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Long chain glycolipids with pentose head groups as biomarkers for marine endosymbiotic heterocystous cyanobacteria

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ABSTRACT

Marine endosymbiotic heterocystous cyanobacteria make unique heterocyst glycolipids (HGs) containing pentose (C_5) moieties. Functionally similar HGs with hexose (C_6) moieties found in free-living cyanobacteria occur in the sedimentary record, but C_5 HGs have not been documented in the natural environment. Here we developed a high performance liquid chromatography multiple reaction monitoring (MRM) mass spectrometry (HPLC–MS^2) method specific for trace analysis of long chain C_5 HGs and applied it to cultures of *Rhizosolenia clevei* Ostenfeld and its symbiont *Richelia intracellularis* which were found to contain C_5 HGs and no C_6 HGs. The method was then applied to suspended particulate matter (SPM) and surface sediment from the Amazon plume region known to harbor marine diatoms carrying heterocystous cyanobacteria as endosymbionts. C_5 HGs were detected in both marine SPM and surface sediments, but not in SPM or surface sediment from freshwater settings in the Amazon basin. Rather, the latter contained C_6 HGs, established biomarkers for free-living heterocystous cyanobacteria. Our results indicate that the C_5 HGs may be potential biomarkers for marine endosymbiotic heterocystous cyanobacteria.

1. Introduction

Cyanobacteria are cosmopolitan oxygenic photoautotrophs that play an important role in the global C and N cycles. Marine cyanobacteria are the major fixers of N_2 in modern tropical and subtropical oligotrophic oceans (Karl et al., 1997; Lee et al., 2002). Because N_2 fixation is sensitive to O_2, cyanobacteria have evolved a range of different strategies in order to combine the incompatible processes of oxygenic photosynthesis and N_2 fixation. One strategy found only in filamentous cyanobacteria is to fix N_2 in differentiated cells known as heterocysts (Wolk, 1973; Rippka et al., 1979). Heterocystous cyanobacteria can be the major source of N in eutrophic lakes and play a role in other freshwater and estuarine systems (Howarth et al., 1988). Free-living heterocystous cyanobacteria are rare in the open sea (Staal et al., 2003), but it recently has become evident that heterocystous taxa are abundant as endosymbionts in diatoms (Villareal, 1991, 2011, 2012; Foster et al., 2011; Luo et al., 2012). These heterocystous cyanobacteria provide the diatom with fixed N_2 (Foster et al., 2011) and can fully support the N needs of both host and symbiont (Villareal, 1990). This N subsidy, which explains the presence of these diatoms in low nutrient environments (Venrick, 1974) such as the tropical southwest North Atlantic Ocean where the symbiotic association produces nearly 70% of total N demand in the surface water (Carpenter et al., 1999).

In all free-living cyanobacteria, the heterocyst cell walls contain glycolipids (Nichols and Wood, 1968; Abreu-Grobois et al., 1977; Gambacorta et al., 1995; Bauersachs et al., 2009a). These heterocyst glycolipids (HGs) comprise a C_6 sugar head group glycosidically bound to long chain diols, triols, or hydroxyketones (**I–VI**; Fig. 1; Bryce et al., 1972; Gambacorta et al., 1998; Bauersachs et al., 2009b, 2011). Studies have found that C_6 HGs are not only biomarkers for heterocystous cyanobacteria and the N fixation process, but show structural diversity, depending on the family level within the divisions (Bauersachs et al., 2009a, 2014). The C_6 HGs are nearly exclusively from freshwater, free-living cyanobacteria. In contrast, the heterocystous cyanobacterium *Richelia intracellularis*, which lives endosymbiotically within the marine diatoms *Hemiaulus hauckii* and *Hemiaulus membranaceus* (Villareal, 1991)

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contained novel HGs with a C_5 sugar head group rather than a C_6 sugar (**VII–IX**; Fig. 1; Schouten et al., 2013). It was speculated (Schouten et al., 2013) that this might be an adaptation to the high O_2 concentration within their diatom host (Walsby, 1985). The only other report of HGs with a C_5 sugar moiety is that of Woermer et al. (2012), who tentatively assigned a compound with a C_5 head group and a shorter C_{26} carbon chain in a culture of the freshwater cyanobacterium *Aphanizomenon ovalisporum* UAM 290 and in suspended particulate matter (SPM) from three freshwater environments in Spain.

In contrast to the established paradigm that lipids with polar head groups are labile compounds which degrade upon cell death (White et al., 1979), C_6 HGs have been found to be preserved in ancient sediments of up to 49 Ma old (Bauersachs et al., 2010). Thus, they have been applied as unique biomarkers for N_2 fixation by heterocystous cyanobacteria in both present and past settings, such as microbial mats (Bauersachs et al., 2009b, 2011; Woermer et al., 2012; Bühring et al., 2014), freshwater lakes (Woermer et al., 2012), Pleistocene sediments from the eastern Mediterranean Sea and Eocene Arctic sediments (Bauersachs et al., 2010). However, it is not known whether C_5 HGs could be used as biomarker lipids in the same manner as C_6 HGs, as they have been only reported in two cultures and not in the natural environment.

In this study, we have developed a specific high performance liquid chromatography/multistage mass spectrometry (HPLC–MS^2) multiple reaction monitoring (MRM) method for trace analysis of long chain C_6 HGs to include analysis of C_5 HGs and carried out further screening of endosymbiotic cultures for the presence of these compounds. The method was then applied to suspended particulate matter (SPM) and surface sediments from the Amazon plume region in the southwest North Atlantic Ocean, which is known to host heterocystous cyanobacteria as endosymbionts with marine diatoms (Carpenter et al., 1999; Foster et al., 2007; Subramaniam et al., 2008). Examination of these and freshwater samples from the Amazon region allowed us to test the hypothesis that long chain C_5 HGs are specific biomarkers for endosymbiotic heterocystous cyanobacteria in the natural environment.

2. Methods

2.1. Culturing of diatoms containing endosymbionts

Rhizosolenia clevei Ostenfeld and its symbiont *R. intracellularis* Lemmerman were isolated from the coastal waters off Port Aransas, Texas in October 2010. Two strains were grown in MET-44 medium (Schöne and Schöne, 2009) as modified by Villareal (1990). Cells were grown in filter sterilized medium at 25 °C, 110 µmol quanta/m^2/s on a 12:12 light:dark cycle. Successive 2–4 l cultures were concentrated by sedimentation and centrifugation, the supernatant discarded, the resulting pellets frozen at −20 °C, and then shipped for analysis. In some cases, the culture was directly filtered onto a 10 µm polycarbonate filter, frozen and shipped. The host cells progressively decreased in diameter over the course of ca. 2 yr [MacDonald-Pfitzer rule MacDonald (1869), Pfitzer (1869)] and, after failing to produce auxospores, became moribund and died. The draft genome was reported by Hilton et al. (2013), NCBI accession number PRJEA104979.

2.2. Collection of environmental samples

Marine SPM and surface sediments from the Amazon shelf and slope encompassing the Amazon plume region were sampled on board of the R/V Knorr 197-4 between February and March 2010 (Table 1 and Fig. 2b; for details see Zell et al., 2014). SPM from the chlorophyll maximum was filtered on 0.7 µm glass fiber (GF) filters with an in situ pump. Sediment cores were collected using a box corer from which the top 1 cm was sub-sampled. In addition, surface sediments ($n = 6$) were collected using a grab sampler of

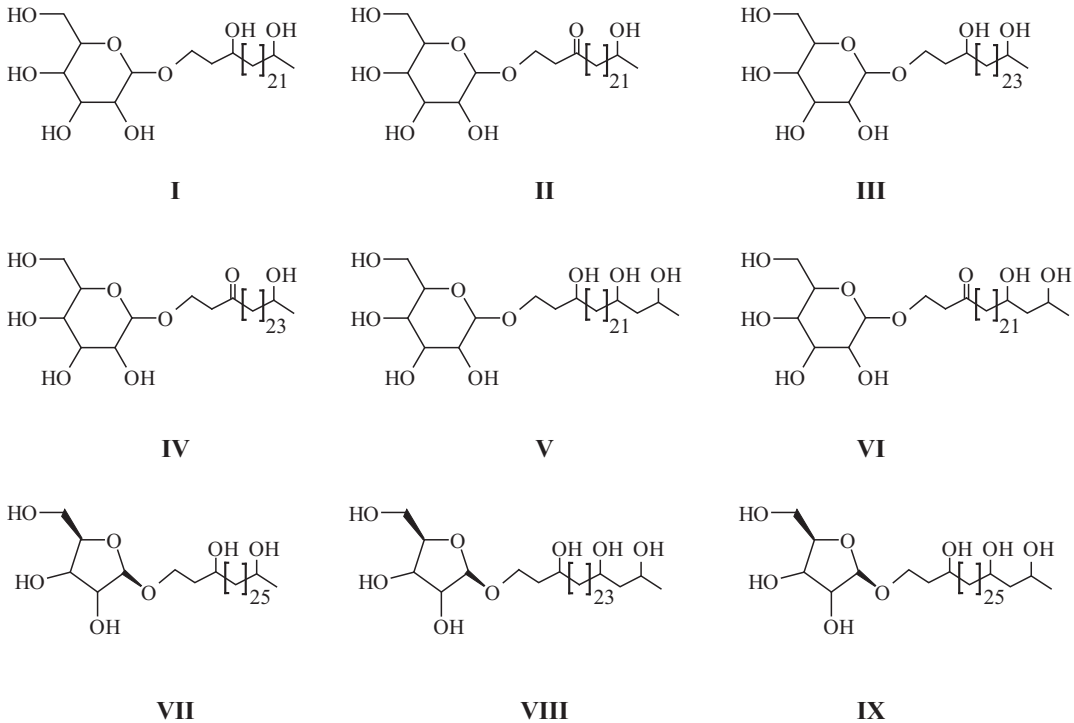

Fig. 1. Structures of heterocyst glycolipids. C_6 glycolipids: I 1-(O-hexose)-3,25-hexacosanediol; **II**, 1-(O-hexose)-3-keto-25-hexacosanol; **III**, 1-(O-hexose)-3,27-octacosanediol; **IV**, 1-(O-hexose)-3-keto-27-octacosanol; **V**, 1-(O-hexose)-3,25,27-octacosanetriol; **VI**, 1-(O-hexose)-3-keto-25,27-octacosanediol. C_5 glycolipids: **VII**, 1-(O-ribose)-3,29-triacontanediol; **VIII**, 1-(O-ribose)-3,29,31-dotriacontanetriol; **IX**, 1-(O-ribose)-3,27,29-triacontanetriol.