Supplemental material

Secretion of Flavins by Three Species of Methanotrophic Bacteria

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MATERIALS AND METHODS

Growth of methanotrophic bacteria. Cultivation of *M. trichosporium* OB3b (5, 6) and *M. capsulatus* (Bath) (7) was carried out as described previously. The protocol for growth of *Methylocystis* sp. strain M was similar to that described for *M. trichosporium* OB3b (4-6). Briefly, batch growth was performed in 250 ml Erlenmeyer flasks fitted with airtight rubber septa. The growth medium contained 50 ml salts solution, 500 μl 0.1 M phosphate buffer (pH 6.8), and 100 μl trace metals solution (4). For growth under default conditions, the medium also included 50 μM $CuSO_4$ and 40 μM $FeSO_4 \bullet 7H_2O$. For copper starved conditions, no additional copper was included, and for iron starved conditions, only 2 μM $FeSO_4 \bullet 7H_2O$ was added. Cultures termed either "copper starved" or "iron starved" were adapted for at least two subculture growths in low copper and low iron conditions. Cultures of *Methylocystis* sp. strain M and *M. trichosporium* OB3b were maintained at 30 $^{\circ}$C in an orbital shaker at 200 rpm protected from light; *M. capsulatus* (Bath) was grown in the same shaker, but at 45 $^{\circ}$C. For each flask, 50 ml of air was replaced with 50 ml of methane upon inoculation and after 12 hrs of growth. After the initial 12 hrs, 50 ml of headspace was withdrawn every 8 hrs and replaced with 50 ml of a methane/air mixture (35:15 ml) until growth was terminated. Large-scale growth was performed in a 1.5 liter or 15 liter fermenter (New Brunswick Scientific) with an agitation rate of 300 rpm. Cells grown to mid-exponential phase (optical density at 600 nm (OD_{600}) of 3-5) were harvested by centrifugation at 7000 x g for 15 min.

***Methylocystis* sp. strain M growth experiments.** All experiments to monitor growth of *Methylocystis* sp. strain M were performed in biological triplicates. The OD_{600}

was measured every 4 hrs. The initial inoculum had an OD_{600} of 0.02-0.04. For each experiment, three separate batches of growths were performed. The first set was harvested during mid-exponential phase (36 hrs), the second set was harvested at late exponential phase (just prior to stationary phase) (60 hrs), and the third set was allowed to reach stationary phase (102 hrs). Cultures grown with no added iron sustained growth only for one growth cycle. Therefore, growth parameters were determined with cultures grown in 2 μM $FeSO_4 \bullet 7H_2O$.

Dry weight of cells was determined for cultures grown under default, iron starved and copper starved conditions to correlate them to optical density. In triplicate experiments, growing cultures were diluted to an OD_{600} of 1.0 and aliquoted into pre-weighed aluminum foil. The cells were allowed to dry for 1 hr at 70 $^{\circ}$C and the differences in weights were calculated (Fig. S3A). The length of cells was also measured using images taken on an optical microscope and tools available in the imageJ software (Fig S3B). Both dry weight and cell lengths are consistent with the conclusion that growth of cells under default, iron starved and copper starved conditions does not drastically affect the cell size or weight.

Isolation and metal loading of spent media. After completion of growth using the batch method, approximately 40 ml of culture was centrifuged at 7000 x g for 15 min to separate cells from the growth medium. The spent medium (cell free growth medium) was frozen in liquid nitrogen and stored at -80 °C or was lyophilized immediately. The lyophilized spent medium was then resuspended in 1 ml of water buffered to pH 5.4 with 20 mM ammonium acetate. Quantitation of flavins and plate assays to detect secreted siderophores were performed using these samples. Un-lyophilized spent media was also

used in plate assays to detect any secreted siderophores. $CuSO_4$ at a concentration of 3 g/l was added to the spent media from the large fermenter, allowed to stir overnight, and the compounds present purified on a Discovery DSC-18 HPLC column (Supelco, PA) using either methanol or acetonitrile as the elution solvent (6). Copper-bound and iron-bound riboflavin 5' phosphate (FMN) were prepared by incubating 10 ml of 0.1 mg/ ml FMN (Sigma Aldrich) with 5 mg of $CuSO_4$ or 5 mg of Fe(II)-EDTA. This mixture was incubated overnight and then purified on a DSC-18 column using methanol as the elution solvent.

Quantitation of extracellular flavins. Quantitation of flavins was performed using a Hewlett Packard HPLC equipped with a diode array detector. A DSC-18 reverse phase or a 25 cm Supelco C-18 reverse phase column was used for purification. For quantitation, an injection volume of 300 μl was used for all samples. The injected samples were normalized based on the optical density of the cells. Flavin quantitation was performed using a standard curve generated by the injection and integration of known concentrations (0.2 μM, 0.6 μM, 1 μM and 2 μM) of FMN. An extinction coefficient of 12,500 cm^{-1} M^{-1} at 450 nm was used to estimate the concentration (3, 9). *Methylocystis* sp. strain M secretes both FMN and riboflavin. The two species elute separately on the DSC-18 column. For quantitation of total extracellular flavins, the sum of the area of the two peaks was compared to known standards. Water and methanol buffered with 20 mM ammonium acetate (pH 5.4) were used for chromatographic analysis. An isocratic run at 5% methanol for 5 min was followed by a linear gradient to 20% methanol at 10 min. This was ramped to 50% methanol at 15 min followed by a

final ramp to 100% methanol at 25 min. All analyses were performed at a constant flow rate of 0.8 ml/min.

To determine the intracellular flavin content, cells were lysed using a previously established procedure (11). The cell pellet was treated with 490 μl of 0.1 M NaOH and mixed thoroughly for 15 sec. HCl (510 μl of 0.1 M) was then added to the solution. This mixture was centrifuged for 5 min at 20,800 x g and the supernatant was analyzed. For detection of FAD, 650 μl of the lysed sample was injected onto the column and the elution times were compared to a known standard of FAD (Sigma Aldrich).

Fe-CAS plate assay for siderophore detection. For Fe-CAS plates, a published protocol (10) was used with the following modifications. Since lyophilized spent medium rather than growing methanotrophic bacteria were spotted on the plates, all the culture ingredients were omitted from the plates. Sodium hydroxide and hydrochloric acid were used to adjust the pH. Briefly, 60.5 mg of chrome azurol S (CAS) (Acros Organics) was added to 50 ml of double distilled water. To this 10 ml of a 1 mM $FeCl_3 \bullet 6H_2O$ (in 10 mM HCl) was added slowly. This solution was added slowly to a third solution containing 72.9 mg of hexadecyltrimethylammonium bromide (HDTMA) in 40 ml double distilled water. This Fe-CAS solution was autoclaved separately from a mixture containing 300 ml double distilled water, 5.0 g Bacto-agar, and 10.4 g PIPES, with the pH adjusted to 6.8. After cooling to 50 °C, 33.3 ml of Fe-CAS solution was added to the agar solution and plates were allowed to solidify. Spots of filter paper were placed on the plates after the plates were dried overnight. Spent medium was added in increments of 20 μl, allowed to dry, and spotted again until a total volume of at least 100

μl was spotted. A positive control using the spent medium from a *Pseudomonas aeruginosa* culture grown under no iron conditions was also performed (1, 2, 8).

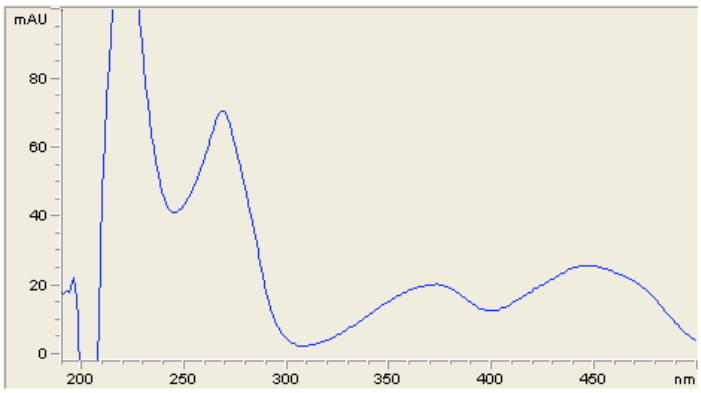
Determination of intracellular iron and copper contents. Total cellular copper and iron contents were measured in biological triplicates by diluting 1 ml of cells at an OD_{600} of 1.0 in 4 ml of 5% metal free nitric acid. The samples were briefly centrifuged for 5 min to remove any precipitate and then analyzed by inductively coupled plasma optical emission spectroscopy (ICP-OES). Copper and iron atomic absorption standards (Sigma Aldrich) were used to generate calibration curves.

Fluorescence spectroscopy. Fluorescence spectra of purified spent media from *Methylocystis* sp. strain M were obtained on an ISS PCI fluorescence spectrometer using a 1 cm path length cuvette. The PMT detector was cooled to 4 °C while the sample was at room temperature. A slit width of 1 mm was used for both excitation and emission wavelengths. A volume of 1000 μl purified spent media was used for all experiments.

Mass spectrometry. Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) was performed using a Bruker Autoflex III smart beam MALDI-TOF. Detection was performed in either the negative or positive ion mode using a 1:1 mixture of the sample and 20 mM *p*-nitroaniline (*p*-NA) (Acros Organics). The mixture was spotted on a plate, allowed to dry, and ionized with a laser power set to 20-70% of the maximum laser intensity of the instrument at a frequency of 10-100 Hz.

SUPPLEMENTARY FIGURES

A)



B)

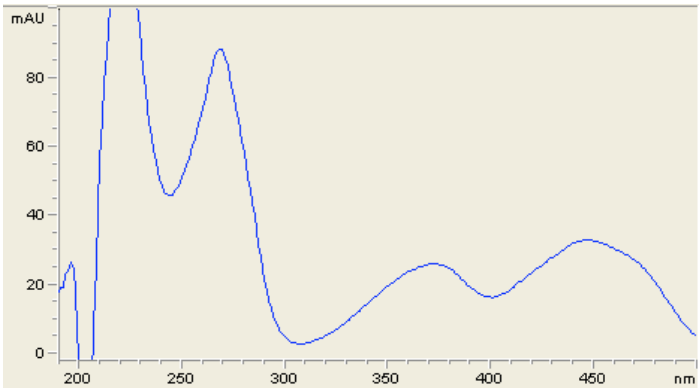


FIG. S1 Diode-array UV-visible profiles of the elution peaks of extracellular flavins (top, A) compared to commercial FMN (bottom, B) purified using a Supelco C-18 reverse phase column.

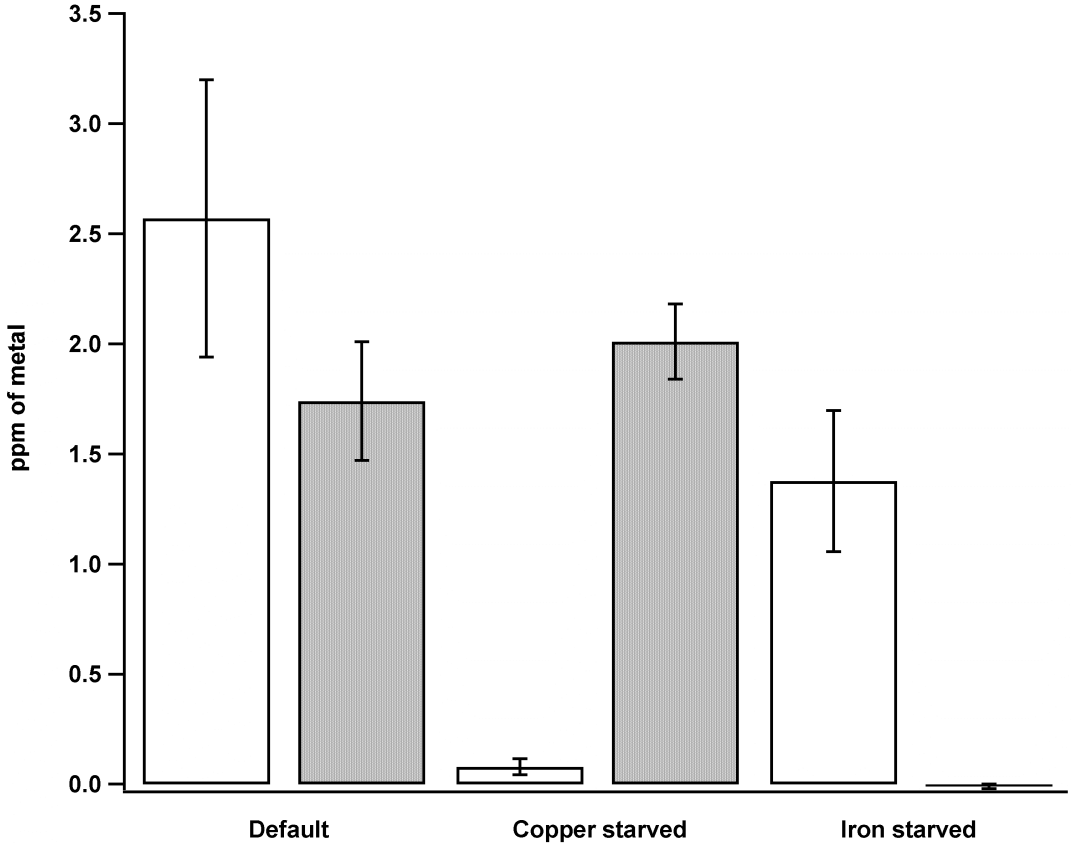


FIG. S2. Total intracellular metal content of copper (white) and iron (gray) in cells grown under default, copper starved, and iron starved growth conditions. Measurements were normalized to an OD_{600} of 1.0. All measurements were performed in biological triplicates with cells that reached stationary growth phase.

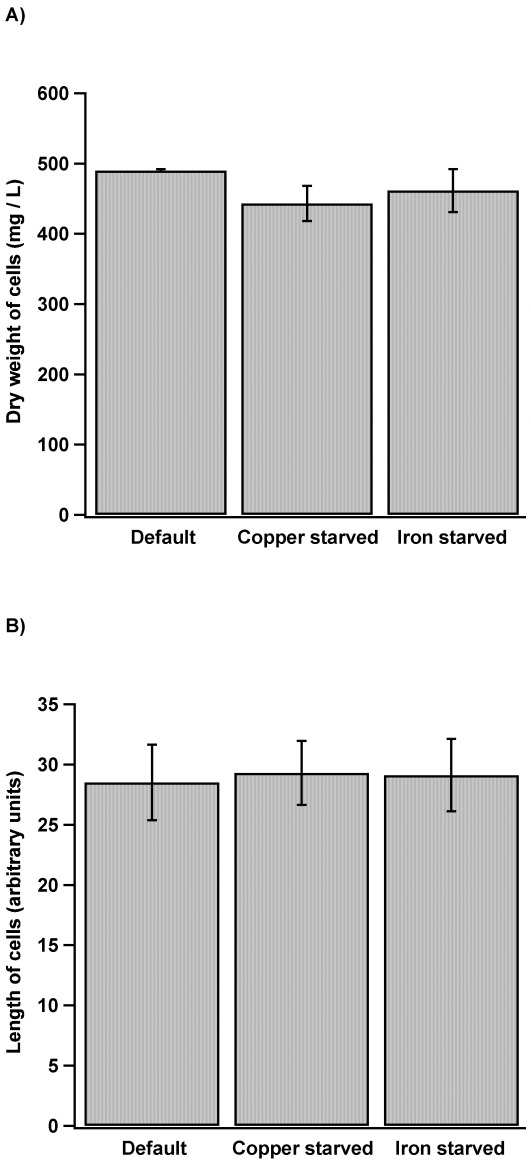


FIG. S3. Dry weight (in mg/ L) (A) and cell length (B) of *Methylocystis* sp. strain M grown under default, copper starved and iron starved growth conditions. The dry weight was normalized based on optical density measured at 600 nm. Cells grown under default, copper starved and iron starved conditions exhibit no significant differences in cell size or weight.

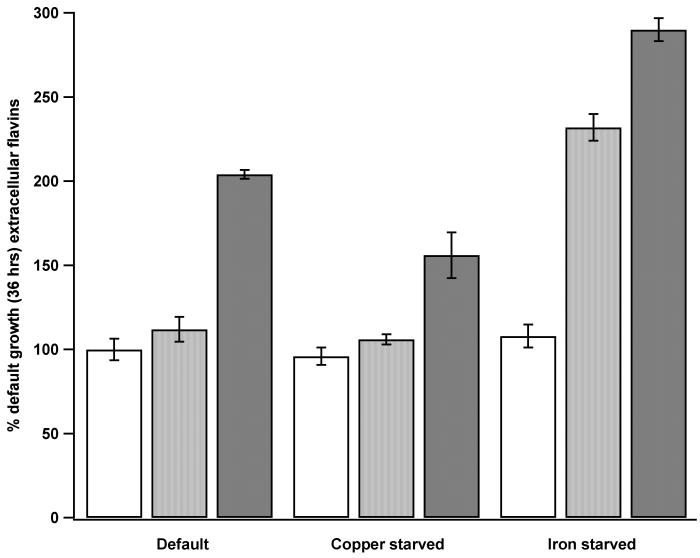


FIG. S4. Quantitation of extracellular flavins secreted by *Methylocystis* sp. strain M grown under default, copper starved, and iron starved growth conditions. Quantitation of extracellular flavins is an average and standard deviation of three biological triplicates. The first set (white) was grown to mid exponential phase (36 hrs), the second set (light gray) was grown to late exponential phase (60 hrs), and the third set (dark grey) was grown to stationary phase (102 hrs). Total flavins are expressed as a percentage normalized to total flavins secreted by cells grown under default conditions for 36 hrs.

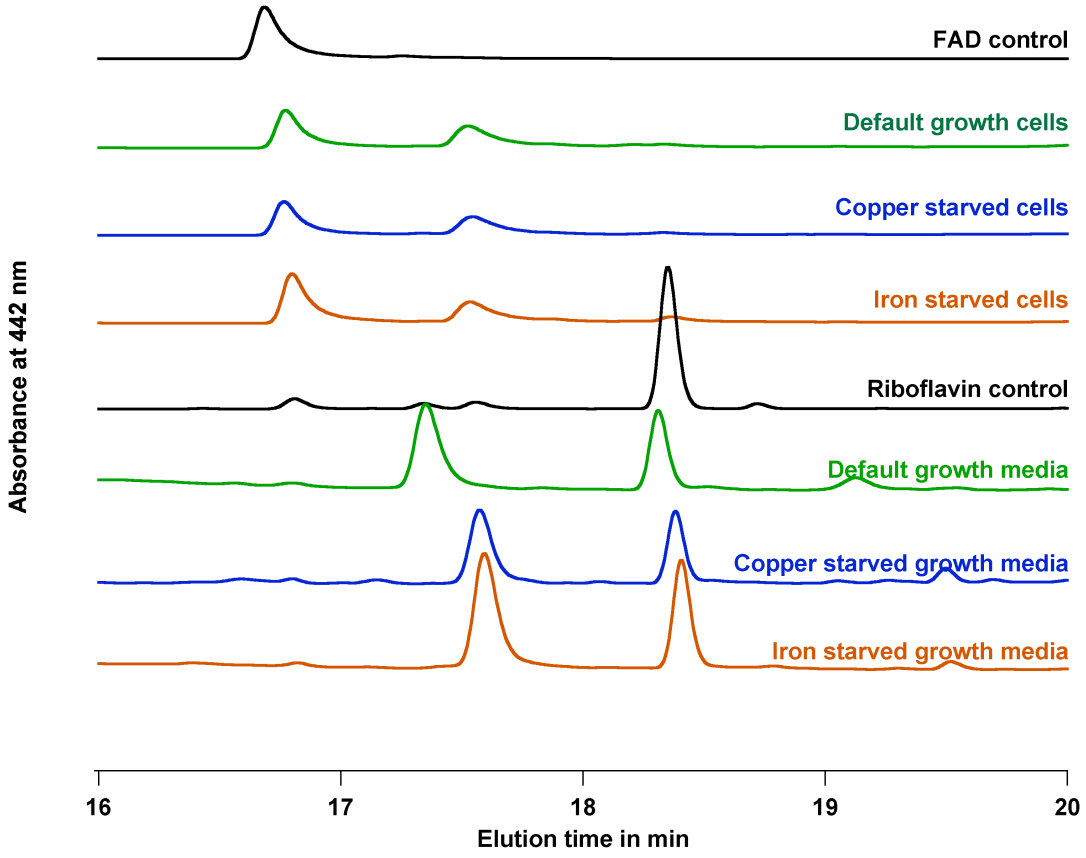


FIG. S5. Elution profiles (DSC-18 column) of intracellular flavins and extracellular flavins from *Methylocystis* sp. strain M grown under default (green), iron starved (red) and copper starved (blue) growth conditions. FAD and riboflavin controls are shown in black. Flavin is present predominantly as FAD inside the cell and as a mixture of FMN and riboflavin outside the cell.

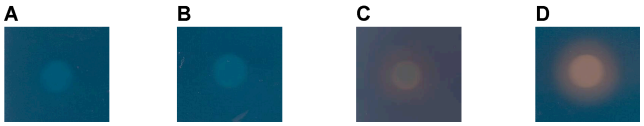
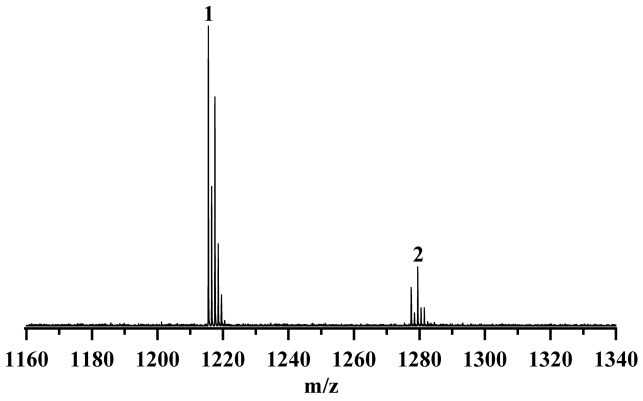


FIG. S6. Fe-CAS assay to detect secretion of siderophores in (A) *Methylocystis* sp. strain M, (B) *M. capsulatus* (Bath), (C), *M. trichosporium* OB3b, and (D), *P. aeruginosa*. All cell cultures were grown under iron starvation conditions. The orange color in (C) and (D) indicates that *M. trichosporium* (OB3b) and *P. aeruginosa* produce siderophores.

A)



B)

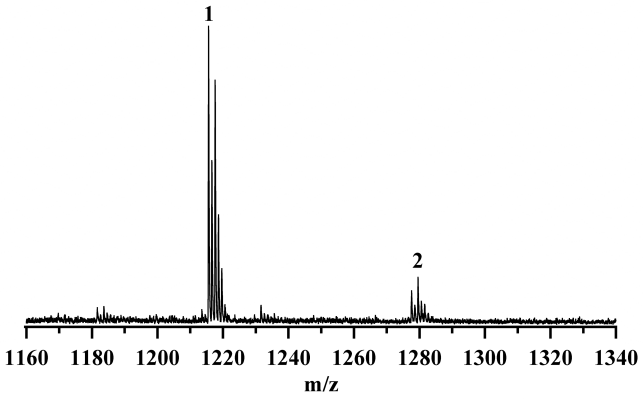


FIG. S7. MALDI-TOF MS of Cu-methanobactin isolated from *M. trichosporium* OB3b (A) and *Methylocystis* sp. strain M (B). The isotopic distribution of the peaks suggests a copper-bound species (peak 1 at ~1218 m/z). Identical m/z values obtained for both *M. trichosporium* OB3b and *Methylocystis* sp. strain M suggest that the two organisms secrete methanobactins that are identical in composition and structure. The peak at ~1280 m/z may be due to adventitious binding of a second copper ion.

A)

B)

C)

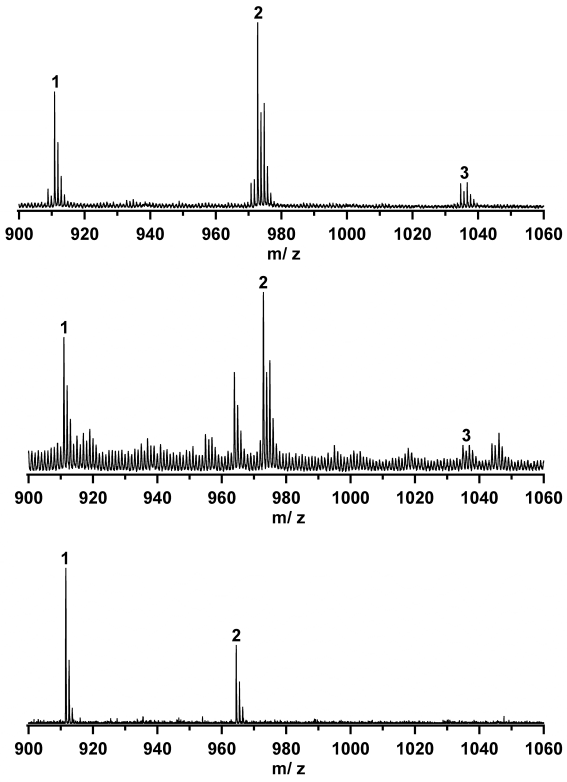


FIG. S8. Mass spectra of copper-loaded extracellular compounds isolated from *Methylocystis* sp. strain M (A), copper-loaded commercial FMN (B) and iron-loaded commercial FMN (C). Peak 1 in all spectra corresponds to $(FMN)_2$ (dimer of FMN). Peak 2 corresponds to Cu-$(FMN)_2$ (A and B) and Fe-$(FMN)_2$ (C). Peak 3 corresponds to Cu_2-$(FMN)_2$.

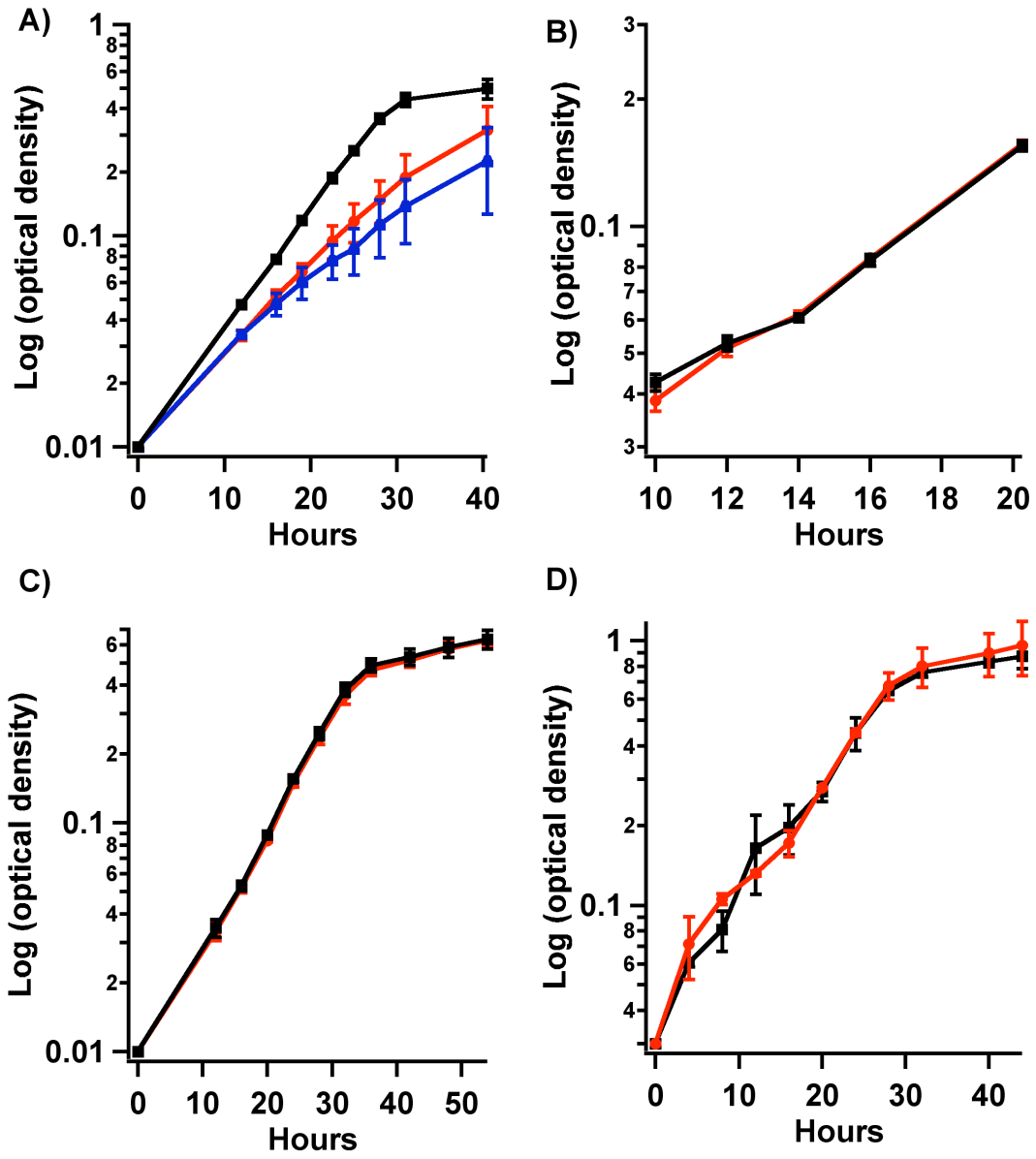


FIG. S9. Growth curves of *Methylocystis* sp. strain M. A) Comparison of growth in 2 μM $FeSO_4 \bullet 7H_2O$ (black squares), 0 μM $FeSO_4 \bullet 7H_2O$ (red circles), and 0 μM $FeSO_4 \bullet 7H_2O$ + 0.5 μM Fe bound to FMN (blue triangles). B) Comparison of growth in 2 μM $FeSO_4 \bullet 7H_2O$ (red circles) and 2 μM $FeSO_4 \bullet 7H_2O$ + 4 μM apo-FMN (black squares). C) Comparison of growth in 10 μM $FeSO_4 \bullet 7H_2O$ (red circles) and 10 μM $FeSO_4 \bullet 7H_2O$

+ 2 μM apo-FMN (black squares). D) Comparison of growth in 40 μM $FeSO_4 \bullet 7H_2O$ (default growth conditions) (red circles), and 40 μM $FeSO_4 \bullet 7H_2O$ + 10 μM apo-FMN (black squares). Addition of FMN, either as apo- or as Fe-FMN, does not alter the growth rates of the cultures. These observations suggest that FMN may not be involved in or does not facilitate acquisition of iron in *Methylocystis* sp. strain M.

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