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Investigation of the presence of an aliphatic biopolymer in cyanobacteria: Implications for kerogen formation

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ABSTRACT

Algaenan has been suggested to be one of the main precursors of certain kerogens. It is a non-hydrolysable and insoluble biomolecule of high molecular weight. It has been found in a limited number of microalgae species. There is considerable uncertainty about its formation and preservation, as well as its role in kerogen formation and the implications for the global C cycle. We tested whether the cyanobacterium *Chlorogloeopsis fritschii* can synthesise a biomacromolecule similar to algaenan with potential to contribute to kerogen via selective preservation. Two freshwater green microalgae, *Pseudochoricystis ellipsoidea* and *Scenedesmus obliquus*, as well as *C. fritschii*, were subjected to harsh solvent extraction and hydrolysis steps to obtain an insoluble and non-hydrolysable macromolecule. The residues from all three species were analysed using pyrolysis–gas chromatography–mass spectrometry and solid-state nuclear magnetic resonance spectroscopy. The analysis revealed that *C. fritschii* indeed contains a resistant biomacromolecule exhibiting the characteristic aliphatic structure of algaenan, similar to the algaenan residues from the two microalgae. Due to the robust nature of *Chlorogloeopsis* compared with eukaryotes, it can prevail in extreme environmental conditions such as freezing, thawing, desiccation and overheating – conditions prevalent on the primeval earth. The presence of a resistant aliphatic biopolymer in *Chlorogloeopsis* suggests that cyanobacteria could have contributed to kerogen via selective preservation.

1. Introduction

Kerogen is commonly defined as the insoluble macromolecular organic matter (OM) in sedimentary rocks (Tegelaar et al., 1989;Vandenbroucke and Largeau, 2007). Until the 1980s it was thought it was formed by random condensation and polymerisation of sugars, lipids and proteins which had been broken down by way of bacterial enzymatic activity (Tissot and Welte, 1984; Tegelaar et al., 1989; Gelin et al., 1997). Today the "selective preservation" hypothesis is also accepted, i.e. resistant biopolymers such as algaenan are selectively preserved over time due to their high resistance to microbial and chemical degradation and therefore contribute to some kerogen fractions of source rock deposits (Allard et al., 2002). Although the levels of resistant biomacromolecules represent a low amount of total biomass, their high preservation potential results in preservation and enrichment during sedimentation and catagenesis, while other biomass constituents such as polysaccharides and proteins are biodegraded. Various resistant biopolymers are found in organisms, of which algaenan from microalgae has a particularly high preservation potential (Tegelaar et al., 1989).

Algaenan is a non-hydrolysable, insoluble, resistant biomacromolecule found in some species of microalgae. Reports have demonstrated its presence in freshwater green microalgae in the Trebouxiophyceae and Chlorophyceae (Versteegh and Blokker, 2004). There are other resistant biomacromolecules found in spores and higher plants and known as sporopollenin, cutin and cutan, respectively (de Leeuw et al., 2006). However, sporopollenin contains oxygenated aromatic building blocks, while cutin is a high molecular weight biopolyester composed of various interesterified hydroxyalkanoic acids and cutan is a non-hydrolysable, polymethylenic biopolymer, distinguishing them from algaenan (Tegelaar et al., 1991; Deshmukh et al., 2005). Two pathways can lead to resistant plant macromolecules: the acetate–malonate pathway, which proceeds via lipid synthesis to algaenan from algae and cutin from plants, and the phenylpropanoid pathway which leads to sporopollenin (Versteegh and Blokker, 2004). Type 1 algal

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kerogens in particular, characterised by high H/C and low O/C ratios, are suggested to be derived from algaenan preservation (Gelin et al., 1997). *Botryococcus braunii* has been identified as a major contributor to this type of kerogen through the preservation of its algaenan, as shown from pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) and identification of acyclic isoprenoid biomarkers (Volkman, 2014; Zhang et al., 2014).

There are no reports of the presence of algaenan in cyanobacteria and only limited reports on the occurrence of resistant biopolymers in cyanobacteria such as a *Schizothrix* sp. (Chalansonnet et al., 1988). However, Allard et al. (1997) suggested that the resistant biopolymers found by Chalansonnet et al. (1988) were melanoidin-like artefacts produced by the isolation procedure used. In our study, we tested for the occurrence of a resistant biopolymer similar to algaenan in the cyanobacterium *Chlorogloeopsis fritschii* and discuss how this could have contributed to the kerogen fraction of source rock deposits.

There is some uncertainty about the importance of the "selective preservation" hypothesis for kerogen formation; this stems mainly from the relatively small number of microalgal species which have been demonstrated to be algaenan producers. Additionally, the majority of these species are freshwater species, thereby introducing some uncertainty with respect to marine kerogen. Approximately 17 species from the Trebouxiophyceae and seven from the Chlorophyceae were listed by Kodner et al. (2009) to contain algaenan. The selective preservation theory and other kerogen formation pathways are of continued debate (de Leeuw, 2007; Gupta et al., 2007). The oxidative polymerisation of low molecular weight lipids is another pathway explaining the presence of the aliphatic content in kerogens (de Leeuw et al., 2006). Gupta et al. (2014) have argued in favour of the formation of geomacromolecules from lipids also contributing to the aliphatic content of kerogens due to algaenan not being as widespread ecologically and phylogenetically. In their experimental studies they obtained macromolecules with aliphatic characteristics from the high T/P pyrolysis of lipids from algae and cyanobacteria that lacked algaenan. They therefore raised the question if bacterial biomass may also have contributed to the insoluble organic fraction of ancient sediments (Gupta et al., 2014). Similarly, we would suggest that, if resistant aliphatic biopolymers are present in cyanobacteria, they could have contributed to kerogen by selective preservation.

Microfossils of the marine microorganism *Gloeocapsomorpha prisca* have been observed as a major contributor to the kerogen of Ordovician sediments (Blokker et al., 2001). Its taxonomic position and biological affinity have been debated and assigned mainly to the cyanobacteria and Chlorophyta, notably *Botryococcus braunii* as this chlorophyte produces structures morphologically similar to *G. prisca* under salt stress (Blokker et al., 2001). However, Versteegh and Blokker (2004) argue that the lack of evidence for *B. Braunii* containing the shorter chain lengths of the *n*-alkyl resorcinol building blocks, indicates it should not be attributed to *G. prisca*. Therefore, a third pathway could exist for the formation and preservation of resistant biomacromolecules (Versteegh and Blokker, 2004). Blokker et al. (2001) state that it is generally accepted that ancient microfossils are often composed of resistant biopolymers which have been selectively preserved over time; cyanobacteria could therefore be a possible source organism for microfossils such as *G. prisca*, if indeed the presence of resistant biopolymers could be shown in cyanobacteria. Foster et al. (1990) suggest *G. prisca* originates from an intertidal, marine, mat-forming benthic cyanobacterium similar to the extant cyanobacterium, *Entophysalis major*. Such properties have recently been described for *Chlorogloeopsis* (Pepe-Ranney et al., 2012).

The presence of algaenan has been suggested to have the function of protecting the alga by strengthening the cell wall and waterproofing the cell, protecting it from dehydration and attack by bacteria (Versteegh and Blokker, 2004). The main microalgae species shown to contain algaenan are *B. braunii*, *Scenedesmus* spp. and *Chlorella* spp. It has been argued that *B. braunii* is one of the main contributors of algaenan to kerogen formation (Audino et al., 2002; Volkman, 2014). Research has demonstrated that the cyanobacterium *Chlorogloeopsis* produces a high yield of oil when processed under hydrothermal liquefaction (Biller et al., 2012). This was not expected from an organism with such a low lipid content (ca. 5%), based on hydrothermal liquefaction studies with model compounds (Biller and Ross, 2011). Algaenan has been shown to contribute to oil formation during hydrothermal liquefaction (Torri et al., 2012). This led to the hypothesis that *Chlorogloeopsis* may contain an algaenan-like, resistant biomacromolecule.

Cyanobacterial cell walls differ from those of eukaryotic microalgae. They are characterised as either gram positive or gram negative using the crystal violet Gram staining method (Hoiczyk and Hansel, 2000). *Chlorogloeopsis* is gram negative, indicating a strong cell wall (Schrader et al., 1982). Its cell walls are made up of an inner, middle and outer layer surrounded by an additional sheath layer outside the cell wall (Peat and Whitton, 1967). The cell walls of gram negative bacteria generally contain an outer membrane and a thin peptidoglycan layer sandwiched between the periplasm and the outer membrane (Beveridge, 1999). Cyanobacterial cell walls have not been shown to contain algaenan and it is unlikely the same biosynthetic pathway for algaenan formation from microalgae also occurs in cyanobacteria. Nevertheless it might be possible that a similar resistant biopolymer to algaenan could be formed by some cyanobacteria via a different pathway. Cyanobacteria played a vital role in evolution to develop a photosynthetic system allowing the evolution of eukaryotic algae and plants and cyanobacteria were already widespread at the beginning of the Proterozoic (2.5×10^9 yr ago). Cyanobacterial matter contributed significantly to stromatolites and amorphous kerogens of supposed cyanobacterial origin (Chalansonnet et al., 1988). If resistant biopolymers exist in cyanobacteria, these could have contributed to these amorphous kerogens via selective preservation. *Chlorogloeopsis* has been shown to be an important contributor to the OM in living stromatolites growing in hot springs in the Yellowstone National Park (Pepe-Ranney et al., 2012). These stromatolites resemble ancient laminated forms, suggesting that *Chlorogloeopsis* could have contributed to ancient stromatolites and amorphous kerogen.

Chlorogloeopsis was originally isolated from soil particles in India (Mitra, 1950; Mitra and Pandey, 1966) and has since been found in a wide range of habitats around the world, including thermal springs (Hindák, 2008). It can differentiate into a broad range of morphological cell types in response to environmental stress, including heterocysts in fixed N depleted environments (Evans et al., 1976). It can survive a wide range of salt concentration (Hindák, 2008) and remains viable following either desiccation or repeated cycles of freeze thawing. All these properties represent a diverse suite of defence mechanisms and survival strategies that would enable *Chlorogloeopsis* to endure constantly fluctuating environmental extremes thought to have prevailed $2\text{–}3.4 \times 10^6$ yr ago.

To investigate the hypothesis that *C. fritschii*, a *cyanobacterium*, contains an aliphatic biopolymer similar to algaenan, it and two microalgae, *Pseudochoricystis ellipsoidea* and *Scenedesmus obliquus*, were investigated for the presence of insoluble, non-hydrolysable residues, which were compared using a range of analytical methods.

2. Material and methods

2.1. Algaenan isolation

S. obliquus (Chlorophyceae) was obtained from the University of Bath, UK and grown outdoors in a raceway bond. *P. ellipsoidea* (Trebouxiophyceae) was sourced from the Denso Corporation (Japan)