Ecophysiological Evidence that \textit{Achromatium oxaliferum} Is Responsible for the Oxidation of Reduced Sulfur Species to Sulfate in a Freshwater Sediment

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\textit{Achromatium oxaliferum} is a large, morphologically conspicuous, sediment-dwelling bacterium. The organism has yet to be cultured in the laboratory, and very little is known about its physiology. The presence of intracellular inclusions of calcite and sulfur has given rise to speculation that the bacterium is involved in the carbon and sulfur cycles in the sediments where it is found. Depth profiles of oxygen concentration and \textit{A. oxaliferum} cell numbers in a freshwater sediment revealed that the \textit{A. oxaliferum} population spanned the oxic-anoxic boundary in the top 3 to 4 cm of sediments. Some of the \textit{A. oxaliferum} cells resided at depths where no oxygen was detectable, suggesting that these cells may be capable of anaerobic metabolism. The distributions of solid-phase and dissolved inorganic sulfur species in the sediment revealed that \textit{A. oxaliferum} was most abundant where sulfur cycling was most intense. The sediment was characterized by low concentrations of free sulfide. However, a comparison of sulfate reduction rates in sediment cores incubated with either oxic or anoxic overlying water indicated that the oxidative and reductive components of the sulfur cycle were tightly coupled in the \textit{A. oxaliferum}-bearing sediment. A positive correlation between pore water sulfate concentration and \textit{A. oxaliferum} numbers was observed in field data collected over an 18-month period, suggesting a possible link between \textit{A. oxaliferum} numbers and the oxidation of reduced sulfur species to sulfate. The field data were supported by laboratory incubation experiments in which sodium molybdate-treated sediment cores were augmented with highly purified suspensions of \textit{A. oxaliferum} cells. Under oxic conditions, rates of sulfate production in the presence of sodium molybdate were found to correlate strongly with the number of cells added to sediment cores, providing further evidence for a role for \textit{A. oxaliferum} in the oxidation of reduced sulfur.

\textbf{Materials and Methods}

Sediment sampling and enumeration and purification of cells from sediment. The sampling site at Rydal, Cumbria, United Kingdom, and methods for purifying \textit{A. oxaliferum} cells by gravity separation have been described previously (15). To obtain bulk samples of \textit{A. oxaliferum} cells, a hand-operated vacuum sampler was used to remove surface sediment from the sampling site (36). This provided sediment containing $10^6$ to $10^7$ cells ml$^{-1}$, and 100- to 200-ml samples were processed when purifying cells for incubation experiments. Sediments were initially screened through a 64-µm-pore-size nylon mesh to remove larger sediment particles, and the cells were subsequently purified by the method of de Boer et al. (11). Briefly, the screened sediment was placed in a sterile flask. The flask was tilted, and the sediment settled to the base of the flask leaving a white line of \textit{A. oxaliferum} cells just below the meniscus. The cells were aseptically removed with a micropipette and were transferred to a sterile microcentrifuge tube (1.5-ml capacity). Cells used for sediment core incubations, for which the purity of cells was crucial, were washed exhaustively in filtered water from the sampling site. The cells were resuspended in filtered water and allowed to settle under the influence of gravity. The dense \textit{A. oxaliferum} cells settled rapidly, leaving smaller bacterial cells in suspension. This procedure was repeated until preparations free of contaminating smaller bacteria (determined by acridine orange staining) were obtained. Depth profiles of \textit{A. oxaliferum} cells were determined in sediment cores (6-cm interior diameter; Perspex core tubes) obtained with a Jenkin surface mud sampler (34). The core was sectioned at 4-mm intervals under oxygen-free nitrogen, and subsamples (100 µl) of core sections were fixed with formaldehyde (2% [vol/vol] final concentration) for enumeration of \textit{A. oxaliferum} cells. The remainder of the sediment slurry was sealed under nitrogen and stored at $-20^\circ$C for analysis of iron, manganese, and sulfur species. \textit{A. oxaliferum} cells were counted with a Sedgwick-Rafter cell (21).
MIMS. Sediment cores were obtained with a modified 25 ml polycarbonate syringe. The cores were returned to the laboratory where depth profiles of dissolved gases were obtained by membrane inlet mass spectrometry (MIMS; Fig. 1) (27). The MIMS probe was constructed from a length of unphased fused silica gas chromatography column (1.5 m long, 0.1-mm interior diameter) linked to the mass spectrometer interface of a Fisons Trio 1000 gas chromatography mass spectrometer. A silicone rubber membrane (Silastic; DuPont) approximately 100 μm thick was gently stretched over the open end of the column. The membrane was then further secured by cementing with more silicone rubber. Measurements of dissolved gases at a resolution of less than 1 mm were made by careful insertion of the probe into sediment cores with a micromanipulator. The probe was allowed to equilibrate for at least 30 min at each depth. Measurements of dissolved oxygen were made by single-ion monitoring at a molecular weight of 32. Calibration of the probe was carried out with air-saturated water at 25°C (258 μmol of O2 per liter) (2) and water sparged with helium (0 μmol of O2 per liter) (27). Cores were sectioned in 4-mm increments immediately after oxygen profiles were taken, and cell counts were conducted on the core sections.

Chemical analyses. The top 10- to 15-cm-thick portions of freshly sampled cores were sectioned under nitrogen in 4-mm increments. A subsample from each depth increment was removed for direct counts of A. oxaliferum; the remainder was sealed under nitrogen and frozen. Sediment samples were assayed for the following sulfur pools: SO4\(^{2-}\), H2S, FeS, MnS, and acid-volatile sulfur (AVS; FeS8). Sediment samples (5 cm\(^3\)) were transferred into 15-ml capacity screw-cap centrifuge tubes under oxygen-free nitrogen and centrifuged at 4,000 rpm for 5 min. Pore waters were removed and stored, frozen, for subsequent SO4\(^{2-}\), FeS8, and MnS analysis. The sediment pellet was resuspended in zinc chloride solution (5% [wt/vol]) to a final volume of 10 ml and stored, frozen, for subsequent AVS, CRS, and elemental sulfur analysis.

When samples containing sodium molybdate were analyzed, the sediment pellet was washed once in 10 ml of zinc chloride (5% [wt/vol]) and resuspended in 10 ml of zinc chloride (5% [wt/vol]). This was done to remove the bulk of the added sodium molybdate, which can form phosphomolybdate complexes that in turn can substantially reduce the recovery of sulfide evolved during the acidification of samples (6, 35).

Analysis of pore water sulfide and sulfate and measurements of sulfate reduction rates. Dissolved sulfide was analyzed by iodometric titration (45) following fixation as ZnS by the addition of zinc chloride (5% [wt/vol]) to pore waters. Sulfate was analyzed by suppressed ion chromatography (Dionex-BIOLC ion chromatograph fitted with an AS5a column), with 40 mM NaOH as the eluent. Sulfate was determined by the ferrozine spectrophotometric method (41). Fe2+ standards were prepared from standard solutions of FeCl3 reduced with a Jones reductor (45). Complete reduction of the Fe3+ was confirmed by measuring the absorption spectra (200 to 400 nm) of the standard iron solutions with a Hewlett-Packard 8452A diode array spectrophotometer. As reduction proceeded, the absorption peak at 338 nm decreased. Mn2+ levels in separate acidified pore water samples (1 ml) were estimated by atomic absorption spectrophotometry with a Varian Spectra AA flame atomic absorption spectrophotometer with an acetylene oxygen flame. Concentrations were determined by reference to external standards.

Sodium molybdate inhibition experiments. Sediment samples, collected with a vacuum sampler (36) were thoroughly mixed. Aliquots (5 ml) of the resultant slurry were placed in 15-ml screw-cap centrifuge tubes with 2 ml of lake water. The tubes were incubated at room temperature for 48 h to allow redox gradients to reform. This was inferred by measurement of oxygen profiles and the distributions of A. oxaliferum cells in replicate sediment samples placed in a modified polycarbonate syringe with the lower end removed and with the bottom sealed with the syringe plunger. Cell distributions and oxygen profiles were found to be similar to those observed in freshly sampled cores. All molybdate inhibition experiments were conducted with cores prepared in this manner. A series of replicate cores was prepared for time course experiments, and cores were sacrificed at 0, 22, 48, 72, 96, and 144 h. In addition, replicate formaldehyde-treated control “core” samples containing buffered formaldehyde (0.01 M sodium phosphate, pH 6.5) to a final concentration of 2% (wt/vol) were sampled as described above. Sodium molybdate solution (175 ml of a 200 mM stock solution) was injected into the cores while the syringe needle was slowly withdrawn from the core. The cores were incubated at 20°C with a 12-h light-dark cycle and were open to the air. Tubes were frozen at regular intervals and stored for subsequent sulfate analysis.

When it had been determined that the increase in sulfate over time was linear, all subsequent experiments were done with 0- and 120-h incubated samples. To determine if the population size of A. oxaliferum affected the rate of sulfate accumulation, reconstituted cores were prepared and increasing amounts of a purified suspension of A. oxaliferum cells were added to duplicate cores. Cell purity was checked by acridine orange staining to confirm the absence of contaminating smaller bacteria. The tubes were treated with sodium molybdate as previously described, and samples containing different numbers of A. oxaliferum cells were analyzed for levels of sulfate, FeS8, MnS, AVS, and CRS. A set of eight identical cores was also prepared and immediately frozen after the addition of sodium molybdate. These cores were then analyzed in the same manner to determine the variability of SO4\(^{2-}\) and reduced sulfur species concentrations at the start of the incubation.

Statistical analyses. Correlations were determined by the method of Spearman. Comparisons of regression lines from time course experiments were performed by using analysis of covariance. All statistical analyses were performed with either SPSS (release 6) or Minitab (release Xtra) computer programs.

RESULTS AND DISCUSSION

A. oxaliferum was first described over a century ago (39). Despite this, very little has been published regarding the biogeochemistry of this distinctive bacterium. There is some information relating to sulfate reduction rates and Eh profiles within sediments inhabited by A. oxaliferum (3). However, these data were not related to the magnitude of the A. oxaliferum population present in the sediments. Until now, nothing was known regarding the distribution of A. oxaliferum in sediments or the redox environment occupied by the bacterium. We now report detailed information on the distribution of A. oxaliferum cells in relation to those of redox-sensitive species in a freshwater sediment and also present evidence that A. oxaliferum has the ability to oxidize reduced sulfur to sulfate.

The sediment environment occupied by A. oxaliferum. The importance of A. oxaliferum in the biogeochemistry of sediments from the Rydal site, studied here, was previously suggested by the dominance of A. oxaliferum in the bacterial population of the sediment. Acridine orange counts had previously
been used to calculate the proportion of the bacterial biovolume in Rydal sediments that was made up of *A. oxaliferum* cells. While in terms of cell numbers, *A. oxaliferum* constituted around 1% of the bacterial population, in terms of biovolume it constituted 90% or greater of the bacterial population (14).

The sediments studied were under less than 1 m of water, and measurements of oxygen profiles in the sediment over a 2-year period demonstrated that the sediment surface remainedoxic throughout the year (data not shown). Ion chromatographic analysis further revealed that nitrate was always depleted in the top 1 to 2 mm of the sediment. The vertical distributions of *A. oxaliferum*, inorganic sulfur species, Mn$^{2+}$ and Fe$^{2+}$ were determined in a sediment core from the Rydal site (Fig. 2) sampled in September 1995. Profiles of NO$_3^-$, Mn$^{2+}$, and Fe$^{2+}$ levels were typical of the succession of respiratory processes in sediments as defined by Berner (7) and as noted in numerous studies of both freshwater and marine environments (28, 40, 44). The Rydal sediments were organically rich (15.31% ± 0.55% total organic carbon, n = 8) and exhibited a steep sulfate level gradient in the top 3 cm. Sulfate reduction rates measured by the ion chromatographic method of Bak et al. (5) were low (27.43 ± 2.92 nmol cm$^{-2}$ day$^{-1}$) compared with rates measured in other marine and freshwater sediments (e.g., Kysing Fjord, 1,410 ± 160 nmol cm$^{-2}$ day$^{-1}$ [17]; Georgia salt marsh, 54,000 to 27,000 nmol cm$^{-2}$ day$^{-1}$ [19]; Lake Vechten, 170 nmol cm$^{-2}$ day$^{-1}$ [16]; Lake Constance, 300 to 2,000 nmol cm$^{-2}$ day$^{-1}$ [4]). However, an important observation was that when sediment was incubated with oxic overlying water, no sulfate reduction levels above that observed in formaldehyde-treated control samples could be measured. We interpreted this as indicating that sulfate depletion by sulfate reduction was balanced by oxidation of reduced sulfur species to sulfate. This suggested that under in situ conditions in which the overlying water column was oxic, rapid cycling of sulfur would occur in the Rydal sediment. This conclusion was supported by the profile of pore water sulfate levels, which exhibited a maximum at approximately 4 mm in the sediment. Sulfate profiles of this nature have previously been interpreted as an indication of rapid redoxation of sulfur in sediments (4). The low rates of sulfate reduction measured in the Rydal sediment were consistent with the observations of Babenzien (3) that *A. oxaliferum* inhabits sediments where sulfate reduction rates are low (in the range of 10 to 300 nmol cm$^{-3}$ day$^{-1}$). However, different methods were used to measure sulfate reduction in the present study, and consequently it was not possible to make a direct comparison with the rates calculated by Babenzien from radiotracer measurements in sediment slurries.

The most abundant pool of reduced inorganic sulfur in the sediment was CRS. Pyrite and elemental sulfur levels were not determined separately in this core, but the pyrite-to-elemental-sulfur ratio in the top 4 cm of the Rydal sediment over a 12-month period was approximately 4:1. CRS levels in the upper 20 mm increased with depth, whereas levels of FeS were comparatively low. FeS concentrations only began to accumulate below 20 mm. These observations are typical of other cores analyzed in this study. The profiles of AVS and CRS suggested that CRS may be precipitating directly from solution without the formation of FeS as a precursor. This mechanism of pyrite formation has been postulated to occur under conditions of low dissolved sulfide (18). The presence of dissolved iron at all depths would indicate that free sulfide levels must be very low (8, 38), promoting direct precipitation of pyrite, and indeed we were unable to detect free sulfide in any of the samples analyzed in this study.

For a sulfide-oxidizing organism to flourish in the environment described as in this study, it must be either an effective scavenger of the available free sulfide or capable of utilizing solid-phase sulfides which accumulate below the oxidized sediment surface. In sediments with low levels of reactive iron and high sulfate reduction rates, the bulk of bacterially produced sulfide can diffuse upwards to more oxidizing zones. In this region it is converted back to sulfide either by chemical oxidation or through the activity of phototrophic and lithotrophic bacteria. It is under these conditions that mat-forming bacteria may flourish (22). However, in sediments where large amounts of reactive iron or Fe$^{2+}$ are present, such as those from Rydal, an organism which can outcompete reactive iron for available sulfide would necessarily need to be dispersed throughout the sulfate reduction zone. Recent studies of mats of marine *Thioploca* spp. have shown that these sulfur bacteria shuttle between the water column, which is rich in electron acceptors (nitrate in the case of *Thioploca*), and sediment layers where high concentrations of sulfide exist (20). We have no evidence to suggest that this might be the case with *A. oxaliferum*. It is, however, intriguing to speculate that in sediments with low rates of sulfate reduction and high levels of reactive iron, such a shuttling strategy might allow *A. oxaliferum* to effectively scavenge sulfide at the site of production in the sediments and migrate to more oxidizing regions of the sediments where electron acceptors may be more abundant. This might to some extent explain why the *A. oxaliferum* population is dispersed over a number of redox zones in the sediment.

**Response of *A. oxaliferum* to dissolved oxygen.** The distribution of *A. oxaliferum* cells in cores taken from Rydal were found to be skewed towards the top few millimeters of the sediment surface when the cores were incubated in the dark for a number of days. On reillumination, the distribution returned to that observed in freshly sampled cores. This behavior was observed repeatedly in cores taken from the Rydal site that were subjected to this illumination regimen. The dissolved oxygen profiles and the distributions of *A. oxaliferum* cells in darkened and illuminated cores (Fig. 3) indicated that the cells...
shown that illumination conditions (Fig. 3). Other authors (31) have responded to movement of the oxycline induced by different within a narrow O₂-H₂S interface in sediments (23) and gradients was attributed to the vertical migration of cells within the sediment core. An alternative explanation, that changes in the distribution of the A. oxaliferum population were due to the death and regrowth of cells, was considered unlikely, since the frequency of replicating cells at all depths remained approximately 5%.

It is clear that A. oxaliferum responds to movement of the oxycline within sediments. Although the organism responds to vertical movement of the oxycline, it is incapable, due to the presence of calcite inclusions, of passing from the sediment into the water column above. This constraint may jeopardize its survival in profundal sediments of lakes prone to summer stratification. Vertical movement of sulfur bacteria in response to illumination conditions was previously demonstrated for marine Beggiatoa spp. (32). Beggiatoa was shown to have a negative phototaxis to white light; this negative phototaxis was not observed with A. oxaliferum, which apparently responded to movement of the oxycline induced by different illumination conditions (Fig. 3). Other authors (31) have shown that Beggiatoa spp. also exhibited a strong response to oxygen concentrations and were able to position themselves within a narrow O₂-H₂S interface in sediments (23) and gradient cultures (31, 33) where they could compete most effectively with abiotic mechanisms of sulfide oxidation.

The distribution of A. oxaliferum in sediments is controlled to some degree by the availability of oxygen. However, it was noted that some A. oxaliferum cells persisted at depths where dissolved oxygen was no longer detectable. These cells living in the suboxic zone may be respiring anaerobically or, as suggested above, may be migrating between redox zones. Besides the use of molecular oxygen as the terminal electron acceptor for oxidation of reduced sulfur, there are a number of mechanisms that facilitate the oxidation of reduced sulfur species using alternative electron acceptors. Nitrate, Mn⁴⁺, and Fe³⁺ are all known to be used for the anaerobic oxidation of reduced sulfur species by bacteria (1, 9, 10, 13, 29, 30, 37, 42).

**Is A. oxaliferum involved in the oxidation of reduced sulfur species?** The profile of pore water sulfate concentration was found to reflect the distribution of A. oxaliferum cells, with a maximum between 5 and 9 mm. This relationship was observed consistently throughout the top 40 mm of eight independently sampled cores over an 18-month period (Spearman correlation, 0.5006; n = 54; P < 0.001) and suggested a possible relationship between A. oxaliferum cells and sulfate production in the sediment. To corroborate our field data, we conducted experiments to determine the effect of the size of the A. oxaliferum population present in a sediment on the rate of reduced sulfur oxidation.

The inhibition of sulfate reduction by sodium molybdate was used to measure rates of sulfate accumulation in sediment cores containing A. oxaliferum cells. The rate of sulfate accumulation in reconstituted cores was linear over the time scale of the experiment at (4.54 ± 0.64) × 10⁻³ mol of SO₄²⁻ • dm⁻³ • day⁻¹ (Fig. 4). Under the conditions of these experiments, in which no bioturbation was observed and in which the oxidation of reduced sulfur was uncoupled from sulfate reduction, the bulk of reduced sulfur oxidation was bacterially mediated, as no increase in the concentration of sulfate was observed in the formaldehyde-treated controls.

Artificially increasing A. oxaliferum numbers in reconstituted sediment cores resulted in an increased rate of sulfate accumulation (Fig. 5), and there was a statistically significant correlation between cell numbers and the rate of sulfate accumulation. An approximate doubling of cell numbers yielded a...
doubled the sulfate accumulation rate. It was thus concluded not only that A. oxaliferum was capable of producing sulfate from reduced sulfur but also that under the conditions of these experiments it was responsible for the bulk of the sulfate produced. The possibility that the addition of A. oxaliferum cells simply provided a sulfur source that could be oxidized by other sulfur-oxidizing bacteria present in the sediment was considered. However, microscopic examination of the A. oxaliferum cells at the end of the incubation period revealed that the cells were intact and exhibiting the rolling motility characteristic of viable A. oxaliferum cells. The fact that highly purified suspensions of A. oxaliferum cells were added to the sediment cores make it likely that it was A. oxaliferum cells and not other fortuitously added sulfide-oxidizing bacteria that were responsible for the increased rates of sulfate accumulation in augmented cores.

The overall increase in sulfate concentration in these experiments (on the order of 10^{-4} mol dm^{-3}) must have resulted from the oxidation of one or more pools of reduced sulfur. Under the conditions of our experiments the potential sources of reduced sulfur included pyrite, amorphous FeS, organic sulfur ([18.88 ± 2.03] × 10^{-3} mol dm^{-3}; n = 3), intracellular and extracellular elemental sulfur, dissolved sulfide, thiosulfate, polysulfides, polythionates, and sulfite. The dynamic nature of the dissolved species and their low concentrations made it impossible to determine which reduced sulfur pool provided the source of sulfate in the incubation experiments. Alternatively, the amount of sulfate accumulation noted could have been supported by the cycling of sulfur through an intermediate other than sulfate, e.g., thiosulfate, bypassing the inhibition of sulfate reduction by molybdate. The key role of thiosulfate as an intermediate in the sulfur cycle that can be both oxidized and reduced has been previously recognized (24, 25), and a thiosulfate shunt might represent a mechanism by which sulfide may be regenerated in our experiments without the need for sulfate reduction. Intracellular elemental sulfur present in A. oxaliferum might also represent a source of the sulfate produced in these experiments. Ultimately, however, our data do not allow us to unequivocally identify the primary reduced sulfur species oxidized to sulfate by A. oxaliferum.

A combination of field data and laboratory studies has allowed us to confirm that A. oxaliferum plays an important role in the oxidation of reduced sulfur in a sediment where it is an abundant member of the bacterial community. These data support the initial assumptions made regarding the metabolism of A. oxaliferum based on the presence of intracellular sulfur inclusions. Furthermore, phylogenetic studies demonstrated that A. oxaliferum was a member of the γ-Proteobacteria and belonged to a lineage dominated by bacteria with physiologies based on sulfur oxidation (15). Within this group a number of morphologically distinct organisms are represented, e.g., Thiotrix spp., Thioploca spp., and Beggiaota spp. (43). However, not all of these bacteria are autotrophic, and a number are known to be mixotrophs or to be capable of heterotrophic growth. We are currently investigating the carbon metabolism of A. oxaliferum to determine whether it is indeed a lithoautotroph.

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