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The Unique Role of Intracellular Calcification in the Genus *Achromatium*

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Abstract *Achromatium* are found in freshwater and brackish sediments, where, as giant sulfur oxidisers, they play a key role in the carbon and sulfur cycles of the sediments they inhabit. The most striking feature of this genus is its enigmatic precipitation of intracellular calcite. Past explanations for this process have included the dissolution of stored calcite to regulate acidity generated by H_2S oxidation, the use of calcite as a buoyancy-regulating mechanism, the use of calcite as an electron acceptor source in "carbonate respiration", and the use of calcification to generate CO_2 for carbon fixation. However, more recent in situ physiological studies and detailed characterisation of the environments inhabited by these organisms have indicated a possible role for intracellular calcification in the dissolution of sulfide minerals. It is proposed that this unique adaptation of *Achromatium* is a means of overcoming a challenge not faced by other giant sulfur bacteria, namely inherently low levels of free sulfide in their sedimentary environment.

1 Introduction

The involvement of bacteria in the precipitation of calcium carbonate (calcification) is widespread. This is because bacterial activities which increase pH and release bicarbonate as a by-product cause oversaturation with respect to carbonate. Extracellular polymeric substances, cell walls and external

sheathes then provide nucleation sites which facilitate the precipitation process (Wright and Oren 2005). Processes such as ammonification, dissimilatory nitrate reduction, degradation of urea or uric acid and sulfate reduction all, in this way, indirectly facilitate extracellular calcification. Photosynthetic bacteria cause extracellular calcification by the uptake of bicarbonate, release of OH^- ions and consequent formation and precipitation of carbonate from solution. However, there is only one bacterial group that produces intracellular precipitates of calcite. These are the giant uncultured sulfur-oxidising bacteria of the genus *Achromatium*.

Achromatium are found in freshwater and brackish sediments, where they play a key role in the carbon and sulfur cycles of the sediments they inhabit (Gray et al. 1997, 1999a; Head et al. 2000). The most striking feature of this genus is its enigmatic precipitation of intracellular calcite. Despite descriptions of *Achromatium* from as early as 1893 (Schewiakoff 1893), the purpose of intracellular calcification has not been satisfactorily explained. In this review, ecological, physiological and geochemical data on *Achromatium* and its habitats are reassessed. By comparison of the data with the ecology of other colourless sulfur bacteria and by reference to intracellular calcification by other organisms, various past theories and one new theory for the role of calcification in *Achromatium* are discussed.

2
Intracellular Calcite Inclusions in *Achromatium* spp.

Intracellular inclusions, which have been shown by X-ray diffraction analysis to contain calcite, constitute a large part of the volume of *Achromatium* cells (Head et al. 1996). With use of confocal microscopy it has been estimated that the mean calcium content of an individual cell is 2.62 ± 0.34 ng cell^{-1} (mean $\pm$ standard error). Scanning electron microscopy images of *Achromatium* cells have revealed that the size, number and distribution of calcite inclusions vary greatly between individual organism (Head et al. 1996); however, even within individual cells, calcite inclusions vary in size (Fig. 1a, b). This variability in inclusion size suggests that calcite precipitation is a continuous process in active *Achromatium*, a finding which was supported by microautoradiographic analysis of bicarbonate uptake, which demonstrated the rapid incorporation of radiolabelled bicarbonate into the calcite component of the cell carbon (Gray et al. 1999b). Thin-section transmission electron microscopy images, which highlight the internal structure of individual inclusions (Fig. 1c), reveal a laminated structure, a possible membrane and an electron-dense central nucleation point. These features, along with the intensity ratios of D spacings in the X-ray diffraction data, suggest that the calcite is not purely crystalline and is thus precipitated under strict biological control.

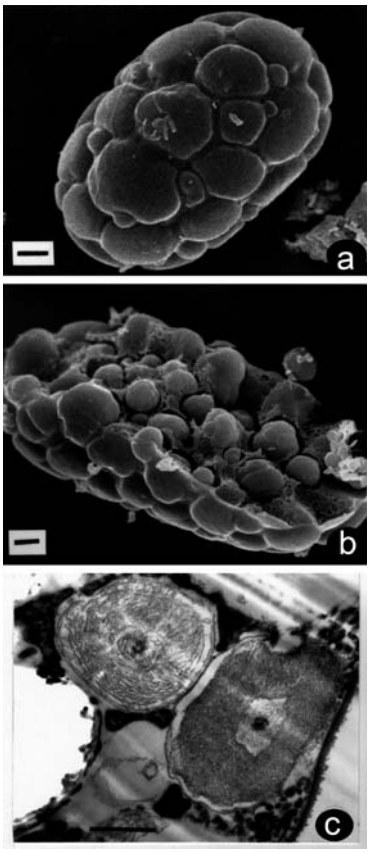

Fig. 1 Scanning and thin-section transmission electron micrographs of *Achromatium* cells from the Rydal Water sediment. **a** Scanning electron micrograph of an oval cell showing calcite inclusions of varying size. The *scale bar* represents 1.25 μm. **b** Scanning electron micrograph of an elongated cell disrupted by grinding in a pestle and mortar showing larger calcite inclusions and smaller sulfur inclusions. The *scale bar* represents 1.25 μm. **c** Transmission electron micrograph of an ultrathin section of an *Achromatium* cell showing two intact calcite inclusions with a characteristic laminated internal structure and probable central nucleation site. The *scale bar* represents 0.625 μm. (Adapted from Head et al. 2000 with the kind permission of Springer)

Although the mechanism of calcification in *Achromatium* is unknown, a number of its features can be deduced from stoichiometric and physiological considerations. For instance, the ability of *Achromatium* cells to precipitate calcite internally is likely to be dependent on the ability to scavenge available Ca^{2+} from the surrounding pore waters (it is assumed that bicarbonate is not limiting in most natural waters). The occurrence of calcite-containing *Achromatium* cells in environments with fairly low dissolved calcium concentrations has been noted in a number of freshwater sediments (Nadson and

Visloukh 1923). Accordingly, it has been inferred that *Achromatium* species must possess an active mechanism for concentrating this cation from the environment (Head et al. 2000). Calcium ATPase mechanisms appear to underlie most biological calcification (McConnaughty and Whelan 1997). For instance, coccolithophorid algae exhibit high Ca^{2+}-dependent V- and P-type ATPase activities (Kwon and González 1994; Araki and González 1998) and a gene sequence of the *vap* subunit of a V-ATPase has been recovered from the calcifying coccolithophorid *Pleurochrysis carterae* (Corstjens et al. 2001). Ca^{2+} ATPases use the energy of ATP hydrolysis to exchange Ca^{2+} ions for protons and, typically, either two or four protons are exchanged for every Ca^{2+} ion transported. In *Achromatium* the formation of calcite most likely occurs by reaction of the accumulated Ca^{2+} with carbonate, but not, it seems, by direct reaction of Ca^{2+} with bicarbonate. This is because calcite is an ionic compound and can only be formed from its component ions (Wright and Oren 2005). On this basis, before calcification can take place, bicarbonate, which is the predominant form of inorganic carbon in near-neutral pH environments, must be converted to carbonate. The possible mechanism and ecological meaning of calcification in *Achromatium* is discussed in more detail later. However, it is clear that the precipitation of intracellular calcite by *Achromatium* is carried out at an energetic cost to the organisms.

3 The Habitat and Physiology of *Achromatium* spp.

An essential requirement for understanding the role of calcification in *Achromatium* is a deeper understanding of the relationship of these organisms with their geochemical environment. *Achromatium* spp., which form a phylogenetically coherent group within the γ-*Proteobacteria* (Gray et al. 1999a), typically, inhabit littoral freshwater sediments. Here, regardless of sulfate-reduction rates and sulfate concentrations, high levels of reactive iron (as oxides of ferric iron and dissolved Fe^{2+}) serve to maintain low levels of dissolved sulfide (less than micromolar quantities) within the sediment (Gray et al. 1997; Gray and Head 1999; Head et al. 2000). These low sulfide conditions are unlike those typically encountered in environments which harbour other genetically related giant sulfur bacteria, where dissolved sulfide is abundant (Schultz et al. 1999).

Studies of the redox chemistry of *Achromatium* sediments have provided an insight into the ecology of these organisms. In one *Achromatium*-dominated freshwater sediment (Rydal Water) in the English Lake District, profiles of pore water sulfate exhibited a maximum at 4 mm. Below this depth sulfate became depleted, accompanied by concomitant increases in precipitated pyrite, sulfur (chromium-reducible sulfur) and iron monosulfides (acid volatile sulfur) (Gray et al. 1997; Fig. 2). This evidence of active sulfate reduc-

tion and the finding that the *Achromatium* constituted 90% of the bacterial biovolume was indicative of the rapid recycling of reduced sulfur (Gray et al. 1997). The role of *Achromatium* in this oxidative process was confirmed by studies which showed that in sediment microcosms, in which sulfate reduction had been inhibited by sodium molybdate, sulfate accumulation was directly proportional to the size of the *Achromatium* population (Gray et al. 1997). Additionally, microautoradiographic studies showed that in uninhibited sediment microcosms ^{35}S-labelled sulfate was rapidly incorporated by *Achromatium* cells into intracellular elemental sulfur (Gray et al. 1999b).

Studies of the depth distribution of *Achromatium* cells in relation to redox-sensitive chemical species showed that they are distributed throughout the zone of sulfate reduction (Gray et al. 1997; Fig. 2). Furthermore, it has been demonstrated, by measurements of sediment oxygen profiles, that a large proportion of *Achromatium* resided below where oxygen was detectable (Head et al. 1996). Recent microcosm experiments (Gray et al. 2004) have confirmed this microaerophilic/anaerobic nature by demonstrating that all the *Achro-*

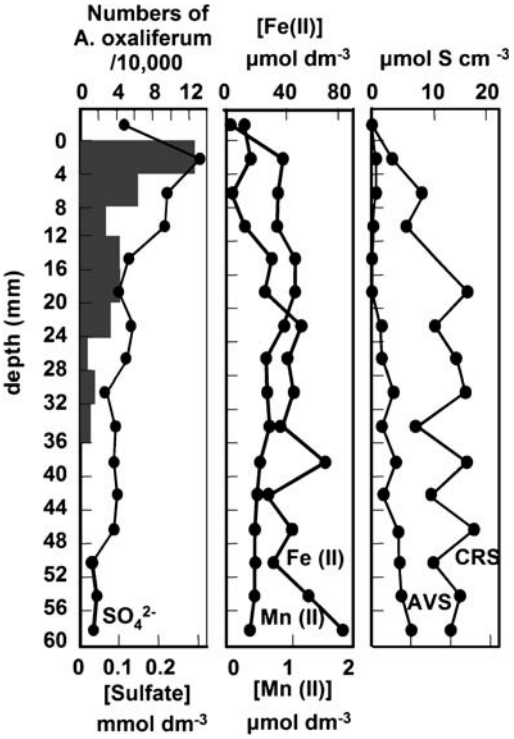

Fig. 2 The vertical distribution of *Achromatium*, dissolved Fe(II), Mn(II), chromium-reducible sulfide (*CRS*) and acid volatile sulfide (*AVS*) in a sediment core from Rydal Water. *Grey bars* denote cell numbers. (Adapted from Gray et al. 1997 with the kind permission of the American Society for Microbiology)

matium spp. present in the Rydal sediment are, at the very least, facultative nitrate reducers.

Ecophysiological studies on *Achromatium* have, therefore, amply demonstrated that despite limitations imposed on their sulfide oxidising potential, i.e. very low levels of dissolved sulfide, *Achromatium* spp. have a clearly defined role in the carbon, sulfur and nitrogen cycles of their environment. However, these organisms cannot rely on the diffusive flux of sulfides as do other giant sulfur bacteria, so it has been suggested that *Achromatium* spp. utilise other reduced sulfur resources (Gray et al. 1997), namely precipitates of iron sulfides that have been shown to form in these iron-rich environments. How this process of sulfide dissolution and take up might be achieved is discussed in more detail later.

4
Past Interpretations of Calcification in *Achromatium* and the Role of Intracellular Calcite Inclusions in the Coccolithophorid Algae

A number of hypotheses relating to the role of intracellular calcification in *Achromatium* have been proposed. For example the dissolution of stored calcite to regulate acidity generated by H_2S oxidation, the use of calcite as a buoyancy-regulating mechanism and the use of calcite an electron acceptor source in "carbonate respiration" (La Rivière and Schmidt 1992; Babenzein 1992; Head et al. 2000). Based on a clearer understanding of the habitat and physiology of *Achromatium* these past interpretations of calcification can now be critically examined. For instance, the use of calcite by *Achromatium* to regulate acidity generated by H_2S oxidation is an unlikely requirement in non-sulfidic, near-neutral, naturally buffered freshwater systems and the precipitation and dissolution of calcite for buoyancy regulation in sediments (Babenzien 1992) is inconsistent with observations which show an inverse relationship between the quantity of calcite and the depth within the sediment (Head et al. 2000). Additionally, the use of calcite as a source of carbonate as an electron acceptor is unlikely when it is considered that *Achromatium* spp. are closely related to other O_2- or NO_3^--consuming sulfur bacteria, not methanogenic Archaea. Perhaps more significantly, these organisms have been shown to carry out nitrate-dependent sulfur oxidation (Gray et al. 1997, 1999a, 2004).

Recently and, more plausibly, it has been proposed that *Achromatium* spp. deposit intracellular calcite to maintain a high internal partial pressure of CO_2 to facilitate carbon fixation by the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) (Head et al. 2000). This proposed use of calcification has also been suggested for the coccolithophorid algae, for example *Emiliania huxleyi* and *Pleurochrysis carterae*. For these unicellular marine algae it is thought that under conditions of high pH, low dissolved CO_2 and high dissolved oxygen concentrations, bicarbonate is converted to CO_2 by the action

of the enzyme carbonic anhydrase (Borowitzka 1982) (Eq. 1) or by export of protons for the extracellular conversion of bicarbonate to CO_2, which is then assimilated (McConnaughty and Whelan 1997). As a consequence, calcification is required to neutralise a raised cytoplasmic pH through the direct or indirect reaction of bicarbonate with hydroxyl ions to form carbonate (Eq. 1), which then precipitates as calcite (Eq. 2). This calcification process occurs within a membrane-bound compartment termed the coccolith-containing vesicle. Support for this mechanism by which calcification is linked to photosynthesis comes from the tight coupling of inorganic and organic carbon produced in the cell, i.e. an approximately one-to-one relationship (Crawford and Purdie 1997) and the fact that calcification rates are affected by light intensity and changes in CO_2 partial pressure. However, an unequivocal link between calcification and photosynthesis has not yet been provided (Paasche 2001) and it has been suggested that calcification and, in some cases its regulation, may impart other benefits to these organisms, for example defence against grazing and parasite invasion or regulation of density and sinking rates based on increased calcification rates (Raven and Waite 2004).

$$HCO_3^- + OH^- \Longleftrightarrow CO_3^{2-} + H_2O \quad (1)$$

$$CO_3^{2-} + Ca^{2+} \Longleftrightarrow CaCO_3 \quad (2)$$

While it has been proposed that calcification is used by *Achromatium* spp. to fix carbon in sedimentary environments (Head et al. 2000), there is now some doubt about the physiological need for such a mechanism. For instance, the pH of an *Achromatium*-containing sediment is typically circumneutral where CO_2 still constitutes a significant component of the dissolved inorganic carbon pool. In addition, recent microautoradiographic studies on carbon metabolism in *Achromatium* populations from different geographical locations have demonstrated that some calcite-precipitating *Achromatium* communities are probably chemolithoheterotrophs and do not utilise bicarbonate for biosynthesis (Gray et al. 1999b). Furthermore, these *Achromatium* populations did not contain homologues of the RuBisCO large subunit gene (*rbcL*), involved in CO_2 fixation (Head et al. 2000). Consequently, the precipitation of calcite by these *Achromatium* populations cannot be linked to carbon fixation.

5
A Proposed Role for Calcification in *Achromatium*: Calcite Precipitation Linked to Sulfide Dissolution

A key question with regard to calcification in *Achromatium* is: What ecological challenge necessitates this unique intracellular process? The answer to this question may lie in the geochemical constraints imposed on *Achro-*

matium spp. by their sedimentary environment, namely the very low levels of free sulfide and, therefore, the necessity for the active dissolution of iron sulfides by these organisms. In sediment pore waters the solubility of iron sulfide is dependent on the concentration of both dissolved Fe^{2+} and pore water pH (Eq. 3). From this equilibrium consideration, it can be deduced that high concentrations of dissolved Fe^{2+} and high pH favour sulfide precipitation, whereas low pH and low concentrations of Fe^{2+} favour sulfide dissolution (Eq. 4):

$$FeS + H^+ \Longleftrightarrow Fe^{2+} + HS^- \quad (3)$$

$$[HS^-] = K[H^+]/[Fe^{2+}]\,. \quad (4)$$

Given the role that *Achromatium* spp. play in the precipitation of carbonate and their likely dissolution and oxidation of iron sulfides, these two processes may be linked, whereby intracellular calcification provides a sink for hydroxyl ions generated as a result of proton export (Fig. 3). In this suggested mechanism the purpose of proton export by *Achromatium* spp. would be to alter extracellular pH sufficiently to mobilise iron sulfides (Eq. 3), with the subsequent oxidation of the liberated HS^- either by oxygen or by nitrate reduction (Eqs. 5–7). Critically, the dissolution of iron sulfide by acidification releases

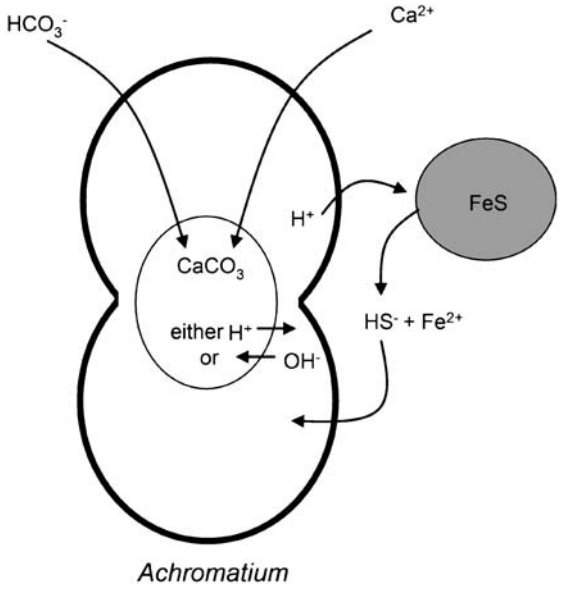

Fig. 3 Putative mechanism for intracellular calcification linked to sulfide mineral dissolution in *Achromatium*. A dividing *Achromatium* cell is shown with just a single calcite inclusion for illustrative purposes. Calcite precipitation occurs as a result of either proton export from or hydroxyl import into the inclusion to facilitate carbonate formation

dissolved Fe^{2+} into solution which would further decrease sulfide solubility (Eq. 4). To maintain the balance of Fe^{2+} concentraton and H^+ concentration at the iron sulfide mineral surface and, therefore, sustain continuous dissolution, an additional proton would be required for each sulfide dissolved. In this proposed mechanism, the export of protons could be achieved either by proton extrusion as a result of chemiosmotic energy generation or by proton-pumping ATPases; however, the exported protons, owing to their reaction with metal sulfides or through their role in maintaining pore water H^+ concentrations, would be unavailable for transport across the cell membrane by ATP synthase. An inevitable consequence of this proton consumption would be an increased cytoplasmic pH. As with calcification in the coccolithophorid, a potential sink for excess hydroxyl ions would be the buffering reaction with bicarbonate, the formation of carbonate and precipitation of calcite. In this putative process of calcification coupled to FeS mobilisation and oxidation (Fig. 3), the complete oxidation of the liberated HS^- to sulfate by consumption of oxygen would regenerate a proton (Eq. 5). This reaction would serve to neutralise half the excess hydroxyl ions. Consequently, for each mole of sulfide liberated and oxidised to sulfate by oxygen, a minimum of 1 mol of precipitated carbonate would be required. The requirement for calcite precipitation would be quantitatively greater if, as is highly likely, *Achromatium* spp. use nitrate (Gray et al. 2004) as their terminal electron acceptor to produce N_2 or NH_3 (Eqs. 6, 7).

$$HS^- + 2O_2 \longrightarrow SO_4^{2-} + H^+ \quad (5)$$

$$5HS^- + 8NO_3^- + 3H^+ \longrightarrow 5SO_4^{2-} + 4N_2 + 4H_2O \quad (6)$$

$$HS^- + NO_3^- + H^+ \longrightarrow SO_4^{2-} + NH_4^+ \quad (7)$$

A key feature of this proposed role for calcification in *Achromatium* is that calcite deposition, with its associated energetic cost, is linked to the energy-generating process of sulfide oxidation. So it remains to be seen from a proper evaluation of the energetics and enzymology of calcification and sulfur oxidation whether this process is feasible. Certainly in environments with high dissolved sulfide concentrations, the additional energy costs of calcification are likely to make *Achromatium* uncompetitive compared with non-calcifying sulfur oxidisers, so it is interesting to note that the only *Achromatium* species known to inhabit sulfidic marine sediments (*Achromatium volutans*) does not precipitate calcite.

6 Conclusion

The unique and enigmatic process of intracellular calcification in *Achromatium* has interested microbiologists for well over 100 years. However, it has

only been through recent in situ physiological studies and through detailed characterisation of the environments inhabited by these organisms that we have gained an understanding of the relationship between their ecology and the geochemical constraints imposed on them. With the advent of genomic technologies it should soon be possible to gain a greater understanding of the genetic basis of calcification in *Achromatium*, an adaptation which is surely critical to this environmental relationship.

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