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Uptake and transformation of arsenic during the reproductive life stage of *Agaricus bisporus* and *Agaricus campestris*

*Michelle M. Nearing, Iris Koch, Kenneth J. Reimer**

Environmental Sciences Group, Royal Military College of Canada, P.O. Box 17000, Station Forces, Kingston, ON K7K 7B4, Canada.
E-mail: michelle.nearing@rmc.ca

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ABSTRACT

Fruiting bodies from the *Agaricus* genus have been found to contain non-toxic arsenobetaine (AB) as a major compound. It is unknown whether AB is formed during the vegetative or reproductive life stages of the fungus, or by the surrounding microbial community, but AB's structural similarity to glycine betaine has led to the hypothesis that AB may be adventitiously accumulated as an osmolyte. To investigate the potential formation of AB during the reproductive life stage of *Agaricus* species, growth substrate and fungi were collected during the commercial growth of *Agaricus bisporus* and analyzed for arsenic speciation using HPLC-ICP-MS. AB was found to be the major arsenic compound in the fungus at the earliest growth stage of fruiting (the primordium). The growth substrate mainly contained arsenate (As(V)). The distribution of arsenic in an *A. bisporus* primordium grown on As(V) treated substrate, and in a mature *Agaricus campestris* fruiting body collected from arsenic contaminated mine tailings, was mapped using two dimensional XAS imaging. The primordium and stalk of the mature fruiting body were both found to be growing around pockets of substrate material containing higher As concentrations, and AB was found exclusively in the fungal tissues. In the mature *A. campestris* the highest proportion of AB was found in the cap, supporting the AB as an osmolyte hypothesis. The results have allowed us to pinpoint the fungus life stage at which AB formation takes place, namely reproduction, which provides a direction for further research.

Introduction

Arsenobetaine (AB) is the only arsenic compound that has no measured toxicity and is found to comprise the majority of arsenic in many marine organisms (Reimer et al., 2010). However, the formation pathway is still unknown. Unlike in the marine environment, AB is found in only a few terrestrial organisms, and in low proportions. The exception to this trend is the fruiting bodies (Reimer et al., 2010), or mushrooms, of many terrestrial fungi species from the class basidiomycetes, where AB can comprise the majority of arsenic in a wide variety of species (Nearing et al., 2014a).

Two of the three current hypotheses for the formation of AB are derived from studies of the marine environment and involve the degradation of arsenosugars, which are found at high levels in food sources, such as algae, for marine organisms. One proposed pathway involves the degradation of dimethylated arsenosugars to an arsenocholine (AC) intermediate, which is then converted to AB. A second proposed pathway also involves the degradation of dimethylated arsenosugars to form a dimethylarsinoylacetic acid (DMAA) intermediate, which is

* *Corresponding author*. E-mail: reimer-k@rmc.ca (Kenneth J. Reimer).

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then converted to AB. The third currently proposed pathway provides a more likely pathway for terrestrial organisms, where dimethylarsinous acid (DMA(III)) reacts with 2-oxo acids, glyoxalate and pyruvate to form a DMAA intermediate, which is then converted to AB (Edmonds, 2000).

AB is structurally similar to the osmolyte glycine betaine and has been found to be adventitiously accumulated in marine organisms as an osmolyte (Clowes and Francesconi, 2004). AB has also been hypothesized to be similarly incidentally accumulated in mushrooms with other osmolytes that are used to help maintain fruiting body structure for effective spore dispersal (Nearing et al., 2014a; Smith et al., 2007). It is unknown whether AB is formed by the fungus, during the vegetative or reproductive life stage, or whether it (or its precursors) is accumulated from the surrounding environment having been produced by the surrounding microbial community.

Terrestrial species in the basidiomycetes class of fungi can grow through asexual reproduction (the vegetative life stage), and through sexual reproduction (the reproductive life stage). The previously mentioned fruiting bodies, or mushrooms, are produced during the reproductive life stage and these mushrooms are used for spore dispersal.

Mushrooms that have been found to contain AB as a major compound include those from the genus *Agaricus* (Nearing et al., 2014a; Šlejkovec et al., 1997). *Agaricus bisporus* is a commonly cultivated edible mushroom, and when cultivated on arsenic treated material, the mature mushrooms also contained a majority of AB, except when grown on material with high arsenic concentrations (Smith et al., 2007; Soeroes et al., 2005).

In the commercial cultivation of *A. bisporus* a compost layer is inoculated with rye grain seeds overgrown with the mycelium. After the mycelium has grown into the compost layer, a casing layer (peat moss and calcium limestone) is added to the surface to facilitate fruiting body development. The growth room is aired to induce the mycelium to differentiate (*i.e.*, to form hyphal aggregations and knots), giving rise to the first growth stage of the fruiting body, the primordium. The temperature is then decreased, leading to the formation of differentiated tissues of the mature fruiting body (Eastwood et al., 2013), the cap and stipe (stalk). Gills containing the spores mature under the cap as the fruiting body enlarges (Umar and Van Griensven, 1997).

Studies on arsenic speciation in mushroom-producing fungi have focused on the mushroom stage because the mushrooms are the part of the fungus that appear above the ground and can be identified and easily picked; this therefore limits studies to the final stage of the reproductive life cycle (Byrne et al., 1995; Koch et al., 2000; Nearing et al., 2014a; Šlejkovec et al., 1997; Slejkovec et al., 1996; Smith et al., 2007; Soeroes et al., 2005). Only a few studies have been carried out at earlier stages. Two studies of *Agaricus* sp. mycelium were carried out under axenic conditions to investigate the formation of AB at this stage and by the fungus alone. *Agaricus placomycetes* mycelium methylated MMA to DMA, and preferentially accumulated tetramethylarsonium (TETRA) and AB, but it could not synthesize AB (Slejkovec et al., 1996). *A. bisporus* mycelium was found to produce trace amounts of MMA and DMA when exposed to As(V) and preferentially accumulate AB, but it also could not produce AB (Nearing et al., 2015). For the previously mentioned *A. bisporus* cultivated on arsenic treated material the growth substrate and compost material were also either collected before inoculation (Soeroes et al., 2005) or after harvesting (Smith et al., 2007). To more comprehensively investigate the formation of AB in *A. bisporus* at each part of the reproductive life cycle from the entire biosphere of the fungus, including the microbial community associated with it and the fungus itself, the present study aims to examine the arsenic speciation in the growth substrate and fungi at different times during the commercial growth of *A. bisporus*.

Additionally, we aimed to study the arsenic distribution in mushrooms at early life stages to interrogate AB formation further. The method used for this was solid state two dimensional X-ray absorption spectroscopy (XAS), but to use this method, higher concentrations were necessary, and these were obtained by cultivating *A. bisporus* on material from a mushroom farm that had been amended with arsenic in a laboratory setting. A mature mushroom from the *Agaricus* genus, collected from arsenic contaminated mine tailings to enable the higher required concentrations, was also mapped to examine the distribution of arsenic in the different tissues of a mature mushroom. Arsenic speciation in the different sections of the primordium and mature mushroom was also determined using micro X-ray absorption near-edge structure (micro-XANES) analysis.

1. Methods

1.1. Chemicals and reagents

Chemicals and reagents used for mushroom growing kit amendments, total arsenic and arsenic speciation analysis are listed in the Appendix A.

1.2. Collection of samples from a commercial growth facility for *A. bisporus*

All samples were collected using a sterile scoop or tweezers. Compost, casing and fruiting bodies samples were collected from the beginning of the commercial growth process to the last harvest of the mushrooms. The commercial growth process is further described in Appendix A. The sampling points were determined by the availability at the commercial facility and the samples collected are summarized in Table 1. Primordia and fruiting bodies were sorted by size. The white strain of *A. bisporus*, the white button mushroom, was collected from throughout the commercial growth process. The brown strain of *A. bisporus*, cremini and Portobello mushrooms, were only available for sampling at time of harvest. Fruiting bodies were washed with deionized distilled water before all samples were frozen, freeze dried, and homogenized. A stainless steel blender was used to homogenize compost samples and a ceramic mortar and pestle was used for the casing and fruiting body samples.

1.3. Cultivation of *A. bisporus*

Mushroom growing kits were purchased from White Crest Mushrooms, Putnam, ON. The kits were delivered with an

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