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Supporting Information

for

A Chemical Reporter Strategy for Detecting and Identifying *O*-Mycoloylated Proteins in *Corynebacterium*

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I. Abbreviations in the manuscript and supporting information

AGM	Arabinogalactan mycolate
Azido-488	Azide-modified carboxyrhodamine 110
CgMyt	*Corynebacterium glutamicum* mycoloyltransferase
CuAAc	Cu(I)–catalyzed alkyne–azide cycloaddition
DI	Deionized water
LC-MS/MS	Liquid chromatography-tandem mass spectroscopy
LB	Luria broth
LDAO	Lauryldimethylamine-N-oxide
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of flight mass spectroscopy
MFI	Mean fluorescence intensity
Myc	Mycoloyl
OD_{600}	Optical density measured by absorbance at a wavelength of 600 nm
PBS	Phosphate-buffered saline
PBS-B	Phosphate-buffered saline with 0.1% bovine serum albumin
PorA	Porin protein A
PorH	Porin protein H
PorB	Porin protein B
PTM	Post-translational modification
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TDM	Trehalose dimycolate
TBTA	Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
TMM	Trehalose monomycolate
xg	Times gravity

II. Methods

Bacterial strain, growth conditions, and reagents

Corynebacterium glutamicum strain 534 was obtained from the Siegrist lab (University of Massachusetts, Amherst). Starter cultures of bacteria were generated by inoculating a single colony from a freshly streaked LB agar plate into 3 mL Luria broth (LB) liquid medium in a culture tube. Starter cultures were incubated at 30 °C with shaking overnight.

A stock solution of O-AlkTMM was prepared in milliQ water at a concentration of 185 mM, sterile filtered (0.45 µM), and stored at –20 ºC. Lower concentration stocks of O-AlkTMM probes (25 mM, 5 mM, and 1 mM) were prepared prior to usage in cell labeling experiments. Other reagent stocks prepared for this study included: Azido-488 (Click Chemistry Tools, 1 mM in DMSO, stored at –20 ºC); Sodium ascorbate (60 mM in milliQ water, freshly prepared); TBTA ligand for CuAAC reactions (Click Chemistry Tools, 6.4 mM in *tert*-butanol/DMSO 3:1, stored at –20 ºC); copper(II) sulfate (50 mM in milliQ water, freshly prepared). Stocks of 10% SDS (stored at room temperatures) and 1% LDAO (stored at 4 ºC) were prepared in milliQ water. Stocks of 0.5 M NaOH and 0.5 M acetic acid were prepared in milliQ water.

Dependence of labeling and growth on O-AlkTMM concentration

Evaluation of O-AlkTMM metabolic labeling and growth effects was performed in triplicate in sterile flat-bottom 96-well plates. First, *C. glutamicum* overnight starter culture was diluted with LB medium to a final volume of 50 mL in a 250 mL Erlenmeyer flask, which was incubated with shaking at 30 °C until an OD_{600} of 0.6 was reached. From this culture, *C. glutamicum* cells were mixed with LB medium and probe stock solution in a 96-well plate to achieve the desired cell density (OD_{600} 0.2–0.3) and probe concentration (0–10 mM) at a final volume of 200 µL. Plates were incubated at 30 °C with shaking in a Tecan plate reader (Infinite F200 PRO operated by Tecan iControl software) for 10 h while recording OD_{600} every 30 min to monitor cell growth.

For secondary labeling of *C. glutamicum* with azido-488 by CuAAC, the cell suspension (200 µL) was transferred to a v-bottom 96 well plate and centrifuged (3,200 xg, 5 min, room temperature). The supernatant was discarded, and the cells were fixed in 4% paraformaldehyde in PBS (180 µL) for 15 min at room temperature. Fixed cells were centrifuged (3,200 xg, 5 min, room temperature) and washed with PBS containing 1 mg/mL BSA (PBS-B) three times. Cells were then reacted with azido-488 fluorophore via CuAAC. A typical CuAAC reaction was carried out by re-suspension of cells in PBS-B (138 uL) and sequential addition of stock solutions of 1 mM azido-488 (3 µL), 60 mM sodium ascorbate (3 µL), 6.4 mM TBTA (3 µL) and 50 mM copper(II) sulfate (3 µL) to give a final volume of 150 µL. The final reagent concentration was 20 µM azido-488; 1.2 mM sodium ascorbate; 128 µM TBTA; and 1 mM copper(II) sulfate. The cell-reagent mixture was thoroughly mixed by pipeting up and down and the reaction proceeded for 30 min at room temperature in the dark. Cells were centrifuged and washed with PBS-B three times as described above, then processed and analyzed by flow cytometry and fluorescence microscopy as previously described.[1]

Protein labeling, extraction, and removal of lipids

C. glutamicum overnight starter culture was diluted with LB liquid medium in duplicate to 1 L in a 2.8 L Fernbach flask. Cells were incubated with shaking at 30 °C until an OD_{600} of 0.6 was reached, then one flask was treated with O-AlkTMM to a final concentration of 100 µM while the other (control) was left untreated. Cells were incubated with shaking (220 rpm) at 30 ºC for 10 h, after which the OD_{600} was determined to be 4.5.

The cells were harvested by centrifugation at 4,000 xg at 25 °C for 10 min. The pellet was re-suspended in 150 mL of chloroform/methanol (1:2, v/v) and stirred overnight at room temperature. The chloroform/methanol suspension was centrifuged at 3,200 xg at 25 °C for 10 min. The supernatant was saved and the pellet was re-suspended in 150 mL chloroform/methanol (2:1, v/v) and stirred overnight at

room temperature, followed by centrifugation at 3,200 xg at 25 °C for 10 min. The supernatants from both extraction steps were combined in a round bottom flask and the solvents were removed by rotary evaporation until near dryness. The resulting residue was then diluted with 150 mL of diethyl ether and kept at –20 °C overnight to allow protein precipitation. The diethyl ether contents were transferred into 50 mL centrifuge tubes and the protein precipitates were collected by centrifugation at 3,200 xg at 25 °C for 10 min. The supernatant was discarded and the precipitate was air-dried.

The air-dried proteins were re-suspended in 500 µL of milliQ water and transferred to a pre-weighed 50 mL conical centrifuge tube. An equal volume (500 µL) of methanol was added. The re-suspended proteins were then diluted with chloroform/methanol (2:1, v/v) to a final volume of 40 mL followed by vigorous shaking. The sample was then cooled and kept at –20 °C overnight to allow proteins to precipitate. The precipitated proteins were recovered by centrifugation at 3,200 xg at 25 °C for 10 min, then the supernatant was discarded and the proteins were air-dried at 37 °C. This procedure was repeated three times to remove the majority of free extractable lipids. The final mass of the extracted proteins was determined (typically, about 30 mg of proteins were obtained). Protein solids were re-suspended in milliQ water, then SDS and LDAO were added sequentially to concentrations of 0.5% and 0.05%, respectively, to a final volume of 1.5 mL and a final protein concentration of 10 µg/µL. The protein contents were then swirled and pipeted up and down gently while avoiding foaming to maximally solubilize proteins in the detergent mixture.

Fluorescence labeling of O-AlkTMM-tagged proteins and NaOH treatment

The SDS/LDAO protein mixture was reacted with azido-488 fluorophore via CuAAC. A typical CuAAC reaction was carried out by transferring 644 µL of the SDS/LDAO protein mixture to a 4 mL screw-cap glass vial followed by sequential addition of stock solutions of 1 mM azido-488 (14 µL), 60 mM sodium ascorbate (14 µL), 6.4 mM TBTA (14 µL) and 50 mM copper(II) sulfate (14 µL) to give a final volume of 700 µL and final concentration of reagents as described above for cell labeling experiments. The reaction was stirred at room temperature for 1 h while protected from light. To remove excess azido-488, the reaction mixture was diluted with an equal volume of methanol (700 µL), transferred to a 50 mL conical centrifuge tube, and diluted with chloroform/methanol (2:1, v/v) to a final volume of 25 mL. The contents were mixed by vigorous shaking, then kept at –20 ºC overnight to allow protein precipitation. Protein solids were recovered by centrifugation at 3,200 xg at 25 °C for 5 min, air-dried, and re-suspended in 350 µL milliQ water containing 0.5% SDS and 0.05% LDAO.

For NaOH treatment experiments, fluorescently-labeled proteins in SDS/LDAO solution (120 µL) were treated with 0.1 M NaOH at 37 °C for 30 min with gentle agitation. The reaction was neutralized by the addition of a stoichiometric amount of acetic acid followed by the chloroform/methanol protein precipitation procedure described in the preceding paragraph to remove the cleaved fluorophore. Typically, the neutralized reaction mixture was diluted with 200 µL of methanol, transferred to a 15 mL conical centrifuge tube, and diluted with chloroform/methanol (2:1, v/v) to a final volume of 5 mL. After storing at –20 ºC overnight to allow precipitation, protein solids were recovered by centrifugation at 3,200 xg at 25 °C for 5 min, air-dried, and re-suspended in 100 µL milliQ water containing 0.5% SDS and 0.05% LDAO.

SDS-PAGE and protein visualization

SDS-PAGE was performed with Mini-PROTEAN TGX precast Tris-Tricene gels (10–20 % cross-linked; Bio-Rad) and Tris-Tricene-SDS running buffer (Bio-Rad). Protein samples were mixed with 4x sample loading buffer (250 mM Tris-HCl, 8% w/v SDS, 0.02% w/v bromophenol blue, 30% glycerol) and loaded into the gel. The proteins were resolved by gel electrophoresis at a maximum voltage of 140 V and a maximum current of 80 mA for 100 min or until loading dye exited the gel. The running buffer was kept cold by using an ice pack. In-gel fluorescence was detected with a Typhoon FLA 7000 (GE Healthcare Life Sciences) using fluorescein excitation/emission filters. For coomassie blue staining, the same gel was fixed in 40% ethanol and 10% acetic acid for 15 min. The gel was washed with 100 mL of DI water and

stained with QC colloidal coomassie stain (Bio-Rad) overnight with gentle agitation. The gel was destained in 100 mL DI water for 3 h, changing the water three times. Imaging of the coomassie-stained gel was performed using a ChemiDoc Touch Imaging System (Bio-Rad) and processed by Image Lab software version 5.2.1 (Bio-Rad).

Approximate labeling distribution of O-AlkTMM in AGM, extractable lipids, and proteins

After culturing *C. glutamicum* in the presence or absence of O-AlkTMM (100 µM) to an approximate OD_{600} of 4.5, 10 mL of cells were centrifuged, washed, and subjected to CuAAC reaction with azido-488 at a final volume of 2 mL using the reagent concentrations described above. Next, the cells were transferred to a 15 mL conical tube, diluted with PBS-B to 10 mL and centrifuged at 3,200 xg at 25 °C for 10 min, then washed three times in PBS-B (10 mL each wash) to remove unreacted fluorophore. The cell pellets were re-suspended in 45 mL of chloroform/methanol (1:2, v/v) and stirred overnight at room temperature. The chloroform/methanol suspension was centrifuged at 3,200 xg at 25 °C for 10 min. The supernatant was saved and the pellet was re-suspended in 45 mL chloroform/methanol (2:1, v/v) and stirred overnight at room temperature, followed by centrifugation at 3,200 xg at 25 °C for 10 min. The pellet (PG–AGM fraction) was saved while supernatants from both extraction steps were combined in a 250 mL round bottom flask and the solvents were removed by rotary evaporation until near dryness. The resulting residue was then diluted with 45 mL of diethyl ether and kept at –20 °C overnight to allow protein precipitation. The suspension was transferred into 50 mL centrifuge tubes and the protein precipitates were collected by centrifugation at 3,200 xg at 25 °C for 10 min. The supernatant containing extractable lipids in diethyl ether was saved in a 250 mL round bottom flask while the precipitated protein residue was air-dried.

The air-dried proteins were re-suspended in 100 µL of milliQ water and transferred to a 15 mL conical tube. An equal volume (100 µL) of methanol was added. The re-suspended proteins were then diluted with chloroform/methanol (2:1, v/v) to a final volume of 5 mL followed by vigorous shaking. The sample was then cooled and kept at –20 °C overnight to allow proteins to precipitate. The precipitated proteins were recovered by centrifugation at 3,200 xg at 25 °C for 10 min, then the supernatant was pooled together with the saved diethyl ether fraction while the proteins were left to air-dry at 37 °C. This procedure was repeated twice to separate the majority of free extractable lipids from proteins. Protein solids were re-suspended in milliQ water, then SDS and LDAO were added sequentially to concentrations of 0.5% and 0.05%, respectively, to a final volume of 2 mL to solubilize the proteins. All of the chloroform/methanol and ether fractions, which contained extractable lipids including trehalose mycolates, were combined and dried down by rotary evaporation, then re-dissolved in 2 mL chloroform/methanol (2:1, v/v) and transferred to a 20 mL scintillation vial. The pellet containing the PG–AGM fraction was allowed to dry in an oven at 37 °C overnight, then thoroughly re-suspended in 2 mL of 0.1% SDS in milliQ water by repeated vortexing and sonication.

From these 2 mL stock solutions, 200 µL were transferred into 2 mL amber glass vials. The extractable lipids fraction was dried down on a rotary evaporator followed by addition of 500 µL of 0.1 M NaOH. The protein and PG–AGM samples were diluted with milliQ water and NaOH stock solution to achieve a final NaOH concentration of 0.1 M and a final volume of 500 µL. The tubes were sealed and stirred at 37 °C for 1 h to saponify and solubilize the samples. The samples were allowed to cool to room temperature. 5 µL aliquots were transferred to a black flat-bottom 96-well plate. The samples were diluted to a final volume of 200 µL with milliQ water and fluorescence analysis was performed using a Tecan Infinite F200 Pro plate reader equipped with a GFP filter (excitation at 485 nm and emission at 535 nm) using the optimal gain setting.

Whole-proteome analysis of protein *O*-mycoloylation using O-AlkTMM

C. glutamicum overnight starter culture was diluted with LB liquid medium in triplicate to 500 mL in 2.8 L Fernbach flasks. Cells were incubated with shaking at 30 °C until an OD_{600} of 0.6 was reached. One flask was treated with O-AlkTMM (100 µM), the second flask was treated with 6-heptynoic acid (100

μM), and the third flask (control) was left untreated. Cells were incubated with shaking (220 rpm) at 30 ºC for 10 h, after which the OD_{600} was determined to be approximately 4.5 for each culture.

The cells were harvested by centrifugation at 4,000 xg at 25 °C for 10 min. The pellet was re-suspended in 150 mL of chloroform/methanol (1:2, v/v) and stirred overnight at room temperature. The chloroform/methanol suspension was centrifuged at 3,200 xg at 25 °C for 10 min. The supernatant was saved and the pellet was re-suspended in 150 mL chloroform/methanol (2:1, v/v) and stirred overnight at room temperature, followed by centrifugation at 3,200 xg at 25 °C for 10 min. The pellet was saved while the supernatants from both chloroform/methanol extraction steps were combined in a round bottom flask and the solvents were removed by rotary evaporation until near dryness. Proteins present in the chloroform/methanol extract were obtained following the protocols described in the protein labeling, extraction, and lipid removal section (page S4). The pellet remaining after chloroform/methanol extraction was dried in an oven at 37 °C, then re-suspended in 3 mL lysis buffer (50 mM Tris, 300 mM NaCl, 5% glycerol, 0.1% SDS, pH 8). The suspension was transferred to a 1.5 mL screw-cap vial containing 0.25 mL of 0.1 mm zirconia/silica beads (BioSpec Products) and mechanically disrupted by bead beating (3 x 1 min) using a Mini-BeadBeater-16 (BioSpec Products). The lysate was transferred to a 15 mL conical tube and centrifuged at 3,200 xg at 25 °C for 10 min. The supernatant containing soluble proteins (expected cytosolic and periplasmic proteins) was saved while the pellet was subjected to sequential extraction with 0.4% LDAO and 2% SDS (1.0 mL each at 60 °C for 1 h). These two detergent-soluble fractions were pooled together to give detergent-soluble proteins (expected membrane proteins).

Next, 200 μL aliquots from the chloroform/methanol extract, bead beating extract, and detergent extract were transferred to 2 mL screw-cap glass vials. CuAAC reactions with azido-488 were performed for each sample at a final volume of 500 μL and final concentration of reagents as described above. Reactions were stirred at room temperature for 1 h while protected from light. To remove excess azido-488, the reaction mixtures were diluted with an equal volume of methanol (500 μL), transferred to 15 mL conical tubes, and diluted with chloroform/methanol (2:1, v/v) to a final volume of 10 mL. The contents were mixed by vigorous shaking, then kept at –20 ºC overnight to allow protein precipitation. Protein solids were recovered by centrifugation at 3,200 xg at 25 °C for 5 min, air-dried, and re-suspended in 200 μL milliQ water containing 0.5% SDS and 0.05% LDAO. Samples were optionally treated with 0.1 M NaOH as described above to evaluate base sensitivity of the fluorescent label. SDS-PAGE and protein visualization was performed as described above.

Mass spectrometry analysis

LC-MS/MS analysis. After an unlabeled protein sample was resolved by SDS-PAGE, the gel was stained with Coomassie Brilliant Blue G (Brilliant Blue G-colloidal concentrate). Protein bands of interest were excised from the gel and subjected to tryptic digestion as reported with slight modifications.[2] Briefly, excised gel slices were dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate buffer, pH ~8, at 56 °C for 45 min. The gel slices were dehydrated again using 100% acetonitrile followed by incubation in the dark for 20 min at room temperature with 50 mM iodoacetamide in 100 mM ammonium bicarbonate buffer. Gel slices were then washed with 100 mM ammonium bicarbonate buffer and subjected to dehydration in 100% acetonitrile. 50 μL of a sequencing-grade modified typsin solution (0.01 μg/μL in 50 mM ammonium bicarbonate buffer; Promega) was added to each gel band so that the gel was completely submerged. Bands were incubated at 37 °C overnight. Peptides were extracted from the gel by water bath sonication in 100 μL of an aqueous solution containing 60% acetonitrile and 1% trifluoroacetic acid. The extraction was repeated two additional times, then the extracts were pooled and vacuum-dried to a volume of ~2 μL. Peptides were re-suspended in 20 μL of an aqueous solution containing 2% acetonitrile and 0.1% trifluoroacetic acid.

5 μL of the tryptic peptide solution were automatically injected by a ThermoFisher EASY nLC 1000 (www.thermo.com) onto a Thermo ACCLAIM PepMap, 0.1 mm x 20 mm C18 peptide trap and washed for approximately 5 min. Bound peptides were then eluted onto a Thermo ACCLAIM PepMap RSLC

0.75 mm x 250 mm C18 column over 35 min with a gradient of 5% B to 30% B in 24 min, ramped up to 90% B at 25 min and held for the duration of the run at a constant flow rate of 0.3 nL/min (A = 99.9% water/0.1% formic acid, B = 99.9% acetonitrile/0.1% formic acid). Eluted peptides were sprayed into a ThermoFisher Q-Exactive mass spectrometer outfitted with a Flex-spray nano-spray source. The top ten ions in each survey scan were subjected to data-dependant zoom scans followed by high energy collision induced dissociation (HCD) and the resulting MS/MS spectra were converted to peak lists using Mascot Distiller, v2.5.1.0 (www.matrixscience.com). Peak lists were then searched against a custom database which included all *C. glutamicum* protein sequences available from NCBI (downloaded 4-06-2016 from www.ncbi.nlm.nig.gov), appended with common laboratory contaminants (downloaded from www.thegpm.org, cRAP project) using the Mascot searching algorithm, v2.5 (www.matrixscience.com). The Mascot output was then analyzed using Scaffold, v4.5.1 (www.proteomesoftware.com) to probabilistically validate protein identifications. Assignments validated in Scaffold with <1% FDR are considered true. Mascot parameters for all databases were set as follows:

- Allowed up to 2 missed tryptic sites
- Fixed modification of Carbamidomethyl Cysteine
- Variable modification of oxidation of methionine, deamidation of asparagine and glutamine
- Peptide tolerance of +/- 10 ppm
- MS/MS tolerance of 0.3 Da
- Peptide charge state limited to +2/+3

MALDI-TOF MS analysis. MALDI-TOF MS analysis was performed on both crude protein mixture following chloroform/methanol extraction (Figure S1) and electrophoretically-resolved intact proteins that were excised from gels (Figure 4). The crude protein sample was obtained using the chloroform/methanol extraction and lipid removal procedure described on page S4. Approximately 1 mg of the crude protein sample was extracted with 100 µL of 0.4% LDAO at 60 °C for 1 h. The mixture was centrifuged at 2,000 xg at 25 °C for 5 minutes, then the supernatant containing dissolved proteins was diluted 10 times with 0.4% LDAO. 1 µL of the protein solution was loaded onto the MALDI plate followed by depositing an equal volume of sinapinic acid (10 mg/mL in an aqueous solution containing 60% acetonitrile and 0.1% trifluoroacetic acid) as the matrix. The protein/matrix mixture was carefully pipeted up and down and air-dried at room temperature. MALDI-TOF mass spectra were acquired on a Bruker autoflex mass spectrometer. Spectra were acquired in the linear positive ion mode using an extraction delay time of 170 ns and an accelerating voltage of 20 kV. The acquired spectra were processed by Bruker FlexAnalysis software version 3.4.

To analyze intact proteins separated by SDS-PAGE, protein bands were excised from the gel, chopped into very small pieces and subjected to 0.4% LDAO extraction. Briefly, the chopped gel slices were dehydrated using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium biocarbonate buffer, pH ~8, at 56 °C for 30 min. Gel slices were then washed once with 100 mM ammonium bicarbonate buffer and dehydrated again using 100% acetonitrile. 30 µL of 0.4% LDAO was added followed by incubation at 37 °C overnight. After overnight incubation, the samples were centrifuged for 30 sec at 110 xg, then the supernatant was collected and subjected to MALDI-TOF MS analysis as described above.

III. Supplementary figures

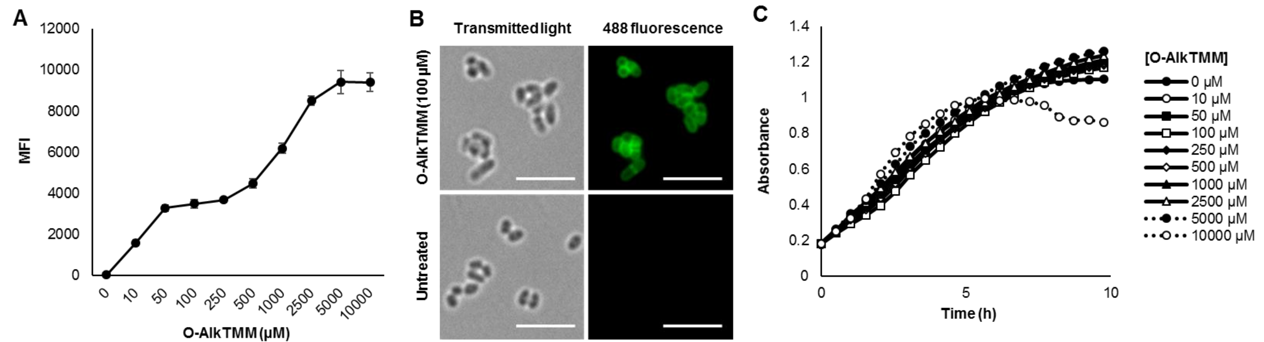

Figure S1. O-AlkTMM efficiently labels *C. glutamicum* and is non-toxic. *C. glutamicum* was incubated in varying concentrations of O-AlkTMM for 10 h, reacted with azido-488 via CuAAC, and analyzed by (A) flow cytometry and (B) fluorescence microscopy. Scale bars, 5 μm. Error bars denote the standard deviation of three replicate experiments. MFI, mean fluorescence intensity. (C) Growth curves of O-AlkTMM-treated *C. glutamicum* obtained by recording optical density at 600 nm during incubation for 10 h. Each data point represents the average of three replicates.

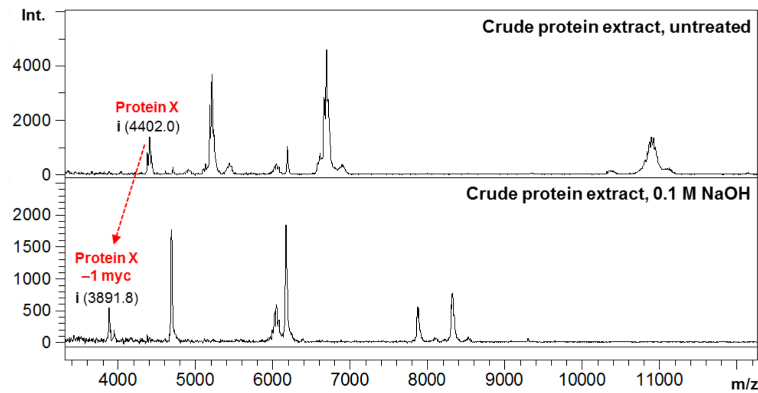

Figure S2. MALDI-TOF MS analysis of crude protein extract prior to electrophoretic resolution. The sample was either treated with 0.1 M NaOH (bottom spectrum) or left untreated (top spectrum), and analyzed by MALDI-TOF MS. Proposed NaOH-induced peak shifts and corresponding number of mycolate (myc) chains are indicated in red for protein X.

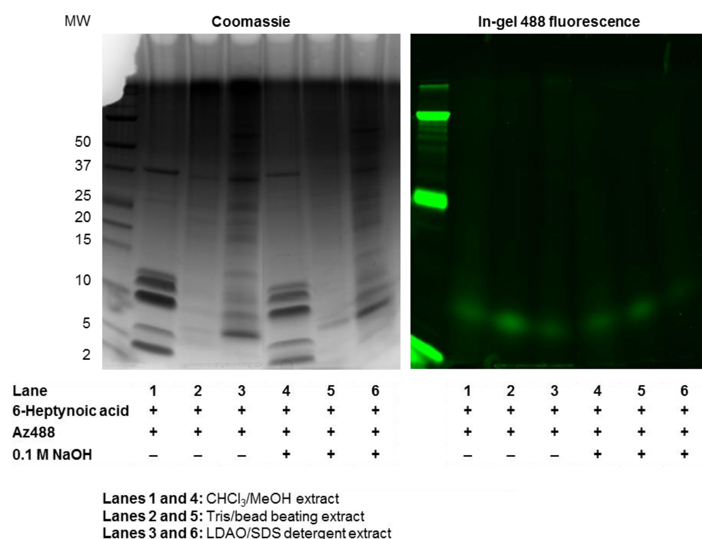

Figure S3. 6-Heptynoic acid does not label proteins in *C. glutamicum*. Bacteria were treated with 6-heptynoic acid (100 µM) or left untreated, then separated into $CHCl_3$/MeOH extract (lanes 1 and 4), Tris extract by bead beating (lanes 2 and 5), and LDAO/SDS detergent extract (lanes 3 and 6) fractions as described on page S6. Samples were either treated with 0.1 M NaOH or left untreated, then analyzed by SDS-PAGE. The Coomassie-stained gel is shown on the left, and in-gel fluorescence is shown on the right. Note: These experiments were run in parallel with O-AlkTMM-treated and untreated samples, the results of which are shown in Figure S5.

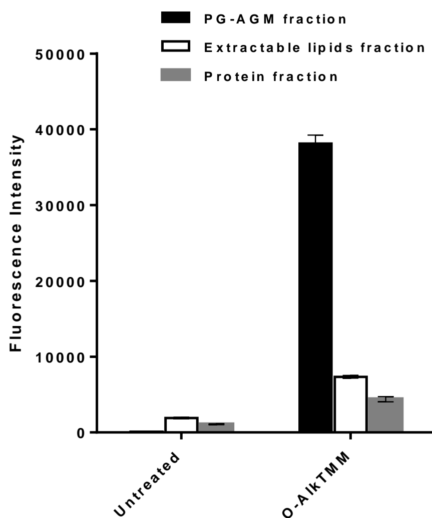

Figure S4. Approximate labeling distribution of O-AlkTMM in AGM, trehalose mycolates, and proteins. *C. glutamicum* was labeled with O-AlkTMM (or left untreated) and reacted with azido-488 via CuAAC. As described on page S6, the cells were then extracted with chloroform/methanol to obtain soluble lipids/proteins and an insoluble pellet enriched for the peptidoglycan–arabinogalactan–mycolate complex ("PG–AGM fraction"). The lipids and proteins in the soluble fraction were separated through protein precipitation to enrich for trehalose mycolates ("extractable lipids fraction") and *O*-mycoloylated proteins ("protein fraction"). All three samples were treated wtih 0.1 M NaOH then analyzed by a fluorescence plate reader. As shown in Figure S5, the PG-AGM fraction does contain some O-AlkTMM-dependent fluorescence corresponding to *O*-mycoloylated proteins. However, this signal

is considered to be quite low compared to labeled AGM (estimated at ≤ 10%) as judged by the similar levels of in-gel fluorescence between the $CHCl_3$/MeOH-extracted proteins (Figure S5, fluorescence, lane 1) and the detergent-soluble proteins (Figure S5, fluorescence, lane 3), the latter of which would be present in the PG–AGM fraction in this experiment. Error bars denote the standard deviation of three separate fluorescence analyses. Fluorescence intensity (y-axis) is given in arbitrary units.

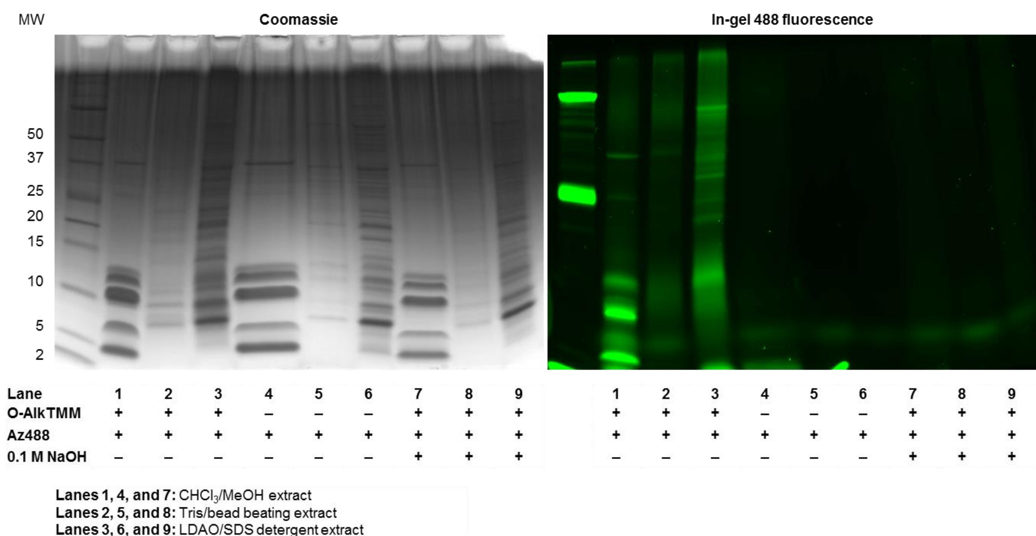

Figure S5. Whole-proteome analysis of protein *O*-mycoloylation in *C. glutamicum*. Bacteria were treated with O-AlkTMM (100 µM) or left untreated, then separated into $CHCl_3$/MeOH extract (lanes 1, 4, and 7), Tris extract by bead beating (lanes 2, 5, and 8), and LDAO/SDS detergent extract (lanes 3, 6, and 9) as described on page S6. Samples were either treated with 0.1 M NaOH or left untreated, then analyzed by SDS-PAGE. The Coomassie-stained gel is shown on the left, and in-gel fluorescence is shown on the right. Note: These experiments were run in parallel with 6-heptynoic acid treatment, results of which are shown in Figure S3.

IV. Supplementary tables

Table S1. Proteins identified by LC-MS/MS

Protein Band	Identified protein[a]	Protein mass (kDa)	Total unique peptide count	Accession number
Band A	Cluster of membrane protein	13	2	GI:499324326
	Cluster of chain A, crystal structure of PorB	11	2	GI:188595886
	Cationic specific porin[b]	6	1 (79%)	GI:57160609
Band B	Cluster of membrane protein	13	4	GI:499324326
	Cluster of chain A, crystal structure of PorB	11	3	GI:188595886
	Unnamed protein product	34	2	GI:111920744
	H+-ATPase c subunit	8	2	GI:10039447
	Multispecies hypothetical protein	6	2	GI:489951708
	Cluster of hypothetical protein	41	1	GI:499323223
Band C	Cluster of anion-specific porin[c]	13	20	CAD79638.1
	Hypothetical protein	12	5	WP_011014297.1
	Hypothetical protein SB89_05105	13	4	AJE66982.1
	Cluster of hypothetical protein	41	4	WP_011013715.1
	Cluster of hypothetical protein	51	4	WP_011013975.1
	Esterase	37	4	AIK86275.1
	Cluster of ABC transporter-binding protein	17	3	AMA00599.1
	Unnamed protein	70	3	CAE06371.1
	Hypothetical protein CGLAR1_04360	109	3	AIK84512.1
	Glutamy-tRNA amidotransferase	11	2	AIK84932.1
	Hypothetical protein CGLAR1_03270	26	2	AIK84298.1
	Iron ABC transporter substrate-binding protein	36	2	WP_011013905.1
	Membrane protein	50	2	AIK83998.1
	Hypothetical protein CGLAR1_05850	25	1	AIK84786.1
	Exodeoxyribonuclease VII small subunit	9	2	AIK84719.1
	Glutamate-binding protein	32	2	AIK85388.1
	Hypothetical protein SD36	32	2	KIH73656.1

	Hypothetical protein APT58_09685	13	2	AMA00480.1
	Unnamed protein product	29	2	CAE06279.1
	Hypothetical protein AC079_11430	37	2	ALP50774.1
	ABC transporter substrate-binding protein	34	2	AIK85285.1
	Hypothetical protein CGLAR1_11745	43	2	AIK85895.1
	Membrane Protein	13	2	AIK85440.1
	Unnamed protein product	13	1	CAE06462.1
	Resuscitation-promoting factor Rpf1	20	1	AIK84537.1
	Hypothetical protein	32	1	WP_011015356.1
	ABC transporter substrate-binding protein	37	1	AJE68670.1
	Hypothetical protein CGLAR1_13890	75	1	AIK86284.1
	Acetyl-CoA carboxylase	9	1	AIK84437.1
	Hypothetical protein CGLAR1_04390	37	1	AIK84517.1
	ABC transporter substrate-binding protein	58	1	AIK85802.1
	Phosphoribosylaminoimidazole synthetase	38	1	AIK85983.1
	Hypothetical protein SB89_13960	10	1	AJE68535.1
Band D	Hypothetical protein SB89_05105	13	4	AJE66982.1
	Cluster of hypothetical protein	41	3	WP_011013715.1
	Hypothetical protein CGLAR1_03270	26	3	AIK84298.1
	Cluster of hypothetical protein	51	3	WP_011013975.1
	Cluster of anion-specific porin	13	3	CAD79638.1
	Hypothetical protein CGLAR1_09965	18	2	AIK85565.1
	Unnamed protein product	13	2	CAE06462.1
	Hypothetical protein	13	2	WP_011014658.1
	Membrane Protein	13	2	AIK85440.1
	Hypothetical protein APT58_09685	13	2	AMA00480.1
	Esterase	37	2	AIK86275.1
	Hypothetical protein CGLAR1_04360	109	1	AIK84512.1
	Hypothetical protein CGLAR1_05850	25	1	AIK84786.1
	Resuscitation-promoting factor Rpf1	20	1	AIK84537.1

	ABC transporter substrate-binding protein	45	1	AIK84457.1
	Hypothetical protein CGLAR1_09845	22	1	AIK85542.1
	Membrane protein	12	1	WP_011014817.1
	Esterase	40	1	WP_011013583.1
	Glutamate binding protein	32	1	AIK85388.1
	Hypothetical protein AC079_11430	37	1	ALP50774.1
	Hypothetical protein	32	1	WP_011015356.1
	Hypothetical protein CGLAR1_08405	12	1	AIK85265.1
	Hypothetical protein	12	1	WP_011014297.1
	Iron ABC transporter substrate-binding protein	36	1	WP_011013905.1
Band E	Cluster of hypothetical protein	41	8	WP_011013715.1
	Cluster of hypothetical protein	51	6	WP_011013975.1
	Cluster of anion-specific porin	13	4	CAD79638.1
	Hypothetical protein CGLAR1_03270	26	3	AIK84298.1
	Iron ABC transporter substrate-binding protein	36	3	WP_011013905.1
	Hypothetical protein SB89_05105	13	2	AJE66982.1
	Unnamed protein product	13	2	CAE06462.1
	Hypothetical protein CGLAR1_09965	18	2	AIK85565.1
	Hypothetical protein	13	2	WP_011014658.1
	Membrane Protein	13	2	AIK85440.1
	Hypothetical protein APT58_09685	13	2	AMA00480.1
	Esterase	37	2	AIK86275.1
	Hypothetical protein CGLAR1_04360	109	2	AIK84512.1
	Hypothetical protein CGLAR1_05850	25	1	AIK84786.1
	Resuscitation-promoting factor Rpf1	20	1	AIK84537.1
	ABC transporter substrate-binding protein	45	1	AIK84457.1
	Hypothetical protein CGLAR1_09845	22	1	AIK85542.1
	Membrane protein	13	1	AIK84550.1
	Serine protease	45	1	AIK84591.1
	Hypothetical protein CGLAR1_13830	71	1	AIK86273.1

Band F	Cluster of hypothetical protein	41	23	WP_011013715.1
	Cluster of anion-specific porin	13	3	CAD79638.1
	Unnamed protein product	29	3	CAE06279.1
	Esterase	40	3	WP_011013583.1
	Glyceraldeyde-3-phosphate dehydrogenase A	36	2	ABB53267.1
	Membrane protein	13	2	AIK85440.1
	Hypothetical protein APT58_09685	13	2	AMA00480.1
	Cluster of hypothetical protein	51	1	WP_011013975.1
	Hypothetical protein CGLAR1_02380	35	1	AIK84130.1
	Hypothetical protein CGLAR1_09965	18	1	AIK85565.1
	Hypothetical protein APT58_04375	27	1	ALZ99524.1

[a] All listed proteins were identified with over 95% probability except PorH, which was identified with 79% probability.
[b] Cation-specific porin was confirmed to be PorH protein by searching the NCBI database using accession number GI:57160609.
[c] Cluster of anion-specific protein was confirmed to be PorB protein by searching the UniProt database using accession number CAD79638.

Table S2. Amino acid sequences for PorA, PorH, and PorB

Protein name	Amino acid sequence			Total number of amino acids	Protein mass (Da)
PorA	MENVYEFLGN	LDVLSGSGLI	GYVFDFLGAS	45	4680
	SKWAGAVADL	IGLLG			
PorH	MDLSLLKETL	GNYETFGGNI	GTALQSIPTL	57	4091
	LDSILNFFDN	FGDLADTTGE	NLDNFSS		
PorB	MKLSHRIAAI	AATAGIAVAA	FAAPASASDF	126	13220
	ANLSSTNKDL	STQYDWVACG	ILEGGLKAAG		
	VLEEGQYNRE	LAEAITAKGE	GFWTTQFPQI		
	GDWNEDQAAA	LADRAQTCGL	VKADTYLSEL		
	SSNFSS				

Table S3. Proteins identified in the current work that overlap with reported mycomembrane-associated proteins from *C. glutamicum*

Previously reported[a,3]			This work		Protein mass (kDa)	BLAST identification match (%)
Protein	NCBI accession number	Reported locus tag[b]	Protein	NCBI accession number		
Esterase (trehalose mycoloyltransferase cMytC)	NP_599594.1	NCgl0336	Esterase	WP_011013583.1	40	100
Esterase (trehalose mycoloyltransferase cMytB)	NP_602069.1	NCgl2779	Esterase	WP_011015457.1	37	100
PorB	NP_600199.1	NCg10933	Cluster of anion-specific porin	WP_011014018.1	13	100
Putative ABC-type cobalamine/Fe^{3+}-siderophore transport system, periplasmic component	NP-600039.1	NCgl0776	Iron ABC transporter substrate-binding protein	WP_011013905.1	36	100
Hypothetical protein	NP_599640.1	NCgl0381	Hypothetical protein CGLAR1_02380	WP_059288658.1	35	98
Hypothetical protein	NP_599774.1	NCgl0513	Cluster of hypothetical protein	WP_011013715.1	41	94
Hypothetical protein	NP_599796.1	NCglo535	Hypothetical protein CGLAR1_03270	WP_01113729.1	26	100
Hypothetical protein	NP_601304	NCgl2024	Hypothetical protein CGLAR1_09845	WP_003856432.1	22	95.4

[a] *C. glutamicum* mycomembrane-associated proteins reported by Bayan and co-workers.
[b] Locus tag of possible overlapping protein reported by Bayan and co-workers.

V. References for supporting information

(1) Foley, H. N.; Stewart, J. A.; Kavunja, H. W.; Rundell, S. R.; Swarts, B. M. *Angew. Chem. Int. Edit.* **2016**, *55* (6), 2053.
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(3) Marchand, C. H.; Salmeron, C.; Bou Raad, R.; Méniche, X.; Chami, M.; Masi, M.; Blanot, D.; Daffé, M.; Tropis, M.; Huc, E.; Le Maréchal, P.; Decottignies, P.; Bayan, N. *J. Bacteriol.* **2011**, *194* (3), 587.