Arsenic-resistant bacteria solubilized arsenic in the growth media and increased growth of arsenic hyperaccumulator *Pteris vittata* L.

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**Abstract**

The role of arsenic-resistant bacteria (ARB) in arsenic solubilization from growth media and growth enhancement of arsenic-hyperaccumulator *Pteris vittata* L. was examined. Seven ARB (tolerant to 10 mM arsenate) were isolated from the *P. vittata* rhizosphere and identified by 16S rRNA sequencing as *Pseudomonas* sp., *Comamonas* sp. and *Stenotrophomonas* sp. During 7-d hydroponic experiments, these bacteria effectively solubilized arsenic from the growth media spiked with insoluble FeAsO 4 and AlAsO 4 minerals (from <5 μg L −1 to 5.04–7.37 mg L −1 As) and enhanced plant arsenic uptake (from 18.1–21.9 to 35.3–236 mg kg −1 As in the fronds). Production of (1) pyochelin-type siderophores by ARB (fluorescent under ultraviolet illumination and characterized with thin layer chromatography) and (2) root exudate (dissolved organic C) by *P. vittata* may be responsible for As solubilization. Increase in *P. vittata* root biomass from 1.5–2.2 to 3.4–4.2 g/plant dw by ARB and by arsenic was associated with arsenic-induced plant P uptake. Arsenic resistant bacteria may have potential to enhance phytoremediation of arsenic-contaminated soils by *P. vittata*.

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1. Introduction

Arsenic (As) pollution causes public health problems in millions of people around the world (Nordstrom, 2002). Chronic exposure to As can cause skin and lung cancers and other non-carcinogenic symptoms such as diabetes, skin diseases, and nervous and cardiovascular problems (Vahter, 2007). The health hazards associated with arsenic-contaminated soil coupled with the high remediation cost makes it necessary to develop cost-effective technologies to restore polluted land.

Phytoremediation is a cost effective technique, which employ plants to remediate polluted soils and to improve soil properties. It is effective in removing metals of low concentrations from surface soils and water, although longer treatment times are required compared to other conventional techniques such as soil excavation and disposal to landfill. The arsenic hyperaccumulator, *Pteris vittata* L. (Chinese Brake fern), is capable of accumulating up to 2.3% As in its dry biomass (Ma et al., 2001), making it a viable candidate for phytoremediation of As-contaminated sites. It is capable of extracting As from both soluble and insoluble forms (Tu et al., 2004a), and has been utilized in field applications to remediate As-contaminated soils (Shermerdine et al., 2009; Kertulis-Tartar et al., 2006) and groundwater (Natarajan et al., 2009). Arsenic uptake in *P. vittata* mainly occurs via the roots, so soil rhizosphere characteristics significantly affect its As accumulation.

The availability of nutrients and As are likely impacted by the microbial community in the rhizosphere of *P. vittata*. Free-living as well as symbiotic plant growth promoting rhizobacteria (PGPR) can enhance plant growth directly by providing available phosphate (P) for plant uptake, lowering plant ethylene levels, and sequestering Fe for plants by producing Fe chelating substances (Huang et al., 2004). The Fe solubilization activity of PGPR can impact As levels in soils. In aerobic soils, arsenate (AsV) is present as HAsO 4 2 − and H 2 AsO 4 −, and is often bound to Fe/Al minerals, making it insoluble (Tu et al., 2004a); however, Fe solubilization also releases the As from the minerals, making it bioavailable to plants and growth microorganisms. Thus As-resistant microorganisms might have a selective advantage to survive under these conditions.

Although some bacteria associated with *P. vittata* have been identified and characterized to a limited extent (Huang et al., 2010), their effects on As uptake have not been examined. In the current study, seven As-resistant bacteria from the rhizosphere soil were isolated and identified, and their As transformation ability to improve plant growth and As uptake via solubilizing Fe and As from insoluble minerals was determined.

2. Methods

2.1. Soil collection and characterization

The soil samples were collected from areas near Gainesville, Florida where *P. vittata* grow naturally, and they were collected from Archer Feed store (AF), Archer Ministorage (AM), Rainbow...
Springs (RS) and Crystal River Quarries (CQ). The sites AF and AM are located in Archer, FL, and RS site in Rainbow Springs State Park, Dunnellon, FL, and CQ site in Crystal River Quarries, FL, which is a dolomite mining site. P. vittata in the AF site grew under an arsenic-treated wood structure. During rain the As may be washed out of the treated wood (Chirenje et al., 2003), which may be the source of As in this site. The AM site is close to an area, which had a previous history of As contamination (Jang et al., 2002). The RS site is located in a park and has no record of As pollution, but P. vittata grew very close to an old wooden bridge. So this may be another example of As pollution from arsenic-treated wood. From that perspective, the site CQ is unique. The fern grew but

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### Table 1

<table>
<thead>
<tr>
<th>Soil Type</th>
<th>pH</th>
<th>Total As (mg kg⁻¹)</th>
<th>Water soluble Fe (mg L⁻¹)</th>
<th>Water soluble Al (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bulk soil</td>
<td>7.25 ± 0.10</td>
<td>235 ± 25.6</td>
<td>6.79 ± 0.33</td>
<td>0.20 ± 0.00</td>
</tr>
<tr>
<td>Rhizosphere soil</td>
<td>7.06 ± 0.03</td>
<td>46.3 ± 1.58</td>
<td>2.50 ± 0.39</td>
<td>1.88 ± 0.07</td>
</tr>
<tr>
<td>Bulk soil</td>
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<td>1.88 ± 0.07</td>
</tr>
</tbody>
</table>

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In addition to As, total Fe and Al concentrations in the plants were measured in the same extracts. To determine the amount of Fe/Al precipitating on root surfaces, plant roots (0.2 g) were washed in 50 mL 1:1 v/v HNO₃ and water for 20 min and Fe and Al concentrations in the supernatants and plant digest were determined using inductively-coupled plasma emission spectrophotometry (PerkinElmer 5300DV, Waltham, MA) via EPA Method 2007.

To confirm the role of root exudate in solubilizing As from FeAsO₄ and AlAsO₄ minerals, dissolved organic C (DOC) in the spent growth medium was analyzed using a total carbon analyzer (TOC-5050A, Shimadzu) equipped with an autosampler (Tu et al., 2004a).

2.4.2. Arsenic solubilization and plant growth effects of ARB and Bacillus sp.

To further test the efficiency of ARB in As solubilization, the experiment described in Section 2.4.1 was repeated with soil samples AF, AM, CQ, and RS, which were purchased from Archer Feed Store, Archer Mineral Storage, Crystal River Quarry, and Rainbow Springs.
bacteria Bacillus sp. (GenBank accession number JN132398) as a treatment, which was grown to an OD600 of 1.5. We selected Bacillus sp. as our control bacterium because it is a commonly found soil bacteria isolated from a non arsenic-contaminated soil, and there has been no report of P. vittata being infected by any bacterial pathogen present in soil. After 7-d of growth, plant biomass (dw) was determined after drying in an oven at 80 °C for 2 d.

To determine the potential role of P in enhancing plant growth, dried plant samples were digested using a H2SO4/H2O2 to analyze P using modified molybdenum blue method. Briefly the pH of the digestion solution was adjusted to ~5 with NaOH and HCl. Ten milliliter of the solution was pipetted into a 20 mL glass test tube, and to this 0.5 mL of t-cysteine (5% w/v in 0.6 M HCl) was added. The test tube was capped tightly to allow AsV reduction to AsIII for 5 min at 80 °C. The solution was cooled to room temperature, and P was determined by the molybdenum blue method.

2.4.3. Plant growth enhancement by arsenic

To separate the effect of As from that of Fe, six-month old P. vittata plants were acclimated in 1 L of 0.25 l/flowerpot of Fe-free Hoagland medium at pH 7 for 2 weeks. The treatment had 0 or 3 mg/l water-soluble AsV with and without the ARB consortium. The fresh plant weight was recorded after 7 d of treatment and corresponding dry weights were measured after drying in an oven at 80 °C for 2 d.

2.4.4. Presence of siderophores

To test the presence of siderophores in spent growth medium, the medium was extracted with chloroform. The water soluble fraction containing the siderophores was dried by injecting air through a nitrogen evaporator (N-EVAP™, Organamation Associates Inc., MA, USA) to increase the concentration of siderophores in the solution. Then the concentrated extract was analyzed using thin layer chromatography (TLC) on cellulose plates developed in a solvent mixture of butanol:acetic acid:water (3:1:2 v/v) system. To test for the presence of pyochelin type siderophores, the fluorescence spots on the TLC were sprayed with 0.1 M FeCl3/HCl solution.

2.5. Statistical analysis

All the experiments followed completely randomized design (CRD) with three replicates for each treatment. The analysis of variance (ANOVA) and Tukey’s mean grouping were used to determine significance of the interactions between the treatment means. All statistical analyses were performed with SAS statistical software (SAS Inst., Cary, North Carolina, USA).

3. Results and discussion

3.1. Soil characterization

The four soils contained different levels of total As (Table 1). The RS soil contained the highest As at 235 mg kg⁻¹, followed by CQ, AM and AF soils (46.3–4.42 mg kg⁻¹). In comparison, the bulk soil, taken 10 feet away from the plant, had very low As concentrations (0.07–4.07 mg kg⁻¹). It is possible that P. vittata may preferentially grow in areas with high As concentration or the presence of P. vittata helped concentrate As near the rhizosphere.

The water-soluble Fe content in the rhizosphere of CQ (1.88 mg kg⁻¹) and AM (1.27 mg kg⁻¹) sites was higher than that in the corresponding bulk soils (0.07–4.07 mg kg⁻¹) (Table 1). This increase in available Fe in the rhizosphere soil may be due to the presence of Fe-solubilizing siderophore produced by rhizobacteria and root exudate by P. vittata. The other two soils had very low water soluble Fe (Table 1). The pH in the rhizosphere soils ranged from 7 to 8, which were higher than the typical acidic soils in Florida (4.5–5.5; Chen et al., 1999). This is consistent with the fact that P. vittata prefers to grow in alkaline soils (Bondada and Ma, 2002). The pH in the bulk soil was 6.81–7.56, but slightly lower than those in the rhizosphere (7.06–7.97; Table 1). This is consistent with Gonzaga et al. (2006) who observed a pH increase from 7.15 in bulk soil to 7.65 in the rhizosphere of P. vittata after growing for 8 weeks in As-contaminated soils. At pH 7–8, most of the nutrients become unavailable with the exception of phosphorus and As, which becomes relatively more available at this pH (Bagayoko et al., 2000).

3.2. AsIII oxidation and AsV reduction in the rhizosphere and bulk soils

When fresh soil was incubated in LEB medium containing 1 mM AsV or AsIII, both AsV reduction and AsIII oxidation occurred. However, their rates differed between the rhizosphere and bulk soils. While both soils had high levels of AsV reduc-

3.3. Isolation and identification of As-resistant bacteria (ARB)

Three ARB were isolated from the RS and CQ soils and one from AM soil. None was obtained from the AF soil likely because the AF rhizosphere soil had the lowest total As concentration (Table 1) and AF bulk soil had the highest AsIII oxidation rate (Fig. 1B). Phylogenetic analyses of the strains suggested that the strains belonged to the group gamma-proteobacteria, and three genera Pseudomonas, Comamonas and Stenotrophomonas sp. (Table 2).
3.4. Solubilization of As from FeAsO₄ and AlAsO₄ by P. vittata and As-resistant bacteria

Both P. vittata and As-resistant bacteria were effective in solubilizing As from insoluble FeAsO₄ and AlAsO₄ minerals added to the hydroponic growth medium (Table 3). In the absence of P. vittata and ARB (no plant and no ARB), neither FeAsO₄ nor AlAsO₄ mineral was solubilized (As = 0.005 mg L⁻¹). In the presence of P. vittata (P. vittata with no ARB), a substantial amount of As was solubilized, with more As from AlAsO₄ (5.04 mg L⁻¹) than FeAsO₄ treatment (2.58 mg L⁻¹) being solubilized (p = 0.01; Table 3). Similarly, ARB inoculation (no plant with ARB) was effective in solubilizing As in the medium, with more from AlAsO₄ than FeAsO₄ treatment (8.02 vs. 4.14 mg L⁻¹). There were two possible reasons for higher As solubilization from AlAsO₄ than from FeAsO₄ treatment. The substantially higher solubility of AlAsO₄ than FeAsO₄ probably makes it more soluble in the medium (Ksp = 1.6 × 10⁻¹⁹ vs. 5.7 × 10⁻²¹; Patnaik, 2004). In addition, it is possible that, under Fe-deficient conditions in the AlAsO₄ treatment (0.25× Fe-free Hoagland solution), the plants and ARB exuded more organic acids to obtain Fe. This idea was supported by the 12–76% more DOC in the AlAsO₄ than the FeAsO₄ containing medium (Table 3). P. vittata produced 1.84 mg L⁻¹ DOC in the FeAsO₄ compared to 3.24 mg L⁻¹ in the AlAsO₄ treatment (Table 3), which is consistent with the data by Tu et al. (2004a).

Both P. vittata and ARB were effective in solubilizing As; however, ARB was 59–60% more effective than P. vittata in As solubilization (Table 3). Though no bacteria were added to the P. vittata treatment, bacteria were present on the roots of P. vittata; however, these bacteria were not as effective as ARB in As solubilization. Inoculation of ARB in P. vittata treatment further increased As concentrations, especially in the AlAsO₄ treatment, which increased by 46% (Table 3). Significantly higher amount of As solubilized by ARB than P. vittata may be due to the fact that more siderophores were produced by ARB under Fe-starvation conditions to complex with Fe or Al, making As more soluble. The presence of siderophores was verified by examination of fluorescence of media extracts. The analysis using thin layer chromatography indicated that there were two UV-positive spots with Rₚ values of 0.44 and 0.00. These spots when sprayed with 0.1 M FeCl₃/HCl generated orange–red coloration (data not shown), suggesting the presence of at least two compounds similar to pyochelin type siderophores (Ankenbauer et al., 1991; Cox et al., 1981).

3.5. Effect of As-resistant bacteria on uptake and translocation of As, Fe and Al by P. vittata

In addition to being effective in As solubilization from FeAsO₄ and AlAsO₄ minerals, P. vittata was also able to take up As from the growth medium (Table 4). However, As concentrations in the plant were low with only small amount of As being translocated from the roots to fronds, resulting in the As translocation factor (TF; concentration ratio in fronds to roots) of 0.9 in FeAsO₄ and 0.5 in AlAsO₄ treatments. Unlike As (Table 3), neither Fe nor Al was detected in the growth medium (data not shown). However, P. vittata was able to take up some Fe and Al, with frond and root concentrations being 19.8 and 385 mg kg⁻¹ Fe in FeAsO₄ treatment, and 2.00 and 39.2 mg kg⁻¹ Al in AlAsO₄ treatment (Table 4). Apparently, P. vittata was ineffective in taking up or translocating Al or Fe (TF < 0.08; Table 4).

Inoculation with ARB significantly increased As concentrations in P. vittata (Table 4). While substantially more As was accumu-

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Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>As concentrations (mg L⁻¹)</th>
<th>DOC concentrations (mg L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No ARB</td>
<td>With ARB</td>
</tr>
<tr>
<td>FeAsO₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plant</td>
<td>0.005 ± 0.05 A</td>
<td>4.14 ± 0.19 BC</td>
</tr>
<tr>
<td>P. vittata</td>
<td>2.58 ± 0.30B</td>
<td>2.89 ± 0.21 CB</td>
</tr>
<tr>
<td>AlAsO₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No plant</td>
<td>0.005 ± 0.00 A</td>
<td>8.02 ± 0.38D</td>
</tr>
<tr>
<td>P. vittata</td>
<td>5.04 ± 0.65C</td>
<td>7.37 ± 1.64 CD</td>
</tr>
</tbody>
</table>

* The values are mean ± SE of three replicates. Same letters following means for As or DOC concentrations indicate no significant difference based on Tukey’s mean separation test.
Inoculation of ARB substantially increased the concentrations of As, Fe and Al in *P. vittata*, with the impacts being more significant in the roots than the fronds (Table 4). For example, ARB increased the As concentrations by 2–11 times in the roots compared to 4–23 times in the roots. A similar pattern was observed for *P. vittata* biomass (Table 5). Little change was observed in frond biomass but ARB increased root biomass by 89% in the FeAsO₄ treatment and 130% in the AlAsO₄ treatment. The release of Fe from the FeAsO₄ + ARB treatment may have helped *P. vittata* biomass, with a larger increase being observed in the roots than the fronds and in the AlAsO₄ than in the FeAsO₄ treatment. For example, the increase in root and frond biomass was 47% and 23% in FeAsO₄ treatment compared to 74% and 60% in AlAsO₄ treatment, respectively (Fig. 2A).

### Table 4

**Effect of As-resistant bacteria (ARB) on As, Al and Fe concentrations in *P. vittata* after growing for 7 d in 0.25× Fe-free Hoagland medium spiked with 0.25 g insoluble FeAsO₄ and AlAsO₄ minerals.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>No ARB</th>
<th>With ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As concentrations (mg kg⁻¹)</td>
<td>Fe concentrations (mg kg⁻¹)</td>
<td></td>
</tr>
<tr>
<td>FeAsO₄</td>
<td>Fronds</td>
<td>18.1 ± 1.2A</td>
<td>35.3 ± 2.6B</td>
</tr>
<tr>
<td></td>
<td>Roots-internal</td>
<td>19.4 ± 1.8A</td>
<td>450 ± 27C</td>
</tr>
<tr>
<td></td>
<td>Roots-external</td>
<td>3.34 ± 0.10D</td>
<td>363 ± 1.26B</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>0.9</td>
<td>0.08</td>
</tr>
<tr>
<td>AlAsO₄</td>
<td>Fronds</td>
<td>21.9 ± 1.8A</td>
<td>236 ± 17E</td>
</tr>
<tr>
<td></td>
<td>Roots-internal</td>
<td>43.4 ± 6.4B</td>
<td>180 ± 12E</td>
</tr>
<tr>
<td></td>
<td>Roots-external</td>
<td>3.85 ± 0.3D</td>
<td>9.99 ± 0.4F</td>
</tr>
<tr>
<td></td>
<td>TF</td>
<td>0.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tissue</th>
<th>No ARB</th>
<th>With ARB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As concentration (mg g⁻¹ dw)</td>
<td>Fe concentration (mg g⁻¹ dw)</td>
<td></td>
</tr>
<tr>
<td>FeAsO₄</td>
<td>Fronds</td>
<td>6.93 ± 0.42</td>
<td>5.83 ± 0.35A</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>3.77 ± 0.07</td>
<td>3.14 ± 0.13A</td>
</tr>
<tr>
<td>AlAsO₄</td>
<td>Fronds</td>
<td>6.23 ± 0.49A</td>
<td>5.23 ± 0.54A</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
<td>1.51 ± 0.36C</td>
<td>3.43 ± 0.07B</td>
</tr>
</tbody>
</table>

a Arsenic solubilized from root surface (roots-external) was obtained by washing in 1:1 HNO₃. The values are mean ± SE of three replicates and the means of As, Fe and Al were analyzed separately. Means followed by the same letters were not significantly different based on Tukey’s mean grouping at *p* = 0.05.

b TF = translocation factor, concentration ratio in the fronds to the roots.

3.6. Effect of As-resistant bacteria and *Bacillus* sp. on growth of *P. vittata*

Inoculation of ARB substantially increased the concentrations of As, Fe and Al in *P. vittata*, with the impacts being more significant in the roots than the fronds (Table 4). For example, ARB increased the As concentrations by 2–11 times in the roots compared to 4–23 times in the roots. A similar pattern was observed for *P. vittata* biomass (Table 5). Little change was observed in frond biomass but ARB increased root biomass by 89% in the FeAsO₄ treatment and 130% in the AlAsO₄ treatment. The release of Fe from the FeAsO₄ + ARB treatment may have helped *P. vittata* growth; however, the increase of *P. vittata* biomass in the AlAsO₄ + ARB treatment may not be due to Al uptake as it is not a nutrient.

Our data clearly demonstrated that ARB was more effective in promoting *P. vittata* growth than the microbes associated with *P. vittata* roots in the no ARB treatment (Table 5). To better understand the role of ARB in promoting plant growth, we repeated the experiment with bacteria *Bacillus* sp. being added as a treatment. No difference was observed between the control and *Bacillus* sp. treatment (Fig. 2A), but ARB increased both root and frond biomass, with a larger increase being observed in the roots than the fronds and in the AlAsO₄ than in the FeAsO₄ treatment. For example, the increase in root and frond biomass was 47% and 23% in FeAsO₄ treatment compared to 74% and 60% in AlAsO₄ treatment, respectively (Fig. 2A).
Since plant biomass increase was larger in the AlAsO$_4$ treatment than that in the FeAsO$_4$ treatment (Fig. 2A), Fe probably did not play a significant role as a nutrient. We hypothesized that As-induced P uptake by *P. vittata* may have helped its growth. In a hydroponic experiment, Luongo and Ma (2005) observed that plant biomass increase after exposure to 1 mg L$^{-1}$ As for 2 weeks was associated with a substantial increase in P uptake by *P. vittata*. Compared to the control, P concentrations increased from 2.33 to 5.19 g kg$^{-1}$ in the fronds, and from 0.91 to 5.76 g kg$^{-1}$ in the roots (Luongo and Ma, 2005). Similar results were obtained by Tu et al. (2004b) as well as in this experiment. It is possible that As stress may have upregulated P uptake by *P. vittata* (Fig. 2B). For example, ARB increased root P by 45% in FeAsO$_4$ treatment and by 266% in AlAsO$_4$ treatments. The much greater increase in root P concentrations (Fig. 2B) was consistent with greater increase in root biomass (Fig. 2A). The fact that P concentrations and biomass in the roots ($r = 0.64$) was better correlated than that in the fronds ($r = 0.44$) also supported our hypothesis that arsenic-induced P uptake was probably responsible for biomass increase in *P. vittata*.

To further examine this hypothesis, the impact of As on the ability of ARB in promoting plant growth was further tested. In this experiment, same cfu/ml of ARB consortium was inoculated as the experiment in Section 2.4.1 to the growth medium with or without 3.0 mg/L As. The level of As concentration in the growth medium was similar to the experiment discussed in Section 2.4.1 (Table 3). Consistent with our hypothesis, higher frond and root biomass were obtained with As (4.8 and 3.5 g/fronds and roots dw, respectively) than without As (3.5 and 1.9 g/plant dw) treatments (data not shown), again supporting arsenic-induced P uptake.

### 4. Conclusions

*P. vittata* and ARB were efficient in solubilizing As from insoluble FeAsO$_4$ and AlAsO$_4$ with ARB being more efficient than *P. vittata*. *P. vittata* was inefficient in taking up Al, and high As and Fe in the roots suggested possible co-precipitation of As and Fe. ARB were efficient in promoting *P. vittata* growth. There was a symbiotic relationship between *P. vittata* and the fluorescent ARB in its rhizosphere. In the presence of ARB and As, the increase in P uptake by *P. vittata* was probably due to arsenic-induced P deficiency, which enabled the plant to grow better in the As-rich environment. In the future, ARB may be applied in field applications to enhance As hyperaccumulation by *P. vittata*.

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### References


