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Chlorobaculum tepidum growth on biogenic S(0) as the sole photosynthetic electron donor

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Summary

The green sulfur bacteria, the *Chlorobi*, are phototrophic bacteria that oxidize sulfide and deposit extracellular elemental sulfur globules [S(0)]. These are subsequently consumed after sulfide is exhausted. S(0) globules from a *Chlorobaculum tepidum* mutant strain were purified and used to show that the wild-type strain of *Cba. tepidum* can grow on biogenic S(0) globules as the sole photosynthetic electron donor, i.e. in medium with no other source of reducing power. Growth yields and rates on biogenic S(0) are comparable with those previously determined for *Cba. tepidum* grown on sulfide as the sole electron donor. Contact between cells and S(0) was required for growth. However, only a fraction of the cell population was firmly attached to S(0) globules. Microscopic examination of cultures growing on S(0) demonstrated cell–S(0) attachment and allowed for the direct observation of S(0) globule degradation. Bulk chemical analysis, scanning electron microscopy, secondary ion mass spectrometry and SDS-PAGE indicate that *Cba. tepidum* biogenic S(0) globules contain carbon, oxygen and nitrogen besides S and may be associated with specific proteins. These observations suggest that current models of S(0) oxidation in the *Chlorobi* need to be revised to take into account the role of cell–S(0) interactions in promoting S(0) degradation.

Introduction

Microbial interactions with solid materials are a critical factor for ecological success as many resources (nutrients, electron donors, electron acceptors) in natural systems are found as insoluble minerals, for example the metal oxides used by *Geobacter* spp. and *Shewanella* spp. as terminal electron acceptors (Reguera *et al.*, 2005; Marsili *et al.*, 2008). Microbial metabolism can also produce insoluble materials that have the potential to entomb cells if interactions with these materials are not controlled, for example iron (Fe) oxides produced by *Zetaproteobacteria* (Saini and Chan, 2013) or calcium carbonate produced by *Cyanobacteria* (Jansson and Northen, 2010). While a number of microbes either precipitate or dissolve extracellular minerals as a consequence of their metabolism, sulfide and zero-valent sulfur [S(0)]-oxidizing bacteria are unique in that they both form and consume S(0) depending on prevailing environmental conditions, primarily the presence or absence of sulfide. Biogenic S(0) can accumulate intracellularly or extracellularly. While S(0) globule metabolism and composition have been studied in *Proteobacteria*, mechanisms of growth and interaction with extracellular S(0) are less well understood in other organisms. Here, we present a detailed examination of growth on extracellular S(0) by the green phototrophic sulfur bacterium *Chlorobaculum tepidum* and on the composition of biogenic S(0) produced by this strain.

Chlorobaculum tepidum is a model organism for the green phototrophic sulfur bacteria (*Chlorobi*) that is genetically amenable and whose complete annotated genome sequence is available (Eisen *et al.*, 2002; Overmann and Garcia-Pichel, 2006). *Cba. tepidum* forms extracellular S(0) globules by oxidizing sulfide under anaerobic conditions then consuming extracellular S(0) globules when sulfide is exhausted, producing sulfate as the terminal oxidized product (Wahlund *et al.*, 1991; Chan *et al.*, 2008; Holkenbrink *et al.*, 2011). The electrons from sulfur oxidation are passed into the photosynthetic electron transport chain, which ultimately reduces ferredoxin (Fd). Reduced Fd is used for CO_2 fixation (Evans *et al.*, 1966) or to reduce $NAD(P)^+$ via a novel Fd:$NAD(P)^+$ oxidoreductase (Seo and Sakurai, 2002) to support anabolism. Proton motive force generated during electron transport is utilized to synthesize ATP and transport other nutrients into the cell. Thus, the oxidation of sulfur compounds is central to the energy metabolism of *Cba. tepidum* and the *Chlorobi*.

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Microbial interactions with S(0) have been studied primarily in *Beta-* and *Gammaproteobacteria*. Microbes that form or consume extracellular S(0), chemolithotrophic *Acidothiobacillus* spp. and *Thiobacillus* spp. produce extracellular hydrophilic S(0) globules that are easily separated from cells and are thought to contain long-chain polymers, though this has not been conclusively demonstrated (reviewed by Kleinjan *et al*., 2003). *Acidothiobacillus* spp. attach S(0) globules during degradation and significantly alter the expression of genes encoding both outer membrane and secreted proteins relative to growth on other substrates (Ramírez *et al*., 2002; 2004; Mangold *et al*., 2011; Chen *et al*., 2012). Unattached cells grow in S(0)-oxidizing cultures suggesting the production of a soluble species by cells attached to S(0) particles (Ceskova *et al*., 2002). Other sulfide-oxidizing bacteria form intracellular S(0) globules including the phototroph *Allochromatium vinosum* and various chemotrophic *Beggiatoa* spp. (Pattaragulwanit *et al*., 1998; Schulz *et al*., 1999). *A. vinosum* is the most well studied system for intracellular S(0) globule synthesis and degradation. In *A. vinosum*, S(0) is stored in the periplasm as globules coated by the sulfur globule proteins SgpA, SgpB and SgpC (Pattaragulwanit *et al*., 1998; Prange *et al*., 2004). Mutagenesis of the *A. vinosum sgpC* gene caused the formation of smaller S(0) globules, suggesting that it plays a role in S(0) globule expansion, while single mutants of *sgpA* and *sgpB* caused no significant defect, suggesting that their gene products may be functionally redundant (Prange *et al*., 2004). *Allochromatium vinosum* can also grow on commercial S(0) provided extracellularly, but growth on either S(0) or sulfide was inhibited by coating cells with polyelectrolyte layers that affect cell surface charge (Franz *et al*., 2010).

The enzymatic machinery for sulfur compound oxidation is similar between the *Chlorobi* and *A. vinosum* (reviewed in Frigaard and Dahl, 2009). Briefly, sulfide is oxidized by sulfide:quinone oxidoreductase to produce S(0), which is further oxidized to sulfate after sulfide is exhausted by a pathway including reverse dissimilatory sulfite reductase complex, adenosine-5′-phosphosulfate reductase and sulfate adenylyl transferase. The localization of S(0) produced by the oxidation of sulfide is extracellular in the *Chlorobi* and periplasmic in *A. vinosum*, perhaps because *Chlorobi* genomes do not contain orthologues of *A. vinosum* sulfur globule proteins (Eisen *et al*., 2002; Gregersen *et al*., 2011). However, the chemical composition of *Chlorobi* S(0) globules has not been examined to determine if they contain other constituents that may be involved in their formation or subsequent utilization.

Here, we present data on the growth of *Cba. tepidum* on S(0), which required that the S(0) be synthesized by *Cba. tepidum*. To the best of our knowledge, this work presents the first quantitative growth data for *Cba. tepidum* or other *Chlorobi* on S(0) in the absence of any other reductant, the first demonstration that cell attachment is required for growth of *Cba. tepidum* or other *Chlorobi* on S(0), and the first evidence that extracellular biogenic S(0) globules made by *Cba. tepidum* contain proteins.

Results

Cba. tepidum *displays robust growth on biogenic S(0) as the sole electron donor*

When *Cba. tepidum* was isolated, 'sulfide was absolutely required for growth' (Wahlund *et al*., 1991). Subsequently, *Cba. tepidum* has been shown in multiple laboratories to grow well on thiosulfate as the sole electron donor (Chan *et al*., 2008; Azai *et al*., 2009; Holkenbrink *et al*., 2011; Eddie and Hanson, 2013). Attempts to grow *Cba. tepidum* on commercially available S(0) powders or flowers as the sole electron donor routinely failed in our laboratory and has not been reported in the literature even though wild-type *Cba. tepidum* clearly consumes extracellular S(0) globules formed from sulfide oxidation (Wahlund *et al*., 1991; Hanson and Tabita, 2001; Chan *et al*., 2008; Holkenbrink *et al*., 2011). Holkenbrink and colleagues (2011) reported that wild-type *Cba. tepidum* oxidized S(0) globules harvested from a *dsrCABL*1/2 mutant strain completely to sulfate in the presence of 0.1 mM sulfide, but no quantitative growth data were reported for this condition. Therefore, we decided to examine whether biogenic S(0) could support the growth of *Cba. tepidum* as the sole electron donor, i.e. in the absence of any added sulfide.

Vigorous and reproducible growth was achieved when biogenic S(0) purified from a *Cba. tepidum* mutant strain was supplied to strain WT2321 as the sole electron donor (Fig. 1). Cultures provided commercial S(0) from a concentrated stock in hexane diluted in medium, i.e. as a hydrophilic colloidal sulfur sol – a Weimarn sol (Steudel, 2003), did not grow. Addition of an equivalent amount of hexane (0.1% v/v) as present in the commercial S(0) cultures to cultures with biogenic S(0) or sulfide plus thiosulfate did reduce the growth yield by about 25% but did not prevent growth of strain WT2321. This suggests that residual solvent did not cause the lack of growth of *Cba. tepidum* on the Weimarn sol. These results are typical of all attempts to grow *Cba. tepidum* on commercial S(0) in our laboratory (data not shown). Growth on biogenic S(0) was not due to stored electron donors in the inoculated cells or carry-over in the inoculum, as strain WT2321 transferred into medium lacking any exogenous electron donor did not grow. The lack of growth in uninoculated tubes of medium with purified biogenic S(0) demonstrated that any cells of the mutant strain present

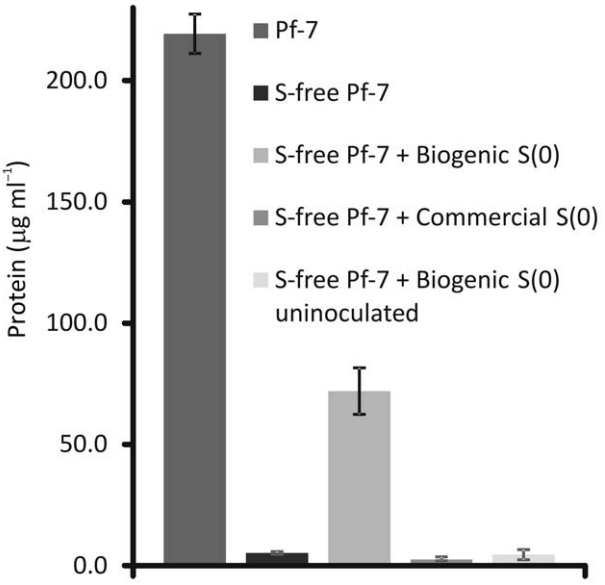

Fig. 1. The growth yield of *Cba. tepidum* was measured after 48 h of growth in the specified medium from an initial inoculation density of 4 μg protein ml^{-1}. Pf-7 medium contains ~ 1 mm HS- and 10 mm $S_2O_3^{2-}$ as electron donors; S-free Pf-7 contains no electron donor. S(0) was supplied at a concentration of 3 mm for both biogenic and commercial types. Data are the mean ($n = 3$) ± standard deviation.

after biogenic S(0) purification were incapable of growth using it as a sole electron donor.

The specific growth rate constant, μ, for *Cba. tepidum* on S(0) was 0.14 ± 0.01 h^{-1} ($n = 4$, ± standard deviation), corresponding to a doubling time of ~ 5.0 h. This value is significantly slower than previous reports for growth in the presence of sulfide (μ ~ 0.35 h^{-1}, doubling time ~ 2 h, Wahlund *et al.*, 1991; Mukhopadhyay *et al.*, 1999; Chan *et al.*, 2008). However, cultures grown with thiosulfate as the sole electron donor in parallel to those grown on S(0) had similar growth rates with μ of 0.12 ± 0.01 h^{-1}.

Biogenic S(0) preparations were routinely assayed to ensure that no other electron donors were present. Concentrated stock solutions of purified biogenic S(0) contained no detectable sulfide or sulfite (detection limit < 1 μM). Thiosulfate was detected in only one batch of concentrated S(0) stock at a concentration of 9 μM relative to a [S(0)] = 0.5 M. Thus, the thiosulfate was diluted to < 0.5 μM at the concentrations of S(0) used for growth in these experiments (3–20 mM) and could not have significantly affected growth.

When the concentration of initial S(0) provided to *Cba. tepidum* was varied, the growth yield was strongly dependent on the amount of S(0) consumed (Fig. 2A). A linear regression of biomass values at low supplied S(0) concentrations (dashed line in Fig. 2A) indicated a maximum yield of 40 g protein [mol S(0)]$^{-1}$. This corresponds to 6.5 g protein (mol e^-)$^{-1}$ assuming the complete oxidation of S(0) to sulfate. When S(0) and sulfate concentrations were measured in cultures grown on biogenic S(0) (Fig. 2B), the sulfur mass balance in this experiment averaged 98.5%. The mass balance observed across a range of experiments with initial S(0) concentrations from 1 to 16 mM was 99 ± 3% ($n = 12$), again when considering only S(0) and sulfate.

Growth requires cell–S(0) contact, but not all cells appear to be attached

S(0) is largely insoluble, on the order of 0.2 μM in aqueous solution at 25°C (Steudel, 2003). Dialysis culture was used to determine if the growth of *Cba. tepidum* on biogenic S(0) could occur solely via soluble, low-molecular-weight intermediates (Fig. 3A). The growth of *Cba. tepidum* on biogenic S(0) was not inhibited by the presence of a microdialysis apparatus where cells and S(0) were on the same side of the dialysis membrane

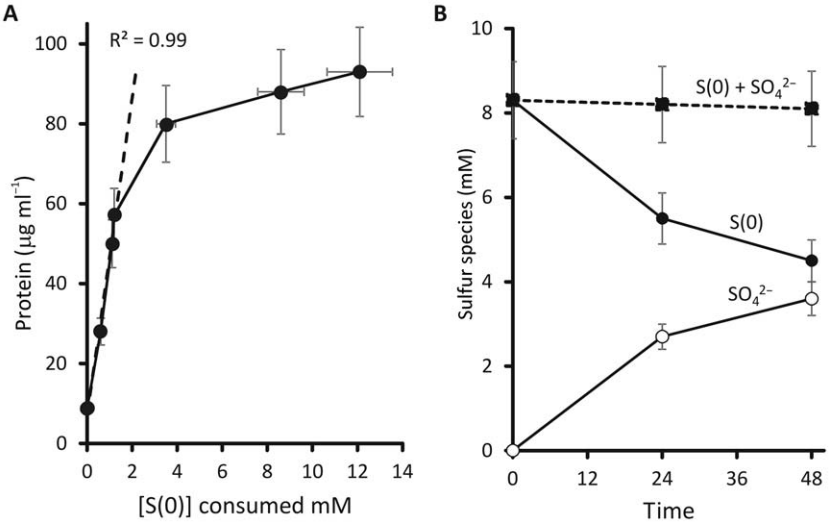

Fig. 2. Characteristics of *Cba. tepidum* growth on biogenic S(0).
A. Growth yield of *Cba. tepidum* with varying S(0). The dashed line is a linear regression of the first four points of data. Data are the mean (n ≥ 2) ± the standard error of measurement for protein or S(0).
B. Sulfur compounds during growth: biogenic S(0), filled circles; sulfate, open circles; summed S(0) and sulfate, filled squares. Data points are the mean ($n = 2$) ± the standard error of measurement for the sulfur compounds.

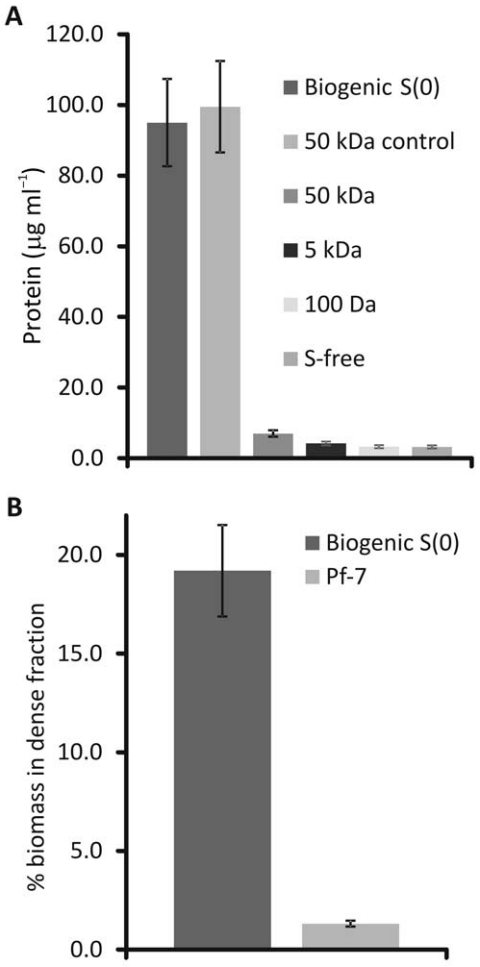

Fig. 3. Evidence that cell–S(0) contact is required for *Cba. tepidum* growth.
A. Growth yield after 48 h of *Cba. tepidum* growth on 4 mm S(0) alone, in the presence of a dialysis membrane (control), or when separated from S(0) by dialysis membranes of varying nominal molecular weight cut-off. Data are the mean ($n \geq 2$) ± the standard error of measurement.
B. The per cent of biomass that passed a sucrose gradient in *Cba. tepidum* cultures grown for 24 h on 4 mm biogenic S(0) as the sole electron donor or in standard Pf-7 medium with sulfide and thiosulfate as electron donors. Data are the mean ($n = 5$) ± the standard deviation.

(50 kDa control). However, when *Cba. tepidum* cells were separated from biogenic S(0) by dialysis membranes ranging in nominal molecular weight cut-off size from 100 Da to 50 kDa, no growth was detected.

Since contact between cells and S(0) appeared to be required for growth, sucrose density centrifugation was used to assess if all *Cba. tepidum* cells were attached to biogenic S(0) (Fig. 3B). Solid S(0) should penetrate the sucrose solution as the mean density of all solid S(0) allotropes stable at the growth temperature of *Cba. tepidum* is 2.09 ± 0.08 g ml^{-1}, not very different from that of cycloocta S(0) at 2.07 g ml^{-1} (Meyer, 1976). In cultures growing on S(0), about 20% of the total protein in the culture could be harvested as dense material [i.e. attached to S(0)] that passed through a sucrose solution of $\rho \sim 1.32$ g ml^{-1} at low centrifugal force ($4000 \times g$). In cultures growing on sulfide plus thiosulfate after sulfide and S(0) were exhausted, only ~ 1% of biomass penetrated the dense sucrose solution suggesting that the sucrose gradient method reliably indicates cellular attachment to dense materials.

Characterization of biogenic S(0) produced by Cba. tepidum

The surface charge of the biogenic S(0) measured as zeta potential was found to be –22 ± 5 mV and –27 ± 5 mV for two independent batches.

Biogenic S(0) was visualized by cryo-scanning electron microscopy (cryo-SEM, Fig. 4). Purified biogenic S(0) was composed of spherical globules over a range of sizes, but most globules were larger than *Cba. tepidum* cells; the globules were generally smooth and intact (Fig. 4A). When globules were imaged from a culture of wild-type *Cba. tepidum* that was actively growing on S(0), cells of *Cba. tepidum* were observed to be attached to S(0) globules (Fig. 4C) and the globules appeared degraded, with large pits or cavities seen on many S(0) globules (Fig. 4B and C).

Several methods were used to determine if purified biogenic S(0) contained any other elements aside from

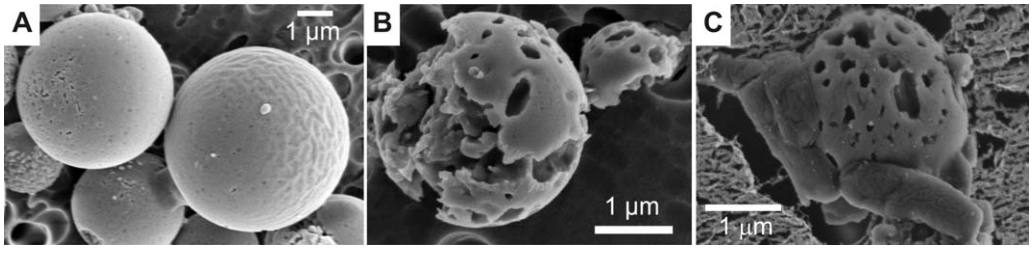

Fig. 4. Representative cryo-fixation scanning electron micrographs of biogenic S(0) produced by *Cba. tepidum*.
A. S(0) globules as isolated from mutant strain C3.
B. S(0) globules from a culture of strain WT2321 growing on biogenic S(0) displaying attached cells.
C. Pitted S(0) globule from a culture of strain WT2321 growing on biogenic S(0).

Table 1. Bulk elemental analysis of two independent samples of biogenic S(0) produced by *Cba. tepidum* or commercial S(0).

	Atom %					
	C	H	N	O	S	Total
Biogenic 1	0.30	0.04	0.00	0.28	99.27	99.89
Biogenic 2	0.23	0.00	0.00	0.36	99.37	99.89
Commercial 1	0.13	0.00	0.00	0.00	99.65	99.89
Commercial 2	0.00	0.00	0.00	0.00	99.66	99.89

sulfur. Bulk elemental analysis of biogenic S(0) indicated that it contained significant amounts of carbon and oxygen that were not found in samples of commercial sulfur analysed in parallel (Table 1). However, these were quite low in terms of absolute abundance; the samples were still > 99.3 atom % sulfur. Electron dispersive X-ray spectroscopy of S(0) globules in cryo-SEM revealed that the surface of purified biogenic S(0) globules had a very high, but variable amount of carbon, averaging 59 ± 17 atom % carbon combined with 41 ± 17% atom % sulfur. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis of purified biogenic S(0) detected molecular ions with formulas consistent of amino acid fragments that, in contrast to the other methods, suggested the presence of nitrogen in biogenic S(0) as well (Table 2).

Direct observation of Cba. tepidum*–S(0) interactions*

Time-lapse phase-contrast microscopy was used to observe the interactions of *Cba. tepidum* cells with biogenic S(0) during growth. In these experiments, cultures were allowed to accumulate biogenic S(0) and were then imaged at 10 min intervals while incubating on a heated and illuminated microscope stage. A montage of images produced from a representative experiment is shown in Fig. 5 and full movies are available as supporting information (Fig. S1 and Movie files 1–3). During S(0) consumption the majority, but not all, of S(0) globules were in contact with cells, but individual S(0) globules were not constantly associated with cells. However, the majority of cells were not attached to S(0) globules. Individual S(0) globules progressively shrink with time until they disappear from view. In the later stages of growth in the time-lapse experiments, no S(0) globules could be observed and cells completely filled the field of view (data not shown).

Table 2. Molecular fragments detected in *Cba. tepidum* biogenic S(0) by ToF-SIMS.

m/z	Formula	Source
28	CH_2N^+	Amino acids
30	CH_4N^+	Amino acids
42	$C_2H_4N^+$	Alanine, glycine, histidine, leucine, serine
54	CH_2Ca^+	
55	CH_3Ca^+	
70	$C_4H_8N^+$	Proline, valine
72	$C_4H_{10}N^+$	Valine

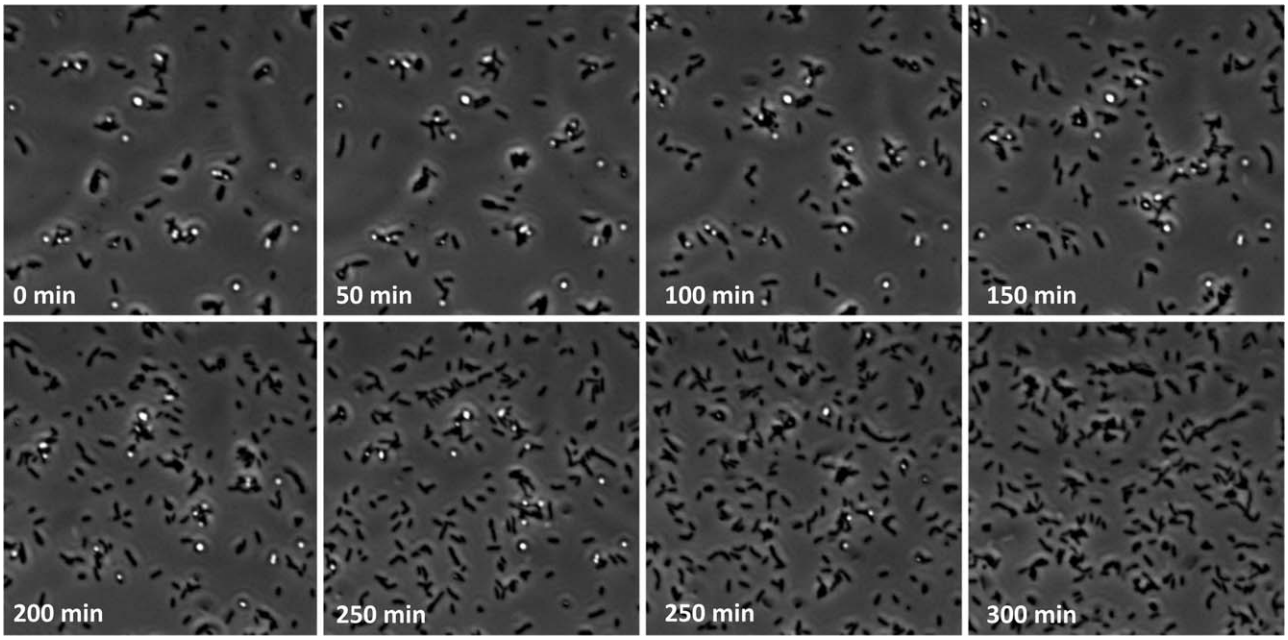

Fig. 5. Time-lapse montage of *Cba. tepidum* growing in standard Pf-7 growth medium at 47°C with ~ 1.5 mm sulfide at the start of the experiment. The capillary was loaded after 4 h of growth in a Balch tube and imaged every 10 min over 16 h. Representative images are from the indicated time points. Phase contrast, 400× total magnification. Cells are dark and S(0) globules are bright under this illumination.

Biogenic S(0) associated proteins

Proteins extracted from biogenic S(0) appeared to contain an enriched subset of the *Cba. tepidum* proteome that was easily distinguished by one-dimensional denaturing gel electrophoresis (Fig. 6A and B). Two proteins were identified in extracts of biogenic S(0) that are poorly functionally annotated, CT1320.1 and CT1305. CT1320.1 was detected with > 99% confidence in two adjacent, broad bands from biogenic S(0) extracts at approximately 11 and 13 kDa (Fig. 6A; black-bracketed bands). Bands corresponding to 11 and 13 kDa were also present in protein extracts from cells of *Cba. tepidum* grown on S(0) (Fig. 6A; bands bracketed in white), but CT1320.1 was not identified in these bands: only two of the four characteristic ions for this protein were detected, but at four- to 20-fold lower signal-to-noise ratios than in the biogenic S(0) extract. CT1305 was observed in a broad band at approximately 27 kDa with > 99% confidence (Fig. 6B; black-bracketed band). This band was enriched and four of five characteristic ions of CT1305 were detected when biogenic S(0) was extracted by heating with detergent and reductant (Fig. 6B; bracketed in white) after the globules had been extracted sequentially with reductant and detergent at room temperature. This provides additional evidence that CT1305 is a protein tightly associated with biogenic S(0). The observed molecular weights for these proteins are significantly smaller than the predicted masses of the open reading frame products: 22.1 kDa for CT1320.1 and 54.1 kDa for CT1305. CT1305 contains a second methionine at residue 45 that is likely the translation initiation site based on homology with related *Chlorobi* proteins (Fig. 6C) and transcript sequence coverage from previous RNA-seq work (Eddie and Hanson, 2013; http://zippo.dbi.udel.edu/gb2/gbrowse/c_tepidum/). CT1320.1 and CT1305 both contain predicted non-cytoplasmic signal peptides (Fig. 6C) that would produce mature, secreted CT1320.1 at 18.2 kDa and CT1305 at 47.2 kDa. Further processing of CT1320.1 and CT1305 to produce the observed masses is supported by the detection of peptides only after residue 98 for CT1320.1 and residue 112 for CT1305 in mass spectrometry (MS) analysis (data not shown).

Discussion

Growth and consumption of biogenic S(0) by Cba. tepidum

The results presented here demonstrate for the first time that *Cba. tepidum* grows well on biogenic S(0) in the absence of any other electron donor or reducing agent. The growth yield on S(0) is similar to that observed for the growth of *Cba. tepidum* on sulfide: 5.8 g protein (mol e^-)$^{-1}$, averaging values from Chan and colleagues (2009), Rodriguez and colleagues (2011) and Mukhopadhyay and colleagues (1999). The S(0) growth yield is greater than that for sulfite: 2.2 g protein (mol e^-)$^{-1}$, calculated from data in Rodriguez and colleagues (2011). All growth yields for *Cba. tepidum* have been measured under conditions where both acetate and CO_2 were provided as carbon sources. Therefore, the yields for growth on S(0) should be higher than those for photoautotrophic growth. This is indeed the case. *Allochromatium vinosum* grown photoautotrophically on S(0) displays a yield of 10–15 g protein [mol S(0)]$^{-1}$ or 2–3 g protein (mol e^-)$^{-1}$ calculated from data in Franz and colleagues (2010), two- to threefold lower than our results. A complete mass balance for sulfur species was found when only considering sulfate and S(0) during S(0) consumption and growth, indicating that any free intermediates in the oxidation pathway were present at low concentrations and confirming that sulfate is the sole oxidation product of S(0) metabolism.

Direct contact of *Cba. tepidum* cells with S(0) was required for growth, as demonstrated by the dialysis culture experiment. However, only a fraction of cells in a culture growing on S(0) contact globules at any given time, as indicated by both the sucrose-gradient fractionation and time-lapse microscopy data. At least two distinct models may explain these observations. Cells may only need to transiently attach to S(0) globules and load a sufficient amount of substrate to fuel subsequent growth. Alternatively, cells attached to S(0) globules may be producing soluble intermediates that diffuse and feed unattached cells. Our current data cannot differentiate between these two models. Efforts are underway to quantify time-lapse microscopy experiments to better understand attachment/detachment kinetics and the nature of observed cellular motions. In parallel, experiments are being conducted to detect potential soluble intermediates, although they are likely to be at low concentrations and transient, i.e. rapidly consumed by unattached cells.

S(0) properties and S(0) globule proteins

The presence of C, H and O in addition to S in bulk chemical analysis, the observation of ions characteristic of amino acids in ToF-SIMS and the ability to extract proteins originating from *Cba. tepidum* from purified biogenic S(0) all indicate that *Cba. tepidum* adulterates S(0) globules that it synthesizes either as a result of depositing a surface coating or by non-specific sorption of cellular material. The specific polypeptides identified in biogenic S(0) globules have no proposed function, i.e. both CT1305 and CT1320.1 are annotated as 'hypothetical protein' in GenBank. These two proteins are representatives of a group that has an extremely limited

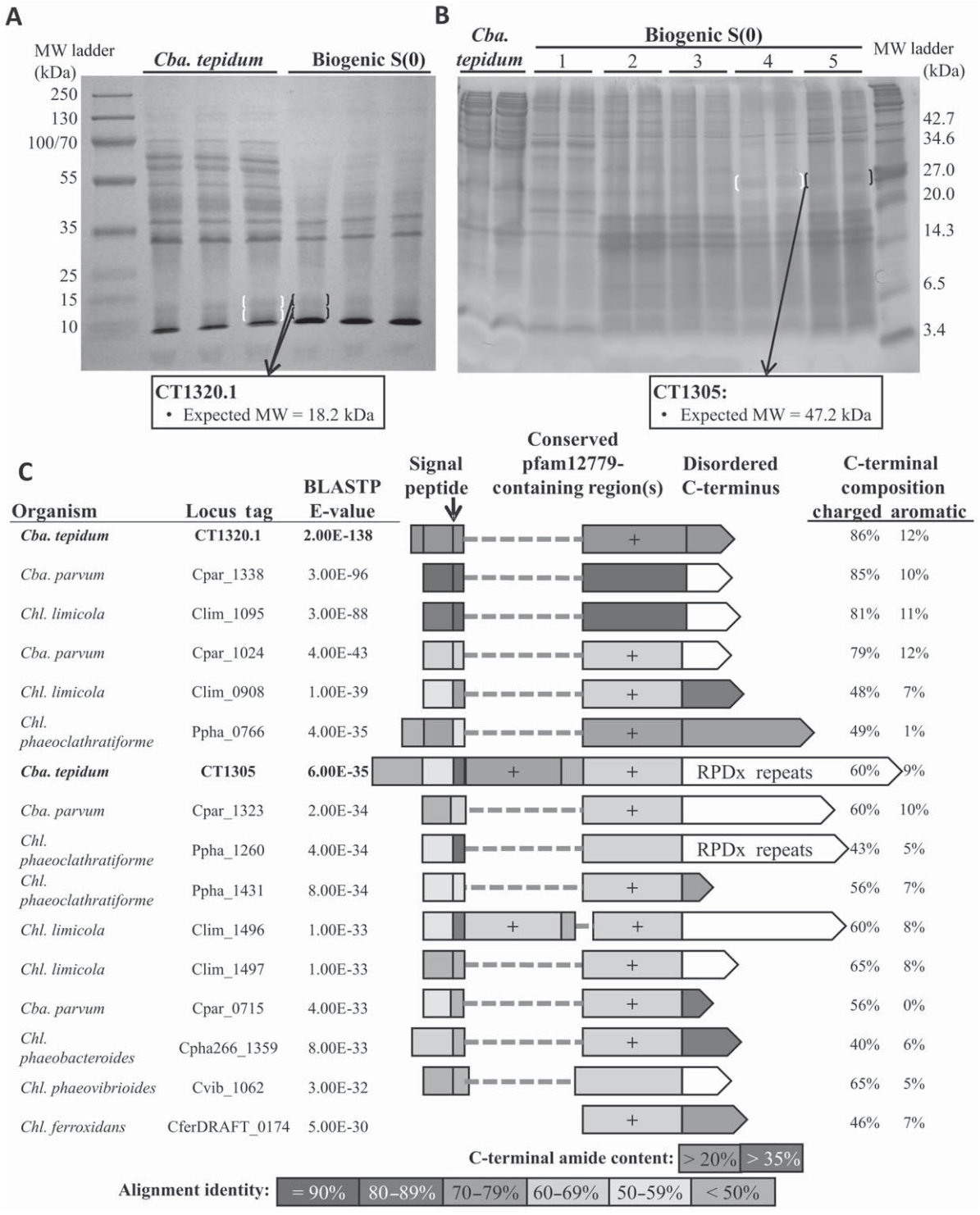

Fig. 6. Biogenic S(0) globules produced by *Cba. tepidum* contain poorly functionally annotated proteins.
A. 15% SDS-PAGE gel stained with Coomassie brilliant blue. *Cba. tepidum* cells after growth on S(0) compared with purified biogenic S(0).
B. 16.5% acrylamide tris-tricine gel stained with SYPRO® Ruby. Lanes were loaded with the supernatant of biogenic S(0) extracted with: 1, S-free Pf-7; 2, pellet from 1 with SDS at 25°C; 3, pellet from 2 with SDS + 2-mercaptoethanol at 25°C; 4, pellet from 3 with SDS + 2-mercaptoethanol at 95°C; 5, pellet from 1 treated as in 4. Black brackets indicate bands where proteins CT1320.1 or CT1305 were detected with > 99% confidence; bands bracketed in white indicate where characteristic ions for these proteins were detected but at < 99% confidence.
C. Annotated schematic alignment CT1320.1 and CT1305 homologues in the *Chlorobi*. The presence of motif pfam12779 (E-value < 0.05) in a domain is indicated by '+'. Alignment quality relative to CT1320.1 and enrichment of amide side chain amino acids in the C-terminus are indicated by shading.

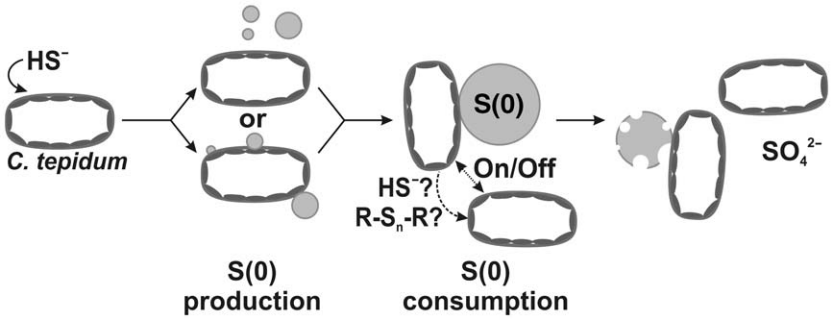

Fig. 7. Working model for *Cba. tepidum* S(0) globule synthesis and consumption. From left to right, *Cba. tepidum* consumes sulfide and produces S(0) globules that have a coating (grey border). It is currently unclear whether globules are in contact with cells during synthesis. S(0) consumption requires cell contact, but cells can become detached and there is likely transfer of soluble species to unattached cells. Pitted/degraded S(0) globules are produced during consumption along with sulfate as the terminal oxidized product. It is unclear whether the proposed coating is breached during consumption.

distribution in prokaryotes. Transcripts for both CT1305 and CT1320.1 were detected at high levels (approximately eightfold higher than mean expression for all genes in all samples) in cultures grown on thiosulfate and after the addition of sulfide to these cultures (Eddie and Hanson, 2013). CT1305, but not CT1320.1, had also been detected in two previous proteomic analyses of *Cba. tepidum* (Aivaliotis *et al*., 2006; 2007).

CT1320.1 and CT1305 are homologues of each other and of proteins in other *Chlorobi* (Fig. 6C and Table S1). There is no detectable homology between these proteins and the *A. vinosum* Sgp proteins. The closest homologues in the UniProt knowledge base (BLASTP e-value < 1E-30) also contain predicted non-cytoplasmic signal sequences and one or two copies of a conserved region that is enriched in charged and aromatic amino acid residues. CT1305 carries two copies of this conserved region that share > 90% amino acid sequence identity with each other, while CT1320.1 and most of the other close homologues carry a single copy. Only one other protein, Clim_1496 from *Chlorobium limicola*, has two copies of this region, with > 60% amino acid sequence identity to the region in CT1320.1. A short conserved motif (Pfam_12779; YXWGXW) of unknown function found in short, secreted bacterial proteins was detected in the central conserved region(s) of CT1320.1, CT1305 and a number of the other homologues (e-values from 4.00E-02 to 3.72E-05). These proteins also have a low-complexity C-terminal region that is predicted to be disordered and is highly enriched in charged residues that often contains RPDx repeats, proline rich regions and/or amide residues. Most *Chlorobi* genomes contain between one and four genes encoding homologues of CT1320.1 and CT1305, of which one is a close homologue (e-value < E-30). Only the most basal member of the *Chlorobi*, *Chloroherpeton thalassium* strain ATCC 35110/GB-78, has no predicted homologues to either CT1320.1 or CT1305.

When searches were performed with less stringent match criteria (BLASTP E-value < 1E-10), a total of 48 sequences similar to CT1305 and CT1320.1 are found in the UniProt knowledge base (Table S1). In addition to those *Chlorobi* sequences described above, weaker homologues are found in the *Chlorobium luteolum* strain DSM 273 (one copy; e-value = 4E-28), *Prosthecochloris aestuarii* strain DSM 271/SK 413 (one copy; e-value = 5E-25), *Chlorobium phaeobacteroides* strain BS1 (one copy; e-value = 2E-24) and *Chlorobium chlorochromatii* strain CaD3 (three copies; e-values from 7E-22 to 8E-19). Beyond the *Chlorobi*, homologues of CT1305 and CT1320.1 are only found in the class *Deltaproteobacteria* (*Geobacter* spp. and *Pelobacter* spp., 18 and two sequences, respectively) and *Alphaproteobacteria* (*Sphingobacteria*, three sequences). That the majority of these homologues are found in *Geobacter* spp. is intriguing as these organisms transfer electrons to extracellular electron acceptors, such as Fe(III) oxides, and must establish direct contact with these solid surfaces in order to reduce them (Nevin and Lovley, 2000; Reguera *et al*., 2005). *Pelobacter propionicus* strain DSM 2379 is the only non-*Geobacter* species within the *Deltaproteobacteria* with CT1305/CT1320.1 homologues.

Summary

Based on the data reported here, we present a working model for S(0) globule metabolism in *Cba. tepidum* (Fig. 7) that is focused on microbe–mineral interactions rather than on specific enzymes and intermediates as in prior models (e.g. Frigaard and Dahl, 2009; Sakurai *et al*., 2010; Gregersen *et al*., 2011). We hypothesize that *Cba. tepidum* coats S(0) globules during synthesis with a peptide or protein coating. It is not clear if the S(0) globule coating is a simple extension of the outer membrane or a compositionally and functionally distinct layer, but we hypothesize the latter. Mechanistically, we hypothesize that *Cba. tepidum* inflates a coated globule with S(0) during sulfide oxidation and that this coating facilitates interactions during S(0) degradation. By demonstrating the ability to grow *Cba. tepidum* on S(0) as the sole electron donor, directly observing cell–S(0) interactions over long time spans, and analysing the composition of S(0) globules, this work sets the stage for multiple experimental routes to test this model.

Experimental procedures

Strains and growth conditions

All growth experiments on S(0) were conducted with *Cba. tepidum* strain WT2321, a plating strain derivative of the original TLS1 isolate (Wahlund *et al*., 1991; Wahlund and Madigan, 1995). S(0) globules were purified (see below) from a mutant strain of *Cba. tepidum* called C3 that can no longer efficiently utilize S(0) globules for growth. The mutant strain was produced by *in vitro* transposition mutagenesis (Chan *et al*., 2008) and will be fully described elsewhere. Cultures were grown in Pf-7 prepared as previously described (Chan *et al*., 2008). For growth with S(0) as the sole electron donor, thiosulfate and sulfide were omitted from the medium to make S-free Pf-7. S(0) was added to autoclaved S-free Pf-7 to the desired concentration from concentrated biogenic S(0) suspensions (see next section) or a S(0) saturated hexane solution. Standard growth conditions were 45°C and a light field of 20 μmol photons m^{-2} s^{-1} measured with a light metre equipped with a quantum PAR sensor (LI-COR, Lincoln, NE). Growth of *Cba. tepidum* was measured by determining protein concentration in culture samples by the Bradford assay as previously described (Mukhopadhyay *et al*., 1999; Chan *et al*., 2008). Unless otherwise stated, growth yields of *Cba. tepidum* were measured after 48 h of incubation, which is in stationary phase for all conditions tested. Growth rates were calculated as the average observed growth rate between 9 and 19 h of culture.

Biogenic S(0) purification

One litre cultures of strain C3 were grown on sulfide as the sole electron donor in narrow-mouth screw cap bottles with an open phenolic cap and butyl rubber septum (Fisher Scientific, Pittsburgh, PA) with the periodic addition of sulfide to a concentration of 3–5 mM from an anoxic, sterile, neutral sulfide solution (Siefert and Pfennig, 1984). A qualitative assay, the precipitation of Cu^{2+} ions from aqueous solution, was used to assess when sulfide was exhausted. Briefly, equal amounts of culture supernatant are mixed with an equal volume of aqueous 10 mM $CuCl_2$. If sulfide is present at greater than 0.2 mM concentration, a distinct grey precipitate is immediately formed. Sulfide was added to cultures when it was no longer detectable by this assay.

S(0) was purified from cultures by sucrose density gradient centrifugation (Donà, 2011). All transfers were carried out in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) using sterile, S-free Pf-7 directly or as the solute for any solutions. S(0)-containing cultures of strain C3 were transferred into sterile 250 ml centrifuge bottles with O-ring sealing caps (Thermo Scientific Nalgene, Waltham, MA). Cells and S(0) were collected by centrifugation at 1000 × *g* for 10 min at 10°C. The supernatant was removed and cells plus S(0) were suspended in a minimal volume. The resulting suspension was layered over 100 ml of sterile 2.5 M sucrose solution ($\rho \sim 1.32$ g ml^{-1}) in sterile 250 ml centrifuge bottles with O-ring sealing caps. The S(0) was pelleted through the sucrose by centrifugation at 4000 × *g* for 10 min at 10°C. The supernatant was removed and the collected S(0) from the pellet was centrifuged through 2.5 M sucrose two more times. Collected S(0) was suspended with S-free Pf-7 and centrifuged at 17 500 × *g* for 5 min at 10°C. This step was repeated twice to remove sucrose. S(0) was suspended again and vortex mixed for several minutes to detach any remaining cells and allowed to settle without centrifugation. The supernatant was aspirated and a working suspension created by adding a volume of S-free Pf-7 to make a concentrated homogeneous suspension that could still be pipetted.

Assessing cell–S(0) attachment

The fraction of cells firmly attached to S(0) globules was determined by a modification of the S(0) globule purification protocol above. Cultures of *Cba. tepidum* WT2321 grown on S(0) or on normal Pf-7 medium containing both sulfide and thiosulfate were sampled for protein concentration and then centrifuged at 8000 × *g* for 10 min at 10°C. The pellet containing both cells and S(0) was suspended in a minimal volume of supernatant and layered over 30 ml of 2.5 M sucrose in a 35 ml centrifuge tube (Thermo Scientific Nalgene, Waltham, MA) and centrifuged at 4000 × *g* for 10 min at 10°C. The supernatant was removed and the pellet was resuspended in a minimal volume of S-free Pf-7. This process was repeated twice more and the pellet from the final centrifugation was assayed for protein concentration. The per cent of S(0)-attached biomass was determined by dividing the total protein recovered in the pellet of the sucrose centrifugation to the total protein in the culture before harvest.

Sulfur species quantification

Sulfur species were quantified with previously described methods (Chan *et al*., 2008) based on published protocols (Rethmeier *et al*., 1997). Standard curves for sulfur species were prepared from S(0) powder (> 99.5% Aldrich, Milwaukee, WI), sodium sulfite (min. 98%, Sigma-Aldrich, St Louis, MO), sodium sulfate (> 99%, Sigma-Aldrich), sodium thiosulfate pentahydrate (> 99.5%, Sigma-Aldrich) and sodium sulfide nonahydrate (Certified ACS, Fisher Scientific, Fair Lawn, NJ).

Dialysis culture

Dialysis cultures were performed in GL-45 screw cap media bottles (VWR Scientific, Bridgeport, NJ) that contained an autoclaveable 1.5 ml spin dialysis chamber (Harvard Apparatus, Holliston, MA). After autoclaving, bottles, #6.5 stoppers, the dialysis chamber and sterile dialysis membranes were taken into an anaerobic chamber. The membranes were rinsed with sterile S-free Pf-7 and the assembled dialysis chamber was loaded with sterile S-free Pf-7 or concentrated biogenic S(0) suspension. The loaded and sealed chambers were placed in media bottles that were then filled with S-free Pf-7 medium to 75% capacity. A stopper was inserted into the bottle opening and the septum cap then threaded and tightened by hand. After bringing the sealed bottles out of the anaerobic chamber, the headspace was exchanged with 5% CO_2 + 95% N_2 passed through heated copper and pressurized to 10 psi (69 kPa) with this gas mixture. Bottles sealed this way were able to maintain pressure for periods of at least

a week. As a control for potential toxicity of the dialysis membrane, growth was assessed in bottles where S(0) suspension was added to bulk medium while the dialysis chamber was loaded with only S-free Pf-7. Growth experiments were inoculated to an initial concentration of 4 μg protein ml^{-1} from early stationary phase cultures and incubated under standard conditions outlined above, except that a magnetic stir plate was used to rotate the spin dialysis chambers at a low rate (< 100 r.p.m.).

Time-lapse microscopy

A culture of *Cba. tepidum* in standard Pf-7 medium was incubated at 47°C for 4 h. A subsample from the culture was then loaded into a borosilicate rectangular capillary tube (VitroCom, Mountain Lakes, NJ), which was then sealed at both ends with epoxy. The filled capillary was then mounted on a glass slide for viewing on a Zeiss AxioImager Z1 light microscope (Zeiss, Oberkochen, Germany). The microscope stage was fitted with a heated glass plate (Tokai Hit, Fujinomiya, Japan) and an objective lens heater (Tokai Hit) was used on a 40x EC Plan NeoFluar lens (Zeiss). The heated stage was set to 47°C, the lens heater to 37°C, and a shield was placed around the microscope to minimize thermal drift. Light needed for standard growth requirements was provided over the course of the experiment by a desktop lamp. Imaging was done under phase contrast at 400× total magnification. The time-lapse experiment was carried out using AxioVision software to capture images every 10 min for 16 h. The photo montage (Fig. 5) and movies for supplementary images were constructed from these files using ImageJ (1.48v, http://imagej.nih.gov/ij).

Characterization of biogenic S(0)

The zeta potential of S(0) particles was measured with a Möbiuζ (Wyatt, Santa Barbara, CA) dynamic light scattering system to measure particle size and electrophoretic mobility. Concentrated biogenic S(0) suspensions were diluted with S-free PF-7 to a concentration of approximately 1 mM. The diluted suspension was loaded into a flow cell and immediately placed into the instrument for analysis. Six zeta potential measurements were averaged for each batch of S(0) analysed.

For bulk elemental analysis and ToF-SIMS, samples of biogenic S(0) were collected by centrifugation at 16 900 × *g* for 2 min. The supernatant was removed and the pellet was washed three times with purified water (> 17.5 MΩ-cm, Barnstead NanoPure system, Thermo Scientific, Waltham, MA). Commercial S(0) powder was incubated in S-free Pf-7 at room temperature for several days then collected and washed as for the biogenic S(0). Samples were then dried in pre-cleaned borosilicate glass vials (Supelco, Bellefonte, PA) at 60–65°C overnight before sealing with a screw cap for transport to analytical facilities. Bulk elemental analysis for C, H, N, O and S was performed at Micro-Analysis Inc. (Wilmington, DE). ToF-SIMS analysis was performed at the University of Delaware Surface Analysis Facility (http://www.udel.edu/chem/beebe/surface.htm).

S(0) globules were tested for associated proteins by extraction and electrophoresis. To extract all associated proteins, S(0) globules prepared as described above were boiled directly with 1X SDS-PAGE sample buffer [1XSB, 31.25 mM Tris–HCl (pH = 6.8), 12.5% v/v glycerol, 1% w/v SDS, 0.015% bromophenol blue, with or without 1% v/v 2-mercaptoethanol as specified], and centrifuged at 16 000 × *g*, 2 min. Cells grown on S(0) were harvested by centrifugation after > 90% of S(0) had been oxidized and treated identically as a control sample. Sequential extractions were performed to extract proteins using conditions that should progressively disrupt hydrophobic and disulfide interactions. S(0) globules prepared as above were collected by centrifugation (0.5 min, 1000 × *g*) and the supernatant saved. The S(0) was washed twice with S-free Pf-7, and the washes were pooled with the original supernatant. This soluble fraction was concentrated with a centrifugal filtration device (Amicon Ultra-0.5 3k NMWL, EMD Millipore, Darmstadt, Germany) and diluted 1:3 with 4XSB. To solubilize weakly associated proteins, the washed S(0) pellet was resuspended in 1XSB with 2-mercaptoethanol omitted and incubated at room temperature for 5 min before centrifugation (16 000 × *g*, 2 min). The supernatant, which constituted weakly associated proteins, was saved. The S(0) pellet was then suspended in 1XSB with 2-mercaptoethanol and again incubated at room temperature for 5 min before centrifugation (16 000 × *g*, 2 min). This supernatant, constituting proteins dissociated by disulfide bond disruption, was saved. Finally, the S(0) pellet was suspended in 1XSB with 2-mercaptoethanol and boiled for 5 min before centrifugation (16 000 × *g*, 2 min).

Solubilized proteins were loaded on either hand-cast 15% acrylamide Laemlli SDS-PAGE gels or precast tris-tricine mini-gels (Bio-Rad Mini-PROTEAN® Precast Gel, 16.5%, Hercules, CA). SDS-PAGE gels were run at 45 mA constant current; tris-tricine gels were run at 100 V constant voltage. After electrophoresis, tris-tricine gels were fixed for 30 min in a solution of 40% methanol, 10% acetic acid on an orbital shaker; SDS-PAGE gels were stained immediately. Gels were stained either with Coomassie (Bio-Safe Coomassie Stain, Bio-Rad, Hercules, CA) or Sypro Ruby (Life Technologies). Gels were imaged by either an AlphaImager® HP (Alpha Innotech, San Leandro, CA) or a FLA-3000 fluorescent imager (Fujifilm Corp., Tokyo, Japan).

Gel plugs were punched out from protein bands of interest, and proteins were identified by mass spectrometry following in-gel trypsin digestion as previously described (Hayduk *et al.*, 2004; Levy *et al.*, 2014). Briefly, gel plugs were subjected to digestion with trypsin (Promega Corporation, Madison, WI). The resulting peptides were desalted and concentrated with C18 ZipTips (EMD Millipore, Bedford, MA), and subsequently spotted onto stainless steel target plates with α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St Louis, MO) as matrix. Analysis was performed by matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometry on an AB Sciex 4800 MALDI-TOF/TOF Analyzer (Framingham, MA). MS and tandem MS spectra were submitted for MASCOT v2.2 (Matrix Science Ltd., London, UK) database searches through GPS EXPLORER software v3.6 (AB Sciex). Spectra were searched against a Uniprot database of the *Cba. tepidum* proteome (downloaded February 26, 2013) with a 50 ppm mass tolerance and oxidation of methionine and carbamidomethylation

of cysteines as variable modifications. Identifications with 95% confidence or greater were accepted.

Protein sequence analysis

Protein sequences were aligned in MEGA6 using the CLUSTALW algorithm with scoring by the BLOSUM62 matrix. The alignment score (Fig. 6C) was determined by calculating the percentage of identical or similar residues in a region of the protein which are identities (exact amino acid matches with CT1320.1) or positives (amino acid mismatches to CT1320.1 that are frequent evolutionary substitutions according to BLOSUM62 scoring). Gaps less than 10 residues in length in the middle of homologous regions are not shown in Fig. 6C, but are accounted for in a penalized alignment score. Signal peptide regions and cleavage sites were predicted with SIGNALP 4.1 (Petersen *et al*., 2011; http://www.cbs.dtu.dk/services/SignalP/) and PHOBIUS (Käll *et al*., 2007; http://phobius.sbc.su.se/). Disordered domains were identified with DISOPRED 3 (http://bioinf.cs.ucl.ac.uk/psipred/).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Representative wide-field 400× phase-contrast image from the beginning of a time-lapse experiment with *Cba. tepidum* growing in standard Pf-7 medium sealed in a capillary on a heated and illuminated microscope stage. Regions of interest that are shown in more detail in the indicated movie files are outlined in a white border.

Movie file 1. Approximately 6 h duration time-lapse video from the region indicated as 'Movie file 1' in Fig. S1. Individual images are at 10 min intervals.

Movie file 2. Approximately 6 h duration time-lapse video from the region indicated as 'Movie file 2' in Fig. S1. Individual images are at 10 min intervals.

Movie file 3. Approximately 6 h duration time-lapse video from the region indicated as 'Movie file 2' in Fig. S1. Individual images are at 10 min intervals.

Table S1. Results of a BLASTP search using CT1320.1 as the query against the UniProt knowledge base (http://www.uniprot.org). Only homologues with an e-value < 1E-10 were considered significant