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Identification of geosmin and 2-methylisoborneol in cyanobacteria and molecular detection methods for the producers of these compounds

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ABSTRACT

Geosmin and 2-methylisoborneol (MIB) are muddy/earthy off-flavor metabolites produced by a range of bacteria. Cyanobacteria are the major producers of the volatile metabolites geosmin and MIB which produce taste and odor problems in drinking water and fish worldwide. Here we detected geosmin and MIB by studying 100 cyanobacteria strains using solid phase microextraction gas chromatography mass spectrometry (SPME GC–MS). A total of 21 geosmin producers were identified from six cyanobacteria genera. Two of the geosmin producers also produced MIB. A PCR protocol for the detection of *geo*A and MIB synthase genes involved in the biosynthesis of geosmin and MIB was developed. The *geo*A and MIB synthase genes were detected in all strains shown to produce geosmin and MIB, respectively. Cyanobacterial *geo*A and MIB synthase sequences showed homology to terpene synthases genes of actinobacteria and proteobacteria. Additional off-flavor compounds, nor-carotenoids β-ionone and β-cyclocitral, were found from 55 strains among the 100 cyanobacterial strains studied; β-ionone was present in 45 and β-cyclocitral in 10 strains. Six of the cyanobacteria which contain off-flavor compounds also produced toxins, anatoxin-a or microcystins. The molecular method developed is a useful tool in monitoring potential cyanobacterial producers of geosmin and MIB.

1. Introduction

Unpleasant taste and odor in fish or drinking water lead to consumer complaints and economic losses (Robin et al., 2006; Burr et al., 2012; Ma et al., 2013). Geosmin and 2-methylisoborneol (MIB) are odorous metabolites that cause muddy and earthy taste and odor problems worldwide in drinking water supplies and fisheries (Lanciotti et al., 2003; Klausen et al., 2005; Westerhoff et al., 2005; Percival et al., 2008; Watson et al., 2008). Geosmin and MIB are the main cause of taste and odor in drinking water (Watson et al., 2008). Identification of the producers of odorous metabolites provides critical insights into the origins of odor episodes.

Geosmin and MIB are produced by a variety of distantly related bacteria including actinobacteria, cyanobacteria and

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proteobacteria (Watson, 2003). Cyanobacteria are the major producers of geosmin and MIB in aquatic environments (Jüttner and Watson, 2007; Watson et al., 2008), while actinobacteria are the main producers of these metabolites in soil (Zaitlin and Watson, 2006). Geosmin and MIB producers have been identified from several cyanobacterial taxa, including benthic and planktonic strains (Izaguirre et al., 1982; Watson, 2003; Jüttner and Watson, 2007; Smith et al., 2008). Geosmin is also produced by other micro-organisms such as proteobacteria (Schulz et al., 2004; Dickschat et al., 2005, 2007) and fungi (Börjesson et al., 1993). Geosmin and MIB are detectable at very low concentrations due to extremely low odor threshold (<10 ng l^{-1}) of humans (Watson et al., 2008) and are difficult to remove by commonly used water purification systems (Srinivasan and Sorial, 2011).

Additional metabolites associated with off-flavor problems have been discovered from cyanobacteria (Jüttner, 1984; Watson et al., 2000; Höckelmann and Jüttner, 2005). These include a range of carotene and carotenoid fermentation products called nor-carotenoids, for example β-cyclocitral and β-ionone (Watson et al., 2000; Höckelmann and Jüttner, 2005; Schulz and Dickschat, 2007). Cyanobacteria are the richest source of nor-carotenoid products (Schulz and Dickschat, 2007). The presence of β-cyclocitral and β-ionone in water environments has been connected to cyanobacterial blooms (Jüttner, 1984; Watson et al., 2000; Watson, 2003; Höckelmann and Jüttner, 2005; Deng et al., 2011).

MIB and geosmin are both terpenoids and synthetized by terpene synthases (Oldfield and Lin, 2012). The genes involved in the biosynthesis of geosmin have recently been discovered from cyanobacteria (Ludwig et al., 2007; Agger et al., 2008; Giglio et al., 2008). Geosmin is synthesized by cyclization of farnesyl diphosphate (Giglio et al., 2008). Cyclization is accomplished by geosmin synthase encoded by the *geo*A gene (Giglio et al., 2008). Genes involved in the synthesis of MIB were first found from actinomycetes (Komatsu et al., 2008) and later from cyanobacteria (Giglio et al., 2011; Wang et al., 2011). The biosynthetic mechanism of MIB is a two-step reaction in which geranyl diphosphate (GPP) is first converted to methyl-GPP by methyltransferase, and in the next step methyl-GPP is cyclized to MIB by MIB synthase (Komatsu et al., 2008).

Molecular ecological methods which target biosynthetic genes are widely used to detect toxin-producing cyanobacteria (Sivonen, 2008; Dittmann et al., 2013). PCR and qPCR methods to detect geosmin producers have been developed for the *Nostoc*, *Anabaena*, *Geitlerinema*, *Pseudanabaena* and *Oscillatoria* genera (Giglio et al., 2008; Su et al., 2013). The detection method available for MIB producers is limited to a few genera in cyanobacteria, including *Anabaena*, *Oscillatoria* and *Phormidium* (Giglio et al., 2011). However, geosmin is produced by at least 20 genera and MIB by eight genera (Watson et al., 2008). Thus, new molecular ecological methods to detect a wide range of the cyanobacterial producers are needed. In addition, the ability to differentiate between cyanobacterial and actinobacterial producers during the taste and odor problems is important. Identification of off-flavor producers may eventually lead to management solutions. Accurate diagnostic tools for the detection of geosmin and MIB producers are important in monitoring the water reservoirs for quality purposes.

Here we report the development of PCR methods that can be used as monitoring tools for the early detection of the cyanobacterial geosmin and MIB producers in aquatic environments. We screened 100 cyanobacterial strains from 20 genera in order to identify producers of geosmin and MIB. Chemical identification was made with solid-phase microextraction coupled with gas-chromatography/mass-spectrometry (SPME GC–MS). Cyanobacteria-specific primers targeting *geo*A and MIB synthase were designed based on biosynthetic genes available in public databases.

2. Materials and methods

2.1. Cyanobacterial strains

Strains used in this study for SPME GC–MS and PCR were grown in the University of Helsinki Cyanobacterial Culture Collection (UHCC) in growth media Z8, Z8x or Z8xs depending on the strain (Table S1). Cultures (40 ml) were grown under continuous illumination (8–12 μE m^{-2} s^{-1}) at 22 °C and were in late logarithmic or early stationary phase during analysis.

2.2. Analysis of odorous metabolites and cyanotoxins

One hundred cyanobacterial strains (Table S1) were analyzed for the production of geosmin and MIB using SPME GC–MS. 4 ml replicate samples were taken from each the strains on two different days and transferred to vials where 1.5 g of NaCl was added. The fiber used in extraction was divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, Supelco, Sigma–Aldrich®). A gas chromatograph (Hewlett–Packard 6890) was equipped with transmission quadrupole mass selective detector (Agilent 5973, Agilent Technologies). The odorous metabolites were extracted using auto-sampler (combiPAL, CTC Analytics). Working conditions for the SPME were agitation (500 rpm) for 5 min at 70 °C and 30 min of extraction in the headspace. The desorption fiber was inserted 10 mm into the vial and after 10 min the fiber was retracted from the injection port and sample was injected into the GC–MS. In GC–MS analysis, the capillary column SPB™-624 (length 30 m, 0.25 mm internal diameter and 1.4 μM of film thickness Supelco, Sigma–Aldrich) was used. The oven temperature was 150 °C with a ramp rate of 5 °C/min for 10 min. The helium carrier gas had a flow rate 1.0 ml/min and split ratio 5:1. Full scan electron impact mass spectra were recorded at a range of 50–200 *m/z* and compared to the spectra from geosmin and MIB standards (Standard for drinking water, Supelco, Sigma–Aldrich). Dilution series from 100 ng ml^{-1} to 0.2 ng ml^{-1} were prepared for the geosmin and MIB standards to test the response and sensitivity of the GC–MS method. The abundance of ions was linear with analyte concentration. Unknown spectra were compared to the Electronic library (Wiley7n.1) in order to identify closest compounds with the highest similarity percentage. Also the β-cyclocitral spectrum from Watson (2003) was compared to unknown spectra. Geosmin and MIB were identified in the GC–MS analysis based on the retention times 12.40 ± 0.013 and 7.58 ± 0.01 min of the MIB and geosmin standards, respectively. The *m/z* of the most abundant ion fragments