

Chlorobaculum tepidum Modulates Amino Acid Composition in Response to Energy Availability, as Revealed by a Systematic Exploration of the Energy Landscape of Phototrophic Sulfur Oxidation

Amalie T. Levy,^{a,d} Kelvin H. Lee,^{a,d} Thomas E. Hanson^{b,c,d}

Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, Delaware, USA^a; School of Marine Science and Policy, University of Delaware, Newark, Delaware, USA^b; Department of Biological Sciences, University of Delaware, Newark, Delaware, USA^c; Delaware Biotechnology Institute, University of Delaware, Newark, Delaware, USA^d

ABSTRACT

Microbial sulfur metabolism, particularly the formation and consumption of insoluble elemental sulfur (S^0), is an important biogeochemical engine that has been harnessed for applications ranging from bioleaching and biomining to remediation of waste streams. *Chlorobaculum tepidum*, a low-light-adapted photoautolithotrophic sulfur-oxidizing bacterium, oxidizes multiple sulfur species and displays a preference for more reduced electron donors: sulfide $> S^0 >$ thiosulfate. To understand this preference in the context of light energy availability, an “energy landscape” of phototrophic sulfur oxidation was constructed by varying electron donor identity, light flux, and culture duration. Biomass and cellular parameters of *C. tepidum* cultures grown across this landscape were analyzed. From these data, a correction factor for colorimetric protein assays was developed, enabling more accurate biomass measurements for *C. tepidum*, as well as other organisms. *C. tepidum*’s bulk amino acid composition correlated with energy landscape parameters, including a tendency toward less energetically expensive amino acids under reduced light flux. This correlation, paired with an observation of increased cell size and storage carbon production under electron-rich growth conditions, suggests that *C. tepidum* has evolved to cope with changing energy availability by tuning its proteome for energetic efficiency and storing compounds for leaner times.

IMPORTANCE

How microbes cope with and adapt to varying energy availability is an important factor in understanding microbial ecology and in designing efficient biotechnological processes. We explored the response of a model phototrophic organism, *Chlorobaculum tepidum*, across a factorial experimental design that enabled simultaneous variation and analysis of multiple growth conditions, what we term the “energy landscape.” *C. tepidum* biomass composition shifted toward less energetically expensive amino acids at low light levels. This observation provides experimental evidence for evolved efficiencies in microbial proteomes and emphasizes the role that energy flux may play in the adaptive responses of organisms. From a practical standpoint, our data suggest that bulk biomass amino acid composition could provide a simple proxy to monitor and identify energy stress in microbial systems.

Microbes that synthesize or degrade insoluble sulfur minerals are instrumental in the biogeochemical sulfur cycle (1) and have been applied for biomining (2, 3) and sulfide remediation (4–7). However, little is known about how these organisms respond to fluctuations in available energy due to shifts in electron donor identity, fixed carbon availability, or light in the case of phototrophic bacteria. A deeper understanding of microbial strategies for coping with energy fluctuations has the potential to improve microbe-catalyzed industrial processes (8) and to impact our understanding of microbial ecology as shaped by energy availability (9). Furthermore, a specific understanding of these adaptations among microbes capable of degrading elemental sulfur could inform new technologies for mitigating the effects of sulfur-metabolizing microbes on disturbed ecosystems (e.g., waste sulfur piles from petrochemical refining), particularly if coupled with recent advances in systems and synthetic biology.

Microbes that produce insoluble elemental sulfur (S^0) as an intermediate of reduced sulfur compound oxidation and subsequently oxidize S^0 to sulfate include both chemotrophs (genera *Acidothiobacillus* and *Beggiatoa*, order *Sulfolobales*) and phototrophs (families *Chromatiaceae* and *Chlorobiaceae*). S^0 may be deposited either extra- or intracellularly (10, 11). The *Chlorobiaceae* are obligate anaerobes that use sulfide and other reduced

sulfur compounds as electron donors for anoxygenic photosynthesis and transiently deposit S^0 as extracellular globules (12–15). Unlike the more metabolically versatile species of the family *Chromatiaceae*, which are capable of dark, aerobic chemotrophic growth in addition to phototrophic sulfur oxidation (16), all of the characterized members of the family *Chlorobiaceae* are obligate anaerobic photolithoautotrophs, although some *Chlorobiaceae* assimilate simple organic carbon compounds (acetate, pyruvate) (17). *Chlorobaculum tepidum* produces and degrades

Received 15 July 2016 Accepted 17 August 2016

Accepted manuscript posted online 26 August 2016

Citation Levy AT, Lee KH, Hanson TE. 2016. *Chlorobaculum tepidum* modulates amino acid composition in response to energy availability, as revealed by a systematic exploration of the energy landscape of phototrophic sulfur oxidation. Appl Environ Microbiol 82:6431–6439. doi:10.1128/AEM.02111-16.

Editor: R. E. Parales, University of California—Davis

Address correspondence to Thomas E. Hanson, tehanon@udel.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.02111-16>.

Copyright © 2016, American Society for Microbiology. All Rights Reserved.

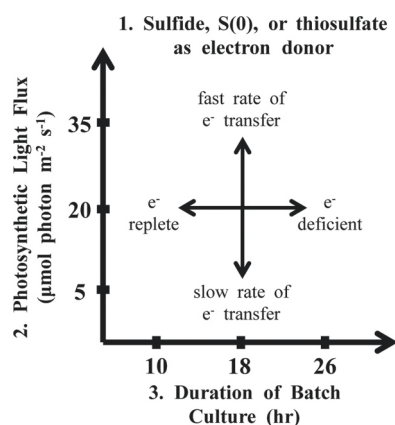


FIG 1 Schematic of the energy landscape of phototrophic sulfur oxidation. The energy landscape of phototrophic sulfur oxidation is constructed from three factors at three levels, i.e., (i) electron donor identity (sulfide, S^0 , or thiosulfate), (ii) light flux (5, 20, or 35 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$), and (iii) duration of batch culture (10, 18, or 26 h).

insoluble extracellular S^0 as an obligate intermediate of sulfide oxidation, grows rapidly (15, 18), is genetically tractable (19–23), and has a sequenced genome (24), making it an ideal platform for studying S^0 metabolism by systems-based methods.

Systems-based methods require reproducible growth under controlled conditions and robust methods of biomass quantitation, both of which are challenging for *C. tepidum*. For example, consistent growth on S^0 as the sole electron donor has only recently been reported (14). Extracellular S^0 complicates growth measurements by standard methods (i.e., optical density and dry weight) because S^0 adds turbidity (18, 25) and mass. Therefore, colorimetric protein assays are the methods of choice for biomass determinations of *C. tepidum* (12, 18, 25). However, differences between the amino acid compositions of biomass and protein standards and interference from a range of compounds (26), including photosynthetic pigments, mean that colorimetric protein assays often do not reflect the absolute protein concentration. While pigment extraction prior to protein measurement is common (12, 18, 25), the effects of extraction and of varying pigment content, induced by changes in light level (27, 28) and electron donor (12), have not been investigated systematically.

Design-of-experiments methodology (29, 30) was used to assess protein-based biomass quantitation methods and the response of *C. tepidum* across a factorial space defined by the electron donor, light flux level, and culture duration. This space—the “energy landscape” of phototrophic sulfur oxidation (Fig. 1)—was designed to alter pigment content by altering energy availability. *C. tepidum* displayed shifts in biomass amino acid composition, cell volume, and storage carbohydrate content across the energy landscape, suggesting that *C. tepidum* alters its physiology in response to energy availability to a greater extent than previously appreciated. While bias in the amino acid composition of highly expressed proteins toward energetically inexpensive amino acids has been inferred by bioinformatic analyses for a range of organisms (31–33), this work reports an experimentally measured shift in bulk amino acid composition for a single organism as a function of growth conditions. This observation implies that relatively simple bulk biomass measurements can be used to infer details about the energy status and adaptation of microbes.

MATERIALS AND METHODS

Experimental design. A three-by-three factorial experiment was employed to examine *C. tepidum* growth and characteristics as a function of the electron donor type, light flux level, and culture duration at three levels per factor. The I-optimal design was created by using JMP Pro (SAS Institute Inc.) and contained 48 independent cultures performed in six culturing blocks (see Table S1 in the supplemental material). This design enabled quantitative analysis of the effects of simultaneous changes in the energy landscape parameters on various measured responses (cell size attributes, total carbohydrate [TC], amino acid composition) by a second-order interaction polynomial model, $Y = \beta_0 + \sum \beta_i x_i + \sum_{i < j} \beta_{ij} x_i x_j + \sum \beta_{ii} x_i^2 + \epsilon$, where Y is the measured response, x_i are experimental factors, $\beta_{0,i,j,ii}$ are coefficients determined from multiple regression, and ϵ represents random error. β_0 is the mean value of the response; main-effect terms (β_i) represent a factor’s direct effect, cross-interaction terms (β_{ij}) represent the synergistic impact of two factors, and polynomial terms (β_{ii}) represent nonlinear effects. Culturing block was investigated as a factor, except for analyses with amino acid analysis (AAA)-derived data as the dependent variable(s), which were conducted on a subset of the full design (see Table S1 in the supplemental material).

Bacterial strains and growth conditions. *C. tepidum* strain WT2321, a plating strain derivative of the original TLS1 isolate (15, 20), was grown in 20-ml cultures with a 10-lb in $^{-2}$ gage (177-kPa) headspace composed of 95% N_2 and 5% CO_2 passed through a heated copper scrubber. Experimental cultures were inoculated to 4 $\mu\text{g protein ml}^{-1}$ from precultures derived from cryogenic stocks and grown at 45 to 46°C in a heated rotisserie culturing system. This system provided improved culture-to-culture consistency in light exposure and mixing relative to stirred water bath cultures (see Text A in the supplemental material for details). A light field of 35 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ was provided from 100 W Reveal incandescent bulbs (GE Lighting) measured with a quantum PAR sensor (LI-COR). To obtain 5 and 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ light, individual cultures were shaded by using printed transparency films. Culture durations (Fig. 1) were selected to capture mid-exponential-, late-exponential-, and early-stationary-phase cells at a light flux of 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$.

Culture medium. Sulfur-free Pf-7 medium (15) was prepared by omitting sulfide and thiosulfate from Pf-7 prepared as previously described (12). Electron donors were added to individual tubes from concentrated, anoxic stock solutions. Sulfide stocks were pH neutralized (34), and biogenic S^0 was purified as previously described (14). The electron donor concentrations in uninoculated medium were sulfide at 3.4 ± 0.2 mM, S^0 at 9.3 ± 0.3 mM, and thiosulfate at 10.2 ± 0.1 mM. Acetate was added to all cultures at an initial concentration of 7.4 ± 0.3 mM.

Quantification of sulfur compounds and acetate. Measurements were performed as described previously (12, 35), with the following modifications. Elemental sulfur was extracted from cell pellets with 10:1 (vol/vol) chloroform-methanol prior to quantitation by reversed-phase high-performance liquid chromatography. Sulfate was quantified by ion chromatography with suppressed conductivity detection (Metrohm) with an A Supp 5 100- by 4-mm column eluted with 3.2 mM Na_2CO_3 –1.0 mM $NaHCO_3$ –6.5% (vol/vol) acetone in ultrapure water. Standard curves were prepared from sodium sulfide nonahydrate (ACS; Fisher), elemental sulfur powder (USP; Fisher), sodium thiosulfate pentahydrate (>99.5%; Sigma-Aldrich), sodium sulfate (>99%; Sigma-Aldrich), and sodium acetate (>99%; EM Science).

Protein and BChl c determinations. *C. tepidum* cells collected by centrifugation ($16,873 \times g$, 5 min) were lysed with 0.25 M NaOH (10 min), neutralized with an equal volume of 0.25 M HCl, centrifuged gently to pellet S^0 ($14 \times g$, 1 min), and diluted with 0.25 M NaCl prior to protein quantitation. Bradford assays were performed with Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories) in accordance with the manufacturer’s instructions by using either the absorbance at 595 nm or the ratio of absorbances at 595 and 470 nm as the measured response (36, 37). Bicinchoninic acid (BCA) assays were performed with the Pierce BCA kit (Thermo Scientific) and incubation at 37°C; the ratio of sample to

working reagent was increased to 1:2 (vol/vol). The observed technical variation for all protein assays is reported in Table S2 in the supplemental material. Bovine serum albumin (>98%; EM Science OmniPur) was used as a protein standard. Pigments were removed from extracted (EX) samples by methanol extraction (-20°C , 10 min) prior to lysis, whereas whole (WH) samples with pigment intact were analyzed without prior extraction. Bacteriochlorophyll *c* (BChl *c*) in the extracts was determined by absorbance at 669 nm ($\epsilon = 86.0 \text{ liters g}^{-1} \text{ cm}^{-1}$ [38]).

AAA. Lyophilized cell samples were hydrolyzed (1% [vol/vol] phenol in 6 N HCl, 110°C , 24 h), and amino acids were separated by ion-exchange chromatography (Hitachi L-8800) with sodium citrate buffer as the mobile phase. The hydrolysis destroys Cys, Met, and Trp and converts the amide amino acids Asn and Gln to Asp and Glu, respectively. Biomass amino acid molar composition was calculated on the basis of the molar quantities of the individual amino acids measured in a sample.

Microscopy and cell volume measurements. Culture aliquots were fixed with 0.37% formaldehyde and stored in the dark (4°C). At least three phase-contrast images at a total magnification of $\times 1,000$ were collected for each culture with an AxioImager Z1 light microscope and a $100\times$ oil immersion objective lens (Zeiss). Images were thresholded and masked by using ImageJ (version 1.49t, <http://imagej.nih.gov/ij>) default settings and a 15.5-pixel μm^{-1} scale. The particle analyzer function in Fiji (<http://fiji.sc/Fiji>) was used to extract area and perimeter measurements of cell-like objects from the images by using predetermined area and circularity criteria. Extracted objects were validated as cells by cross-referencing with the original images. Dividing, clumped, or out-of-focus cells and other debris were manually excluded so that only well-resolved cells were retained in the final data set. Cell images were modeled as the two-dimensional projection of a cylinder with hemispherical end caps to calculate cell diameter, length, and volume from area and perimeter measurements. Diameter, length, and volume measurements displayed lognormal distributions across the data set and within cultures and were log transformed and averaged by culture prior to analysis by least-squares linear models.

Cellular carbohydrate analysis. Cellular carbohydrates were measured by the anthrone-sulfuric acid assay (39) adapted to a 96-well format (40, 41). Cell pellets were extracted with acetone (10 min, -20°C) to remove interfering pigments (42). Extracellular carbohydrates were removed by resuspension in a solution of 0.85% NaCl, centrifugation ($16,873 \times g$, 5 min), and removal of the supernatant (39). Pellets were resuspended in water, and sample aliquots (45 μl per well) were transferred to a polypropylene 96-well plate (VWR). Samples were mixed with 150 μl anthrone reagent (2 g liter $^{-1}$ anthrone [Acros Organics] in 98% sulfuric acid [EM Science]). For each sample, one aliquot was mixed with sulfuric acid without anthrone as a negative control and one aliquot was spiked with 5 μg glucose as a positive control. The covered plate was incubated at 4°C for 10 min at 95 to 105°C for 20 min and cooled at room temperature for 20 min. The absorbance at 620 nm was measured along with a glucose standard curve (0.5 to 10 μg glucose per well).

Statistical analysis. JMP Pro was used for all statistical analyses and preparation of box-and-whisker plots. In box-and-whisker plots used throughout, the middle line indicates the median value, boxes span the 25th to 75th quantiles, and the whiskers represent either $1.5\times$ the interquartile range from the end of the box or the upper and lower data points (excluding outliers).

The effect of the energy landscape on univariate responses was analyzed by least-squares fitting of the data to the second-order interaction polynomial model described above in the paragraph on experimental design. Log-transformed and averaged cell dimensions were fitted by weighted least-squares analysis, using the inverse of the variance of the cell dimension measurement as the weighting variable, to account for the large range of cell sizes observed within each population. Analysis of variance (ANOVA) was used to assess overall model significance by *F* test, which can assess multiple coefficients simultaneously, and to estimate parameter coefficient values, where the significance of individual parameters was determined by *t* test. Insignificant parameters, defined as $P >$

0.2, were sequentially eliminated to improve model significance and parameter estimates, and only parameters with *t*-test probabilities (*P* values) of <0.01 were considered to have a significant effect on the measured response. Light and culture duration factors were coded between -1 and 1 before model fitting. In some cases, using culture attributes (e.g., growth rate, etc.) as factors provided more meaningful relationships than the original energy landscape parameters, and the factors identified as producing significant effects are specified in the relevant sections below.

Amino acid composition data were fitted by multivariate ANOVA (MANOVA), which enabled analysis of the effects of the energy landscape on amino acid composition as a whole, as well as on individual amino acids. The statistical significance of effects in MANOVA is determined by *F* test, to enable the assessment of multiple coefficients simultaneously; approximated *F* ratios for categorical factors in MANOVA were calculated by Wilk's lambda.

RESULTS

Calibration of indirect protein assays provides accurate prediction of absolute biomass protein concentration. As intended in the experimental design, cultivation of *C. tepidum* across all 27 energy landscape treatments (see Table S1 in the supplemental material) produced biomass with BChl *c* contents spanning 0.10 to 0.24 μg of BChl *c* μg of protein $^{-1}$. Photosynthetic pigment interference in indirect protein assays, specifically, the Bradford and bicinchoninic acid (BCA) methods, was assessed by comparing protein measurements of WH and methanol-extracted (EX) samples. Two versions of the Bradford assay were used: standard (BrS), which measured A_{595} , and ratio (BrR), which measured the A_{595}/A_{470} ratio to provide better sensitivity and linearity than the standard assay (36, 37). The performance of the indirect assays was benchmarked against direct quantitation of protein by AAA measurements on WH samples, which displayed $<5\%$ error with bovine serum albumin as a standard (data not shown).

Indirect protein assays were highly correlated with AAA measurements (Table 1; Fig. 2), with the highest accuracy exhibited by BCA assay of EX samples (normalized root mean square error [nRMSE] = 16%; Table 1; see Text B in the supplemental material for calculations of accuracy metrics). The BCA assay overpredicted protein in WH samples (nRMSE = 37%), particularly for early- to mid-exponential-phase cultures (Fig. 2A, inset). Both versions of the Bradford assay underpredicted protein (nRMSE = $>25\%$), regardless of whether pigments were removed, with BrR-WH measurements the least accurate (nRMSE = 38%).

The accuracy of all assays except BCA-WH was improved by a linear correction function as follows: Corrected = (Indirect $-\beta_0$) $\times \beta_1^{-1}$. Parameters β_0 and β_1 were obtained from the intercept and slope, respectively, of least-squares linear regressions of indirect protein measurements against AAA measurements (Table 1; see Text B in the supplemental material for details). Although BCA-WH exhibited the smallest absolute RMSE when corrected by a linear correlation function, it had the highest nRMSE due to proportionally large deviations for low-density cultures (Table 1; Fig. 2A, inset). Corrected Bradford assay measurements of EX samples demonstrated the smallest nRMSE (13% for BrS and 12% for BrR), a slight improvement in prediction accuracy over the uncorrected BCA-EX measurements.

Thus, for routine use with highly pigmented organisms like *C. tepidum*, BCA-EX measurements should provide the best results without the need for a correction factor. For the subsequent experiments, however, we employed corrected BrR-EX measurements based on the improvement in overall accuracy.

TABLE 1 Accuracy of Bradford and BCA (indirect) protein assay measurements in predicting direct protein quantitation by AAA

Assay correction, pigment status, and assay method	R ²	β ₁	β ₀	RMSE (μg/ml)	nRMSE (%)
Without correction					
WH cell pellet					
BCA	0.989			26	37
BrS	0.966			38	25
BrR	0.969			56	38
EX cell pellet					
BCA	0.983			12	16
BrS	0.971			40	29
BrR	0.983			43	33
After correction ^a					
WH cell pellet					
BCA		1.07	15.0	10	24
BrS		0.734	2.61	18	15
BrR		0.604	1.52	17	15
EX cell pellet					
BCA		0.981	3.30	12	18
BrS		0.734	−0.57	16	13
BrR		0.714	−2.11	12	12

^a Corrected = (indirect − β₀) × β₁^{−1}.

Comparisons of WH and EX protein measurements suggest characteristics of the internal metabolite pools of *C. tepidum*. While methanol extraction improved the accuracy (i.e., lowered the nRMSE) of the Bradford and BCA assays, this step could remove protein and/or soluble pools from biomass samples. Indeed, methanol extraction reduced the measured protein mass by 9%, on average (see Fig. S1A in the supplemental material; $P = 0.001$, one-tailed test), as measured by AAA of 16 pairs of WH and EX samples (see Table S1 in the supplemental material). Furthermore, the amino acid composition was altered between WH and EX samples, indicating that extraction preferentially removed Ala, Pro, and Glx (see Fig. S1B in the supplemental material; see Text C in the supplemental material for details). This was interpreted as loss of the soluble pools of these amino acids. However, there was no obvious relationship between the extent of depletion of Ala, Pro, or Glx and the energy landscape (data not shown).

Comparing BCA-WH and BCA-EX measurements revealed an interesting bias in BCA-WH error. While there was no relationship between BCA-EX error and energy landscape parameters, the tendency of BCA-WH to overpredict protein was greatest among samples from sulfide- and S^0 -grown cultures, at early times or low light levels, but not with thiosulfate-grown cultures (see Fig. S2 in the supplemental material). These observations suggest that *C. tepidum* possesses one or more internal pools of methanol-soluble metabolic reductants that artificially increase the signal of the BCA-WH assay, which is based on the reduction of Cu(II) to Cu(I).

C. tepidum cell size variations across the energy landscape are associated with the production of storage carbohydrates. As cell size provides an indicator of the physiological state of microbes (43), we assessed changes in *C. tepidum* cell dimensions across the energy landscape and found that the growth rate positively correlated with cell length ($R^2 = 0.38$; $P < 0.0001$) and cell volume ($R^2 = 0.34$; $P < 0.0001$) but did not correlate with cell diameter (see Fig. S3 in the supplemental material). Increases in cell volume with higher growth rates is a well-established phe-

nomenon (44), but the large variability in cell volume at a particular growth rate suggested that additional factors were influencing cell size (43). Adding parameters for electron donor identity and acetate availability revealed that these factors affected cell size beyond the effect of growth rate (Fig. 3A). Growth on sulfide led to larger (21%, $P < 0.0001$) and longer (17%, $P < 0.0001$) cells than growth on S^0 and thiosulfate but had a negligible effect on cell diameter (see Fig. S4 in the supplemental material). Acetate depletion decreased the cell volume by 29% through the combined effects of decreased length (17%; $P < 0.001$) and decreased diameter (8%; $P < 0.0001$).

As the accumulation and depletion of storage carbohydrates are known to influence cell volume (39, 40), TC was measured in biomass samples from across the energy landscape. Growth on sulfide increased the TC-to-protein ratio of *C. tepidum* by 72%, on average ($P < 0.0001$). The corresponding increase in the cell volume and TC-to-protein ratio of sulfide-grown cultures (Fig. 3B) provides evidence that growth on sulfide leads to enhanced glyco-

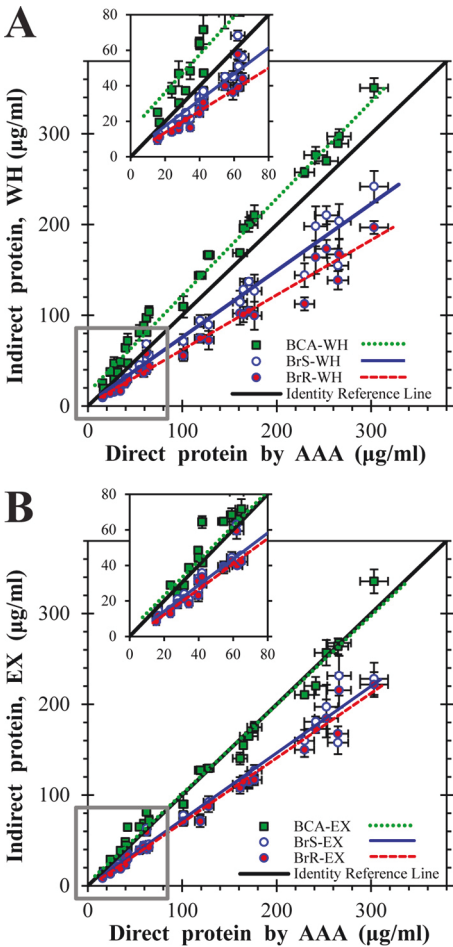


FIG 2 Calibration of indirect protein measurements against AAA. Values from BCA and Bradford assays are plotted versus direct protein quantitation by AAA for WH samples (A) and EX samples (B) along with the linear least-squares regression. Insets show low-concentration regions. The solid black identity reference line indicates equality between indirect and AAA protein determinations. The vertical error bars for indirect assay measurements represent the standard errors of triplicate determinations; horizontal error bars on AAA measurements represent propagation of the pooled standard error for four replicated AAA analyses.

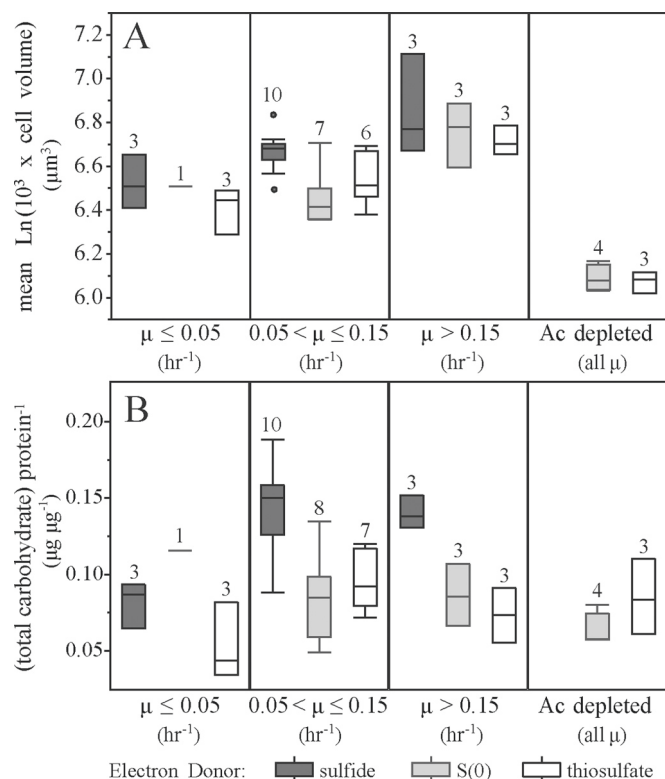


FIG 3 Effects of the electron donor and acetate depletion on cell volume and storage carbohydrate accumulation across cultures at different growth rates. Box-and-whisker plots of culture-averaged log-transformed cell volumes (A) and total culture carbohydrate (B) normalized to culture protein binned by growth rate and classified by electron donor identity and acetate (Ac) availability. The number of cultures in each grouping is indicated above the box. Acetate was fully depleted by the 26-h time point for S^0 - and thiosulfate-grown cultures at the medium ($20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and high ($35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) light levels.

gen production relative to *C. tepidum* growth on S^0 or thiosulfate. Interestingly, the TC-to-protein ratio was not correlated with acetate depletion; thus, decreased cell volume in acetate-depleted cultures is not a result of storage carbohydrate degradation.

***C. tepidum* amino acid composition varies across the energy landscape.** The bulk amino acid composition of *C. tepidum* biomass, as determined from the AAA measurements (see Fig. S5 in the supplemental material), exhibited statistically significant changes across the energy landscape. MANOVA produced a highly significant ($P \leq 0.0001$) second-order interaction model (see Materials and Methods), revealing that the electron donor ($P = 0.0006$), light level ($P < 0.0001$), and culture duration ($P = 0.002$) affected the abundance of specific amino acids (where the interaction of the light level and culture duration had a weak effect, $P = 0.04$). The electron donor had the most significant effect on Gly ($P < 0.0001$; see Fig. S6A in the supplemental material), which was enriched on sulfide and depleted on thiosulfate. Increasing light decreased the Asx content ($P = 0.0003$; see Fig. S6D in the supplemental material) and enriched for Lys ($P = 0.0015$; see Fig. S6F in the supplemental material). Increased culture duration enriched for Pro ($P = 0.0008$; see Fig. S6C in the supplemental material) and decreased Arg ($P = 0.002$; see Fig. S6B in the supplemental material) and Gly ($P = 0.007$; see Fig. S6A in the supplemental material).

Light flux affects biomass amino acid composition according to amino acid biosynthetic cost. A closer inspection revealed that light induced subtler changes in nearly all amino acids. High light flux enriched for amino acids with high biosynthetic ATP requirements, while low light flux enriched for amino acids with low biosynthetic ATP costs (Fig. 4A; values for photosynthetic bacteria from reference 45). When the cost of amino acid synthesis for *C. tepidum* biomass ($\sim P_{\text{avg}}$) was calculated by using the amino acid composition data and the energetic costs of synthesizing each amino acid in terms of the ATP required per amino acid (45), $\sim P_{\text{avg}}$ was positively correlated with light flux (Fig. 4B; $P = 0.002$ for light as a single factor, $P = 0.0008$ when including culture duration as a nonlinear effect). The mean change in $\sim P_{\text{avg}}$ between the low- and high-light conditions was 0.14 ± 0.04 ATP per amino acid. Different amino acids did not contribute equally to the change in $\sim P_{\text{avg}}$ —for example, Lys was the largest contributor, while Pro and Thr slightly opposed the overall trend (see Table S3 in the supplemental material). However, excluding individual amino acids from the $\sim P_{\text{avg}}$ calculation did not eliminate the re-

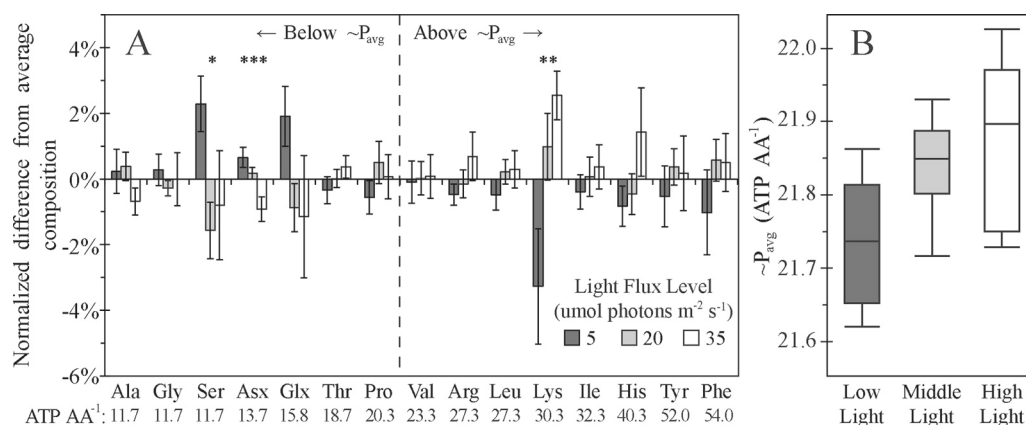


FIG 4 Light level affects variation in amino acid abundance according to biosynthetic cost. (A) Mean values of the normalized difference from the average composition are plotted as a function of light. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Amino acids are arranged in order of increasing biosynthetic cost (ATP required per amino acid) using values for phototrophic bacteria (45). The average amino acid biosynthetic cost for *C. tepidum* biomass (21.8 ATP molecules per amino acid) is indicated by the vertical dashed line. Error bars represent the standard error of the mean within each light level. (B) Box-and-whisker plot representation of the distribution of the measured $\sim P_{\text{avg}}$ values for low, medium, and high light levels.

relationship between $\sim P_{\text{avg}}$ and light flux (see Table S4 in the supplemental material), consistent with a globally coordinated alteration of biomass composition.

As *C. tepidum* is known to increase its BChl *c* content and chlorosome volume fraction in response to decreased light flux (27, 28), we considered the possibility that the observed bias in amino acid composition at low light levels could be the result of increased abundance of chlorosome-associated proteins. The BChl *c*-to-protein ratio increased with decreasing light for sulfide- and S^0 -grown cultures, as expected (see Fig. S7A in the supplemental material), although this was not the case for thiosulfate-grown cultures. We found that the BChl *c*-to-protein ratio strongly correlated with the overall amino acid composition ($P = 0.0003$ by MANOVA), and cultures with similar BChl *c*-to-protein ratios clustered together in a principal-component analysis plot (see Fig. S6B in the supplemental material). However, the BChl *c*-to-protein ratio did not correlate with $\sim P_{\text{avg}}$ ($P = 0.8$; see Fig. S6C in the supplemental material), suggesting that the bias in composition toward amino acids with lower biosynthetic costs is not merely the result of increased chlorosome and chlorosome protein abundance.

There was no correlation between $\sim P_{\text{avg}}$ and growth rate ($P = 0.8$; data not shown). All cultures achieved exponential growth, with maximal rates observed from 0 to 10 h. Growth rates of low-light cultures were lower with all electron donors ($\mu = 0.09$ to 0.11 h^{-1}) than those of medium-light (0.19 to 0.22 h^{-1}) and high-light ($\mu = 0.23$ to 0.25 h^{-1}) cultures. Growth rates of cultures grown at medium (by 37 to 62%) and high (by 65 to 76%) light levels were lower after 10 h, but cultures continued to grow through 26 h.

DISCUSSION

Prior extraction of biomass samples improves the accuracy of indirect protein assays. Bradford and BCA protein assays are simple, rapid, and inexpensive yet exhibit protein-to-protein variability, measure protein indirectly relative to a chosen protein standard, and are subject to interference from a range of compounds, including photosynthetic pigments (26). As *C. tepidum* pigment content changes in response to the electron donor (12), light intensity (27, 28), and culture duration, we used cultures grown across the energy landscape to examine this interference by using AAA as the “gold standard” for protein quantitation (46).

This work established that BCA-EX measurements provided good overall accuracy in predicting *C. tepidum* protein without correction. This method should provide a convenient and more generally applicable method of protein determination for photosynthetic biomass samples. Slightly improved accuracy was obtained from BrR-EX and BrS-EX measurements with an empirical correction function (Table 1; Fig. 2). However, we expect that the correction factor will vary between organisms based on different pigment contents and this will need to be determined for each organism independently. This study provides a road map for how to carry out such a correction, if desired.

Extractable pools inferred from protein analyses. Pigments are typically removed by methanol extraction, and we found that this introduced changes in biomass amino acid composition by preferential extraction of alanine, proline, and glutamate/glutamine. This result was interpreted as the loss of free intracellular amino acid pools in *C. tepidum*, as these amino acids are observed as free pools in other microorganisms (47, 48). Prior studies of *C.*

tepidum indicate that glutamine synthase/glutamine aminotransferase (GS/GOGAT) is the main route of ammonium assimilation in *C. tepidum* (49), which would be expected to contribute to free pools of glutamate and glutamine. Alanine dehydrogenase (ADH) has been suggested as an alternative ammonium assimilation pathway in “*Chlorobium chlorochromatii*” (50), which is consistent with our observation of an extractable alanine pool. This is further supported by *C. tepidum* transcriptome sequencing data (51), which showed that genes for GS/GOGAT (CT1411, CT0401, CT0402) and ADH (CT0650, CT0706) were expressed at similar levels. The significance of free proline in *C. tepidum* is not clear, but free-proline pools have been associated with osmotic stress (44, 47, 52), where converting glutamate to proline enables an increase in the intracellular solute concentration without requiring additional positively charged solutes to balance the charge (52).

The overestimation of protein by BCA-WH, but not BCA-EX, measurements for a subset of the energy landscape treatments provides evidence for the transient accumulation of an intracellular, methanol-extractable compound that increases the reduction of Cu(II) to Cu(I) in the BCA assay. The increase in the BCA-WH protein signal was predominantly observed in cells grown on sulfide or S^0 , particularly at short culture durations or low light levels (see Fig. S3 in the supplemental material), and was not present in cultures grown with thiosulfate as the electron donor or where the electron donor was largely exhausted. The increase in BCA-WH could be due to intracellular pools of sulfide or polysulfide, which have been detected in *C. tepidum* (unpublished data). Intracellular pools of polysulfides but not sulfide have also been observed in the purple sulfur bacterium *Allochrochromatium vinosum* (53). While intracellular pools of thiosulfate and sulfite have been previously quantified in *C. tepidum* (54, 55), these compounds are not soluble in methanol and would be unlikely to be extracted.

***C. tepidum* cell size changes suggest storage compound formation.** A positive relationship between the growth rate and cell size of heterotrophic bacteria such as *Escherichia coli* and *Salmonella enterica* serovar Typhimurium is textbook material (44), but whether this property translates to less-well-studied systems like *C. tepidum* is not clear. Growth rate and cell size correlate across different species of cyanobacteria and heterotrophic bacteria, but data on changes within a given species are lacking (56). Cell size was observed to correlate with the growth rate of *Chromatium minus*, a purple sulfur phototrophic bacterium, where large variations in cell volume among cells of a constant growth rate were attributed to intracellular storage of sulfur and glycogen (57). The *Chlorobiaceae* synthesize glycogen in the light as a carbon storage compound and ferment the stored glycogen in the dark to provide maintenance energy (17, 58–61).

After accounting for growth rate differences, *C. tepidum* cells grown on sulfide were larger than on S^0 or thiosulfate, a difference that appears to be due to the accumulation of glycogen in sulfide-grown cells (Fig. 3B). While degradation of glycogen was previously described upon the transition from sulfide oxidation to S^0 oxidation for members of the family *Chromatiaceae* (62, 63), this work directly compared glycogen production levels during the oxidation of different electron donors in the family *Chlorobiaceae*. Enhanced glycogen storage on sulfide suggests that *C. tepidum* may use glycogen synthesis in part to cope with and/or store excess reducing power available during sulfide oxidation. This is consistent with the induction of a high-velocity, low-affinity sulfide:

quinone oxidoreductase (CT1087) whose physiological role seems to be primarily sulfide tolerance (51, 64, 65).

The parallel effect of culture parameters on cell length and volume, but not on cell diameter, suggests that *C. tepidum* modulates cell size by altering length rather than diameter. Because *C. tepidum* chlorosomes are arranged at the perimeter of cells, the chlorosome concentration per unit of cell volume will be maximized by a smaller diameter. Thus, maintaining the cell diameter may be advantageous for maximizing the light-harvesting potential of the cells.

Amino acid composition changes induced by energy landscape parameters suggest that biosynthetic streamlining at low energy can be observed with bulk measurements. At the outset, we did not expect to observe significant changes in amino acid composition across the energy landscape because we felt that protein expression changes would be too subtle to be detected in bulk biomass measurements. However, the data clearly indicate that *C. tepidum* biomass composition detectably shifts across the energy landscape. Furthermore, these shifts appear to be sensible in the context of the energy landscape when interpreted on the basis of the energetics of amino acid biosynthesis. The $\sim P_{\text{avg}}$ decreased in response to decreased light flux, amounting to a difference of 0.14 ± 0.04 ATP per amino acid from the high-light to the low-light conditions (Fig. 4B), which translates to a savings in amino acid synthesis costs of 0.64% from 21.87 ATP molecules per amino acid at high light levels. As noted above, tryptophan, the most energetically expensive amino acid (76.3 ATP molecules per amino acid [45]), is not measurable by the AAA method employed. Trp shares chorismate as a biosynthetic intermediate with Phe and Tyr, which both contribute positively to the $\sim P_{\text{avg}}$ change in response to light. Thus, we expect that Trp would also positively contribute to an observed change in $\sim P_{\text{avg}}$.

This bias of *C. tepidum* toward using less energetically expensive amino acids at reduced light flux could potentially be an evolved response for coping with ATP limitation, as members of the family *Chlorobiaceae* are adapted to lower light flux than other phototrophs (16, 66). Interestingly, the first- and third-lowest-cost proteins in the *C. tepidum* proteome on a per amino acid basis (calculated by using the approach of Smith and Chapman [45]) are associated with light harvesting (chlorosome proteins CsmD and CsmF), and the fourth-lowest-cost protein is associated with ATP generation (ATP synthase subunit c).

Efficiency in the primary sequence of proteins has been observed previously; highly expressed bacterial genes (as assessed by major codon usage) tend to code for proteins that use less energetically expensive amino acids (31–33), and extracellular proteins tend to be composed of less energetically expensive amino acids than intracellular and membrane proteins (45). Furthermore, a survey of yeast transcriptomic data found that resource limitations (e.g., salt stress, reduced exogenous amino acid availability, etc.) induced gene expression changes that varied inversely with the length of the coded proteins (67). However, whereas these previous studies were based on inferences from bioinformatic analyses and omics data sets, this work reports an experimentally measured shift in an organism's bulk amino acid composition in response to growth conditions. That the bias toward amino acids with lower biosynthetic cost appears to be independent of BChl *c* content (see Fig. S7C in the supplemental material) suggests that this effect is the result of global changes in *C. tepidum* protein expression, and possibly evolved efficiency in the primary

sequence of proteins important at low light levels, rather than a mere consequence of increased chlorosome expression.

While a 0.64% cost savings in the energy requirements of amino acid synthesis at first glance appears quite small, long-term evolutionary success can be built on such incremental advances. Estimating that that amino acid biosynthesis accounts for 57% of the cellular energy budget of a reverse tricarboxylic acid cycle autotroph (68), this change in $\sim P_{\text{avg}}$ would translate to a 0.37% increase in the growth rate, enabling a 10^{-6} initial proportion of the population to achieve >99% of the population within 7,300 doublings. This is roughly 20 years, assuming a doubling time of 8 h, which is consistent with the measured growth rate of *C. tepidum* under the low-light conditions of this study. While this time frame is long compared to typical laboratory growth experiments, 20 years is short compared to evolutionary time scales; for example, there is evidence that populations of green sulfur bacteria have consistently occurred in the Mediterranean Sea for >200,000 years (69) and potentially >2,000,000 years (70) and that purple sulfur bacteria have inhabited Mahoney Lake for >9,000 years (71). Furthermore, the energy savings and selection coefficient for this advantage (2.5×10^{-3} generation⁻¹, calculated in accordance with reference 72) are similar to the advantage for switching from low-frequency to high-frequency codons in highly expressed proteins (31, 73).

The fact that the biases in the cost of amino acid synthesis were detectable by bulk analysis of biomass rather than by fine-grained omics-type analyses emphasizes the role that energy flux may play in the evolved adaptations of organisms and provides experimental evidence for evolved efficiencies in microbial proteomes. Thus, future systems-based studies that explore the response of *C. tepidum* and other environmental microbes to changing energy flux have the potential to provide deeper insight into the environmental pressures that have shaped proteins critical for life in low energy flux environments.

Concluding remarks. The factorial approach employed in this work, coupled with efforts to reduce culturing variability and establish robust biomass quantitation, enabled a systematic analysis of the effects of electron donor, light flux level, and culture duration, as well as their interacting effects, on the physiology of *C. tepidum*. Through the range of treatments produced by this approach, protein-based biomass quantitation methods were calibrated and previously unidentified traits of *C. tepidum* were revealed. These include changes in glycogen storage as a function of electron donor and overall biomass amino acid composition in response to growth conditions. Our observation of a bias in the amino acid composition toward less energetically expensive amino acids under low-light conditions provides a “real-time” experimental observation of whole-biomass amino acid compositional shifts in response to energy limitation. Thus, measurements of biomass amino acid composition have the potential to provide a simple, useful means to assess and diagnose energy stress or adaptations in applications such as bioreactor process monitoring. The experimental design approach employed here enabled simultaneous examination of multiple factors that revealed subtle and interacting-factor effects that would have remained hidden in studies with single-factor designs. The generalized approach of carefully designed “landscape”-type studies can be extended to many other systems to enable the identification of previously undetected interactions in problems impacted by multiple interacting factors.

ACKNOWLEDGMENTS

We thank Cassandra Marnocha and Clara Chan for assistance with microscopy techniques and use of equipment. We are grateful to Jacob Hilzinger for sharing information on *C. tepidum*'s internal metabolite pools and to Kathleen Scott (University of South Florida) for help estimating amino acid synthesis cost in the context of the cellular energy budget. AAA was performed by the Molecular Structure Facility at the University of California at Davis.

FUNDING INFORMATION

This work, including the efforts of Thomas E. Hanson, was funded by National Science Foundation (NSF) (MCB-1244373). This work, including the efforts of Amalie Tuerk Levy, was funded by National Science Foundation (NSF) (GRFP 0750966). This work used infrastructure resources funded by National Science Foundation (NSF) (EPS-0814251). This work used infrastructure resources funded by HHS | NIH | National Institute of General Medical Sciences (NIGMS) (P20 GM103446). This work, including the efforts of Thomas E. Hanson, was funded by University of Delaware Research Foundation (UDRF).

REFERENCES

- Lavik G, Stührmann T, Brüchert V, Van der Plas A, Mohrholz V, Lam P, Mussmann M, Fuchs BM, Amann R, Lass U, Kuypers MMM. 2009. Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. *Nature* 457:581–584. <http://dx.doi.org/10.1038/nature07588>.
- Johnson DB. 2014. Biomining—biotechnologies for extracting and recovering metals from ores and waste materials. *Curr Opin Biotechnol* 30:24–31. <http://dx.doi.org/10.1016/j.copbio.2014.04.008>.
- Rawlings DE. 2005. Characteristics and adaptability of iron- and sulfur-oxidizing microorganisms used for the recovery of metals from minerals and their concentrates. *Microb Cell Fact* 4:13. <http://dx.doi.org/10.1186/1475-2859-4-13>.
- Fortuny M, Guisasola A, Casas C, Gamisans X, Lafuente J, Gabriel D. 2010. Oxidation of biologically produced elemental sulfur under neutrophilic conditions. *J Chem Technol Biotechnol* 85:378–386. <http://dx.doi.org/10.1002/jctb.2333>.
- Janssen AJH, Ruitenberg R, Buisman CJN. 2001. Industrial applications of new sulphur biotechnology. *Water Sci Technol* 44:85–90.
- Syed M, Soreanu G, Falletta P, Bédard M. 2006. Removal of hydrogen sulfide from gas streams using biological processes—a review. *Can Biosyst Eng* 48:2.1–2.14.
- Lens PNL, Kuenen JG. 2001. The biological sulfur cycle: novel opportunities for environmental biotechnology. *Water Sci Technol* 44:57–66.
- Sturman PJ, Stein OR, Vymazal J, Kröpfelová L. 2008. Sulfur cycling in constructed wetlands, p 329–344. *In* Vymazal J (ed), *Wastewater treatment, plant dynamics, and management in constructed and natural wetlands*. Springer Science+Business Media BV, Berlin, Germany.
- Macalady JL, Hamilton TL, Grottenberger CL, Jones DS, Tsao LE, Burgos WD. 2013. Energy, ecology and the distribution of microbial life. *Philos Trans R Soc Lond B Biol Sci* 368:20120383. <http://dx.doi.org/10.1098/rstb.2012.0383>.
- Kleinjan WE, de Keizer A, Janssen AJH. 2003. Biologically produced sulfur, p 167–188. *In* Steudel R (ed), *Elemental sulfur and sulfur-rich compounds I*. Springer-Verlag, New York, NY.
- Dahl C, Prange A. 2006. Bacterial sulfur globules: occurrence, structure and metabolism, p 21–51. *In* Shively JM (ed), *Inclusions in prokaryotes*. Springer-Verlag, Berlin, Germany.
- Chan L-K, Weber TS, Morgan-Kiss RM, Hanson TE. 2008. A genomic region required for phototrophic thiosulfate oxidation in the green sulfur bacterium *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*). *Microbiology* 154(Pt 3):818–829. <http://dx.doi.org/10.1099/mic.0.2007/012583-0>.
- Holkenbrink C, Barbas SO, Møllerup A, Otaki H, Frigaard N-U. 2011. Sulfur globule oxidation in green sulfur bacteria is dependent on the dissimilatory sulfite reductase system. *Microbiology* 157:1229–1239. <http://dx.doi.org/10.1099/mic.0.044669-0>.
- Hanson TE, Bonsu E, Tuerk A, Marnocha CL, Powell DH, Chan CS. 31 July 2015. *Chlorobaculum tepidum* growth on biogenic S(0) as the sole photosynthetic electron donor. *Environ Microbiol* <http://dx.doi.org/10.1111/1462-2920.12995>.
- Wahlund T, Woese C, Castenholz R. 1991. A thermophilic green sulfur bacterium from New Zealand hot springs, *Chlorobium tepidum* sp. nov. *Arch Microbiol* 156:81–90. <http://dx.doi.org/10.1007/BF00290978>.
- Imhoff JF. 2006. The Chromatiaceae, p 846–873. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), *The prokaryotes—volume 6: proteobacteria: delta and epsilon subclasses*. Deeply rooting bacteria, 3rd ed. Springer Science+Business Media, LLC, Singapore.
- Overmann J. 2006. The family Chlorobiaceae, p 359–378. *In* Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (ed), *The prokaryotes—volume 7: proteobacteria: delta and epsilon subclasses*. Deeply rooting bacteria, 3rd ed. Springer Science+Business Media, LLC, Singapore.
- Mukhopadhyay B, Johnson EF, Ascano M. 1999. Conditions for vigorous growth on sulfide and reactor-scale cultivation protocols for the thermophilic green sulfur bacterium *Chlorobium tepidum*. *Appl Environ Microbiol* 65:301–306.
- Chan L-K, Morgan-Kiss RM, Hanson TE. 2008. Genetic and proteomic studies of sulfur oxidation in *Chlorobium tepidum* (syn. *Chlorobaculum tepidum*), p 357–373. *In* Hell R, Dahl C, Knaff DB, Leustek T (ed), *Sulfur metabolism in phototrophic organisms*. Springer Science & Business Media, Dordrecht, The Netherlands.
- Wahlund TM, Madigan MT. 1995. Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. *J Bacteriol* 177:2583–2588.
- Frigaard N-U, Bryant DA. 2001. Chromosomal gene inactivation in the green sulfur bacterium *Chlorobium tepidum* by natural transformation. *Appl Environ Microbiol* 67:2538–2544. <http://dx.doi.org/10.1128/AEM.67.6.2538-2544.2001>.
- Hanson TE, Tabita FR. 2001. A ribulose-1,5-biophosphate carboxylase/oxygenase (RubisCO)-like protein from *Chlorobium tepidum* that is involved with sulfur metabolism and the response to oxidative stress. *Proc Natl Acad Sci U S A* 98:4397–4402. <http://dx.doi.org/10.1073/pnas.081610398>.
- Azai C, Harada J, Oh-oka, H. 2013. Gene expression system in green sulfur bacteria by conjugative plasmid transfer. *PLoS One* 8:e82345. <http://dx.doi.org/10.1371/journal.pone.0082345>.
- Eisen JA, Nelson KE, Paulsen IT, Heidelberg JF, Wu M, Dodson RJ, Deboy R, Gwinn ML, Nelson WC, Haft DH, Hickey EK, Peterson JD, Durkin AS, Kolonay JL, Yang F, Holt I, Umayam LA, Mason T, Brenner M, Shea TP, Parksey D, Nierman WC, Feldblyum TV, Hansen CL, Craven MB, Radune D, Vamathevan J, Khouri H, White O, Gruber TM, Ketchum KA, Venter JC, Tettelin H, Bryant DA, Fraser CM. 2002. The complete genome sequence of *Chlorobium tepidum* TLS, a photosynthetic, anaerobic, green-sulfur bacterium. *Proc Natl Acad Sci U S A* 99:9509–9514. <http://dx.doi.org/10.1073/pnas.132181499>.
- Frigaard N-U, Voigt GD, Bryant DA. 2002. *Chlorobium tepidum* mutant lacking bacteriochlorophyll *c* made by inactivation of the *bchK* gene, encoding bacteriochlorophyll *c* synthase. *J Bacteriol* 184:3368–3376. <http://dx.doi.org/10.1128/JB.184.12.3368-3376.2002>.
- Sapan CV, Lundblad RL, Price NC. 1999. Colorimetric protein assay techniques. *Biotechnol Appl Biochem* 29:99–108.
- Morgan-Kiss RM, Chan L-K, Modla S, Weber TS, Warner M, Czymmek KJ, Hanson TE. 2009. *Chlorobaculum tepidum* regulates chlorosome structure and function in response to temperature and electron donor availability. *Photosynth Res* 99:11–21. <http://dx.doi.org/10.1007/s11120-008-9361-7>.
- Borrego CM, Gerola PD, Miller M, Cox RP. 1999. Light intensity effects on pigment composition and organization in the green sulfur bacterium *Chlorobium tepidum*. *Photosynth Res* 59:159–166. <http://dx.doi.org/10.1023/A:1006161302838>.
- Mead R, Gilmour SG, Mead A. 2012. Statistical principles for the design of experiments: applications to real experiments. Cambridge University Press, New York, NY.
- Ogunnaike BA. 2010. Random phenomena: fundamentals of probability and statistics for engineers, p 787–849. CRC Press, Boca Raton, FL.
- Akashi H, Gojobori T. 2002. Metabolic efficiency and amino acid composition in the proteomes of *Escherichia coli* and *Bacillus subtilis*. *Proc Natl Acad Sci U S A* 99:3695–3700. <http://dx.doi.org/10.1073/pnas.062526999>.
- Raiford DW, Heizer EM, Miller RV, Doom TE, Raymer ML, Krane DE. 2012. Metabolic and translational efficiency in microbial organisms. *J Mol Evol* 74:206–216. <http://dx.doi.org/10.1007/s00239-012-9500-9>.
- Heizer EM, Raiford DW, Raymer ML, Doom TE, Miller RV, Krane DE. 2006. Amino acid cost and codon-usage biases in 6 prokaryotic genomes:

- a whole-genome analysis. *Mol Biol Evol* 23:1670–1680. <http://dx.doi.org/10.1093/molbev/msl029>.
34. Siefert E, Pfennig N. 1984. Convenient method to prepare neutral sulfide solution for cultivation of phototrophic sulfur bacteria. *Arch Microbiol* 139:100–101. <http://dx.doi.org/10.1007/BF00692721>.
35. Rethmeier J, Rabenstein A, Langer M, Fischer U. 1997. Detection of traces of oxidized and reduced sulfur compounds in small samples by combination of different high-performance liquid chromatography methods. *J Chromatogr A* 760:295–302. [http://dx.doi.org/10.1016/S0021-9673\(96\)00809-6](http://dx.doi.org/10.1016/S0021-9673(96)00809-6).
36. Ernst O, Zor T. 2010. Linearization of the Bradford protein assay. *J Vis Exp* 38:1918. <http://dx.doi.org/10.3791/1918>.
37. Zor T, Selinger Z. 1996. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 236:302–308. <http://dx.doi.org/10.1006/abio.1996.0171>.
38. Stanier RY, Smith JHC. 1960. The chlorophylls of green bacteria. *Biochim Biophys Acta* 41:478–484. [http://dx.doi.org/10.1016/0006-3002\(60\)90045-7](http://dx.doi.org/10.1016/0006-3002(60)90045-7).
39. Hanson RS, Phillips JA. 1981. Chemical composition, p 328–364. In Gerhardt P, Murray RGE, Costilow RN, Nester EW, Wood WA, Krieg NR, Phillips GB (ed), *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, DC.
40. Leyva A, Quintana A, Sánchez M, Rodríguez EN, Cremata J, Sánchez JC. 2008. Rapid and sensitive anthrone-sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: method development and validation. *Biologicals* 36:134–141. <http://dx.doi.org/10.1016/j.biologics.2007.09.001>.
41. Zuroff TR, Xiques SB, Curtis WR. 2013. Consortia-mediated bioprocessing of cellulose to ethanol with a symbiotic *Clostridium phytofermentans*/yeast co-culture. *Biotechnol Biofuels* 6:59. <http://dx.doi.org/10.1186/1754-6834-6-59>.
42. Turnbull TL, Adams MA, Warren CR. 2007. Increased photosynthesis following partial defoliation of field-grown *Eucalyptus globulus* seedlings is not caused by increased leaf nitrogen. *Tree Physiol* 27:1481–1492. <http://dx.doi.org/10.1093/treephys/27.10.1481>.
43. Sánchez O, Van Gernerden H, Mas J. 1998. Utilization of reducing power in light-limited cultures of *Chromatium vinosum* DSM 185. *Arch Microbiol* 170:411–417. <http://dx.doi.org/10.1007/s002030050661>.
44. Neidhardt FC, Ingraham JL, Schaechter M. 1990. *Physiology of the bacterial cell*. Sinauer Associates, Inc., Sunderland, MA.
45. Smith DR, Chapman MR. 2010. Economical evolution: Microbes reduce the synthetic cost of extracellular proteins. *mBio* 1:e00131–10. <http://dx.doi.org/10.1128/mBio.00131-10>.
46. Smith AJ. 1997. Postcolumn amino acid analysis. *Methods Mol Biol* 64:139–146.
47. Brown CM, Stanley SO. 1972. Environment-mediated changes in the cellular content of the “pool” constituents and their associated changes in cell physiology. *J Appl Chem Biotechnol* 22:363–389. <http://dx.doi.org/10.1002/jctb.5020220307>.
48. Clark VL, Peterson DE, Bernlohr RW. 1972. Changes in free amino acid production and intracellular amino acid pools of *Bacillus licheniformis* as a function of culture age and growth media. *J Bacteriol* 112:715–725.
49. Wahlund TM, Madigan MT. 1993. Nitrogen fixation by the thermophilic green sulfur bacterium *Chlorobium tepidum*. *J Bacteriol* 175:474–478.
50. Cerqueda-García D, Martínez-Castilla LP, Falcón LI, Delaye L. 2014. Metabolic analysis of *Chlorobium chlorochromatii* CaD3 reveals clues of the symbiosis in ‘*Chlorochromatium aggregatum*’. *ISME J* 8:991–998. <http://dx.doi.org/10.1038/ismej.2013.207>.
51. Eddie BJ, Hanson TE. 2013. *Chlorobaculum tepidum* TLS displays a complex transcriptional response to sulfide addition. *J Bacteriol* 195:399–408. <http://dx.doi.org/10.1128/JB.01342-12>.
52. Ellar DJ. 1978. Some strategies of osmoregulation and ion transport in microorganisms, p 125–150. In Schmidt-Nielsen K, Bolis L, Maddrell SHP (ed), *Comparative physiology: water, ions, and fluid mechanics*. Cambridge University Press, New York, NY.
53. Franz B, Gehrke T, Lichtenberg H, Hormes J, Dahl C, Prange A. 2009. Unexpected extracellular and intracellular sulfur species during growth of *Allochromatium vinosum* with reduced sulfur compounds. *Microbiology* 155:2766–2774. <http://dx.doi.org/10.1099/mic.0.027904-0>.
54. Rodríguez J, Hiras J, Hanson TE. 2011. Sulfite oxidation in *Chlorobaculum tepidum*. *Front Microbiol* 2:112. <http://dx.doi.org/10.3389/fmicb.2011.00112>.
55. Hiras J. 2012. Characterization of a novel redox active thiol from *Chlorobaculum tepidum*. Ph.D. dissertation. University of Delaware, Newark, DE.
56. Kirchman DL. 2016. Growth rates of microbes in the oceans. *Annu Rev Mar Sci* 8:285–309. <http://dx.doi.org/10.1146/annurev-marine-122414-033938>.
57. Montesinos E. 1987. Change in size of *Chromatium minus* cells in relation to growth rate, sulfur content, and photosynthetic activity: a comparison of pure cultures and field populations. *Appl Environ Microbiol* 53:864–871.
58. Sirevåg R, Ormerod JG. 1977. Synthesis, storage, and degradation of polyglucose in *Chlorobium thiosulfatophilum*. *Arch Microbiol* 111:239–244. <http://dx.doi.org/10.1007/BF00549360>.
59. Sirevåg R. 1975. Photoassimilation of acetate and metabolism of carbohydrate in *Chlorobium thiosulfatophilum*. *Arch Microbiol* 104:105–111. <http://dx.doi.org/10.1007/BF00447309>.
60. Badalamenti JP, Torres CI, Krajmalnik-Brown R. 2014. Coupling dark metabolism to electricity generation using photosynthetic cocultures. *Biotechnol Bioeng* 111:223–231. <http://dx.doi.org/10.1002/bit.25011>.
61. Habicht KS, Miller M, Cox RP, Frigaard N-U, Tonolla M, Peduzzi S, Falkenby LG, Andersen JS. 2011. Comparative proteomics and activity of a green sulfur bacterium through the water column of Lake Cadagno, Switzerland. *Environ Microbiol* 13:203–215. <http://dx.doi.org/10.1111/j.1462-2920.2010.02321.x>.
62. van Gernerden H, Beeftink HH. 1978. Specific rates of substrate oxidation and product formation in autotrophically growing *Chromatium vinosum* cultures. *Arch Microbiol* 119:135–143. <http://dx.doi.org/10.1007/BF00964264>.
63. Schmidt GL, Kamen MD. 1970. Variable cellular composition of *Chromatium* in growing cultures. *Arch Mikrobiol* 73:1–18. <http://dx.doi.org/10.1007/BF00409947>.
64. Chan L-K, Morgan-Kiss RM, Hanson TE. 2009. Functional analysis of three sulfide:quinone oxidoreductase homologs in *Chlorobaculum tepidum*. *J Bacteriol* 191:1026–1034. <http://dx.doi.org/10.1128/JB.01154-08>.
65. Shuman KE, Hanson TE. 2016. A sulfide:quinone oxidoreductase from *Chlorobaculum tepidum* displays unusual kinetic properties. *FEMS Microbiol Lett* 363:fnw100. <http://dx.doi.org/10.1093/femsle/fnw100>.
66. Biehl H, Pfennig N. 1978. Growth yields of green sulfur bacteria in mixed cultures with sulfur and sulfate reducing bacteria. *Arch Microbiol* 117:9–16. <http://dx.doi.org/10.1007/BF00689344>.
67. Vilaprinyo E, Alves R, Sorribas A. 2010. Minimization of biosynthetic costs in adaptive gene expression responses of yeast to environmental changes. *PLoS Comput Biol* 6:e1000674. <http://dx.doi.org/10.1371/journal.pcbi.1000674>.
68. Mangiapia M, Scott K. 2016. From CO₂ to cell: energetic expense of creating biomass using the Calvin-Benson-Bassham and reductive citric acid cycles based on genome data. *FEMS Microbiol Lett* 363:fnw054. <http://dx.doi.org/10.1093/femsle/fnw054>.
69. Coolen MJL, Overmann J. 2007. 217 000-year-old DNA sequences of green sulfur bacteria in Mediterranean sapropels and their implications for the reconstruction of the paleoenvironment. *Environ Microbiol* 9:238–249. <http://dx.doi.org/10.1111/j.1462-2920.2006.01134.x>.
70. Passier HF, Bosch H-J, Nijenhuis IA, Lourens LJ, Böttcher ME, Leenders A, Sinninghe Damsté JS, de Lange GJ, de Leeuw JW. 1999. Sulphidic Mediterranean surface waters during Pliocene sapropel formation. *Nature* 397:146–149. <http://dx.doi.org/10.1038/16441>.
71. Coolen MJL, Overmann J. 1998. Analysis of subfossil molecular remains of purple sulfur bacteria in a lake sediment. *Appl Environ Microbiol* 64:4513–4521.
72. Dykhuizen DE. 1990. Experimental studies of natural selection in bacteria. *Annu Rev Ecol Syst* 21:373–398. <http://dx.doi.org/10.1146/annurev.es.21.110190.002105>.
73. Brandis G, Hughes D. 2016. The selective advantage of synonymous codon usage bias in *Salmonella*. *PLoS Genet* 12:e1005926. <http://dx.doi.org/10.1371/journal.pgen.1005926>.