin *et al.*, 1970). The Csaky screening assay for hydroxamate siderophores, however, gave negative results for *P. spartinae* vs. positive results for a) *R. piminae* grown in iron limited media and, b) hydroxylamine hydrochloride standard solutions. Based on an absorbance maximum at $\lambda = 520$ nm and standard curves of hydroxylamine hydrochloride, the hydroxamate level in the supernatants of *R. piminae* cultures was on the order of 1 $\mu g\,ml^{-1}$ after 72 hours (Fig. 4). The detection limit for the Csaky assay was remarkable: 38 $ng\,ml^{-1}$ hydroxylamine hydro- chloride in distilled water. The CAS and $HClO_4$ hydroxamate assays also did not detect siderophores in *P. spartinae* cultures.

The nonspecific nature of iron uptake by *P. spartinae* (i.e., divalent, trivalent, and organic-complexed forms), and the lack of siderophores in iron limited cultures suggested that a redox mechanism was involved in iron transport. It is known that *S. cerevisiae* and other fungi have nonreductive and reductive iron transport mechanisms, but lack siderophores (Lesuisse and Labbe, 1989; Lesuisse *et al.*, 1987). The reductive system is bound to the plasma membrane, and is induced by low available iron while the nonreductive system transports organic-Fe complexes and operates constitutively. In the current study, experiments with the redox probe INT showed active membrane redox

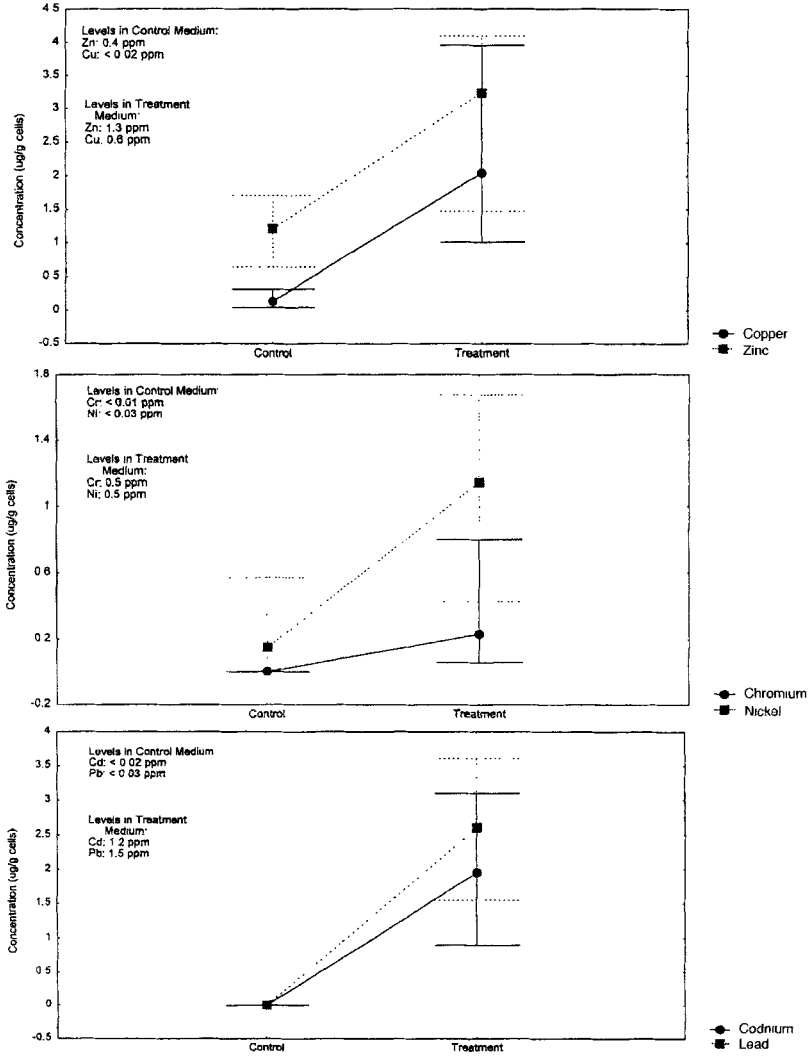


FIGURE 3 Results of metal uptake studies in control (yeast extract) and treatment (metal-amended yeast extract) media. Means and error bars reflect $n = 5$ replicate samples. All control vs. treatment differences were highly significant in t-tests ($p = 0.005$) with the exception of chromium, which showed no significant difference.

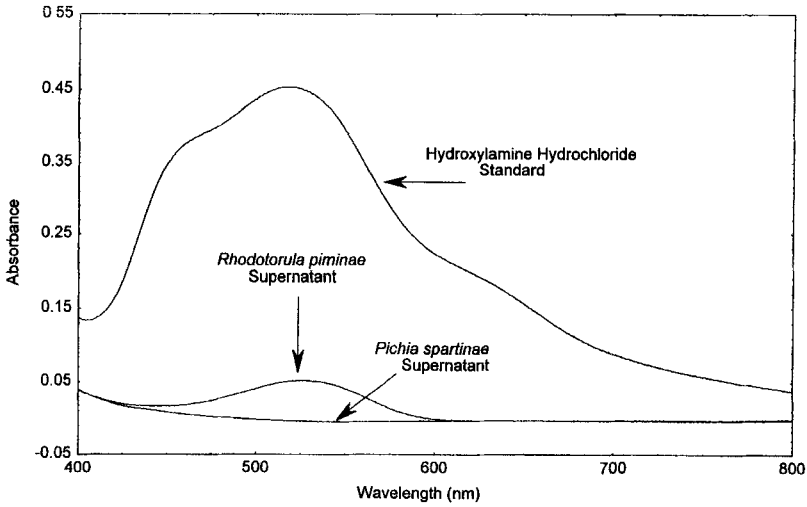
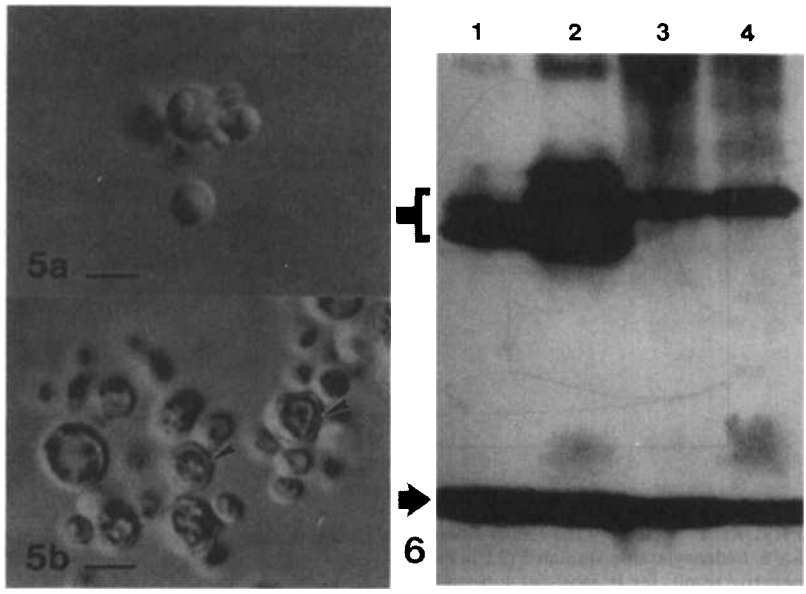


FIGURE 4 Results of the Csaky siderophore screening assay showing positive results for a hydroxylamine standard (22 μg ml^{-1}) solution and *R. piminae* supernatants and negative results for *P. spartinae* supernatants.

systems in *P. spartinae*. No activity was observed at the cell capsule or ascus (sexual reproductive body), but the plasma membrane and mitochondrial membrane sites showed rapid (5 min) and dense staining (Fig. 5). Spectrophotometric assays (Catallo *et al.*, 1990) indicated that the rate of INT reduction was enhanced in cells grown under iron limited conditions vs. those grown in iron replete conditions. The growth of *P. spartinae* on Fe (III)-dextran (average MW = 5000) indicated that the cell capsule was permeable to large organic compounds and particulates, which would allow for contact between bulky solutes (e.g., iron oxyhydroxide colloids, exogenous siderophores) and plasma membrane associated redox sites. Thus, it was not surprising to find that iron-complexed siderophore from *R. piminae* cultures could be utilized by *P. spartinae* for growth in iron-deficient media (cf. Plessner *et al.*, 1993).

The uptake of trace metals, including copper, zinc, and perhaps other toxic metals (Fig. 3) indicated that nonspecific metal scavenging mechanisms were present in *P. spartinae*. These mechanisms also could be redox-mediated, but the many similarities between *P. spar-*



FIGURES 5 and 6 Photomicrographs of live *P. spartinae* cells. 5a: normal cells. 5b: normal cells after 10 min incubation with 13 mM INT showing intracellular and plasma membrane-associated reduction activity (arrows 1100 X).
6. Autoradiogram of ^{35}S-cysteine labelled low molecular weight proteins. Lane 1. *S. cerevisiae* cells without pre-exposure to 50 μM $CuSO_4$. Lane 2. *S. cerevisiae* cells after incubation with $CuSO_4$. Lane 3. *Pichia spartinae* cells without pre-exposure to $CuSO_4$. Lane 4. *P. spartinae* cells incubated with $CuSO_4$. Arrow is free ^{35}S-cysteine; bracket shows position of *S. cerevisiae* metallothionein.

tinae and *S. cerevisiae* suggested that LMWCRPs also might be detected in the former. Electrophoretic assays of cell extracts showed constitutive expression of LMWCRPs (i.e., non copper-inducible) in *P. spartinae* that had electrophoretic properties similar to metallothionein from *Saccharomyces cerevisiae* (Fig. 6). These data suggested constitutive rather than inducible production of metal scavenging polypeptides by *P. spartinae*, an adaptation that would be beneficial in environments (e.g., ocean water, *S. alterniflora* rhizospheres) containing trace levels or unavailable forms of nutritive metal cations. Although the electrophoretic behaviour of the LMWCRPs from *P. spartinae* was very similar to metallothionein from *S. cerevisiae*, the LMWCRPs cannot be identified as metallothionein until the polypeptides have been isolated and sequenced.

DISCUSSION

The data presented on *Pichia spartinae* suggest: a) the yeast can assimilate divalent and trivalent forms of inorganic iron, as well as large organic-Fe (III) complexes; b) the uptake of inorganic trivalent iron under otherwise iron deficient conditions proceeds by a different mechanism than uptake of soluble iron; and the intracellular loadings of iron are much increased under the former conditions; c) trivalent iron uptake is not mediated by hydroxamate siderophores at levels detectable by the Csaky assay; d) the assimilation of some trace metals is likely to be mediated by constitutively expressed LMWCRPs, possibly metallothionein; e) siderophores from other fungi can provide iron for *P. spartinae*. The data on iron assimilation suggest that multiple mechanisms were involved, and these were influenced by the concentration and speciation of iron in the system. In general, iron assimilation mechanisms seemed very similar to mechanisms described for closely related yeasts, such as *S. cerevisiae*. This involves a) endocytosis of trivalent iron followed by vacuolar storage/transport and reduction to divalent iron, or b) reduction of trivalent iron bound to organic compounds, such as siderophores or fulvic matter. The presence of LMWCRPs in *P. spartinae* was of great interest from a number of perspectives, including a) such proteins also could be involved in iron assimilation and b) the range of trace metals found in *S. alterniflora* tissues (Zn, Cu, Cd, Ni, Fe, Mn) is very similar to that of metals assimilated by *P. spartinae in vitro* (Lima *et al.*, 1989; Lee *et al.*, 1981; Huiskes and Nieuwenhuiza, 1985; Alberts *et al.*, 1990). It seems that complexation of metals with soluble organic and inorganic disulphides is a significant transport mechanism in salt marshes, particularly within detritus (Boulegue and Church, 1981). Thus, organisms secreting polypeptides rich in disulphide bonds provide a biochemical analogue to a central chemical transport process in salt marshes.

These observations, and previous work on the plant-fungi assemblage in salt marshes suggest that *P. spartinae* can participate in mobilization, transport, and cycling of micronutrient and toxic metals in *S. alterniflora*. A plausible scenario would involve biological uptake of iron and other trace metals from deposits on the rhizosphere, followed by transport and deposition to interior plant spaces. This metal "shuttle" would involve numerous species: *P. spartinae* colonizes

marsh sediments, the rhizosphere, and especially the aerial vascular spaces of *S. alterniflora* at high levels, while *K. drosophilarum* and *Rhodotorula sp.* (both of which produce siderophores) are largely confined to the rhizosphere. Thus, iron would be available to *P. spartinae* as a) $Fe(OH)_3$ precipitated at the rhizosphere, b) iron bound to siderophores from other fungi in the rhizosphere and /or c) inorganic or organic iron from lysed cells or disulphide complexes in vascular plant fluids.

The plant has been shown to assimilate zinc, copper, iron, manganese, cobalt, mercury and chromium salts from sediments, hydroponic solution, and dredge spoil, but not all studies agree on the degree of uptake for each metal (Lima *et al.*, 1989; Lee *et al.*, 1976; Lee *et al.*, 1981; Lee *et al.*, 1983; Huiskes and Nieuwenhuiza, 1985; Alberts *et al.*, 1990; DeLaune *et al.*, 1990; Folsom *et al.*, 1981; Giblin *et al.*, 1980; Gambrell, 1994; Catallo, 1993). The significance of the plant to metal chemistry in marshes continues after death: as detrital plant matter decays, the concentrations of metals increase via a) removal of labile carbon leaving lighter residues of refractile compounds and metals and, b) scavenging of allochthonous metals by carboxyl, phenol, disulphide, and amine moieties on the plant matter, also promoted by microbial transformation (Breteler *et al.*, 1981, Drifmeyer *et al.*, 1982). As a result, plant-incorporated metals may enter the ecosystem via grazing, detrital, and/or aquatic food chains (Pellenbarg, 1978).

We are continuing with molecular and plant biological studies of *P. spartinae* with respect to growth, productivity, and trace metal assimilation in *S. alterniflora* reared in greenhouses. It is clear from the present work that the effects of symbiotic microbes on plant properties of biological and ecological interest (growth/productivity, ecophysiology, metal cycling, biochemistry, toxicology, natural products) must be considered in future studies of salt marsh plant systems.

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