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A bioorthogonal monomycolate of trehalose disclosed the *O*-mycoloylation of mycoloyltransferases and other cell envelope proteins in *C. glutamicum*

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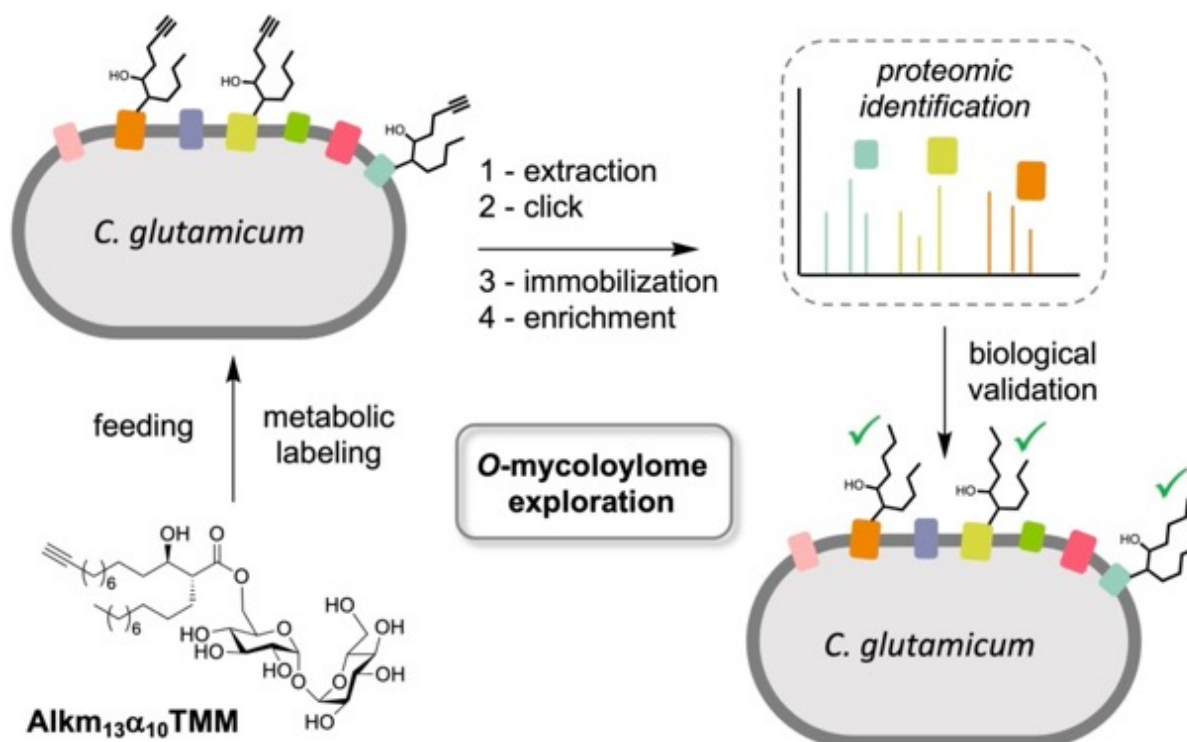
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Abstract

Protein mycoloylation is a recently identified unusual post-translational modification (PTM) exclusively observed in Mycobacteriales, an order of bacteria that includes several human pathogens. These bacteria possess a distinctive outer membrane, known as the mycomembrane, composed of very long-chain fatty acids called mycolic acids. It has been demonstrated that a few mycomembrane proteins undergo covalent modification with mycolic acids in the model organism *Corynebacterium glutamicum*, through the action of mycoloyltransferase MytC. This PTM represents the first example of protein *O*-acylation in prokaryotes and also the first example of protein modification by mycolic acid. Many questions about the specificity of protein *O*-mycoloylation remain crucial for understanding its evolutionary significance in Mycobacteriales and its role in cell physiology. We have developed the first bioorthogonal mycolate donor featuring the natural mycolic acid pattern, enabling direct unambiguous transfer of the lipid moiety to its acceptors and efficient metabolic labeling and enrichment of MytC protein substrates. Mass spectrometry analysis of the labeled proteins and comparative proteomic analysis of the cell envelope proteome between wild-type and $\Delta mytC$ strains identified an unbiased list of 21 proteins likely mycoloylated in the cell. The robustness of our approach is demonstrated by the successful biological validation of mycoloylation in 6 candidate proteins within wild-type cells, revealing the characteristic profile of proteins modified with natural mycolates. These findings provide interesting insights into the significance of this new lipidation pathway and pave the way for understanding their function, especially concerning the mycoloyltransferase family that includes the essential Antigen85 enzymes in Mycobacteria.

Introduction

Protein lipidation is an important post-translational modification (PTM) that occurs in all living cells. In eukaryotic cells, this process can take many forms, varying in terms of the hydrophobic chains added, the type of bonding, and the nature of the proteins involved. In prokaryotic cells, however, this process is restricted to cell envelope proteins and always involves the addition of acyl chains (one to three) at the *N*-terminal amino acid of the protein.¹ This addition generally occurs to the conserved *N*-terminal cysteine of the protein but can also exceptionally occur at an *N*-terminal glycine, as described in pathogenic *Escherichia coli*.^{2,3} In 2010, an additional protein lipidation modification was discovered in the order of Mycobacteriales, which includes numerous important pathogens such as *Mycobacterium tuberculosis*, *Mycobacterium abscessus* or *Corynebacterium diphtheriae*.⁴ This novel PTM was first described in *Corynebacterium glutamicum*, a model organism for Mycobacteriales. It involves the *O*-acylation of one or several serine residues of some proteins by very atypical α -branched and β -hydroxylated fatty acids, known as mycolic acids. This reaction depends on MytC, a member of the mycolyltransferase family,⁵ which also includes the Ag85 complex of *M. tuberculosis* involved in the biogenesis of the major glycolipids of the outer membrane (also called the mycomembrane) of this order of bacteria.^{6,7} So far, the only characterized examples of *O*-mycoloylated proteins have been identified in *C. glutamicum* and involve the two outer membrane porins, PorAH (an oligomer of PorA and PorH) and PorBC (an oligomer of PorB and PorC) as well as ProtX of unknown function.⁸⁻¹⁰ All these small polypeptide chains (ranging from 38 to 123 residues) are either mono or di-mycoloylated with the various mycoloyl chains mainly produced by the cell i.e. C32:0, C34:1 and C36:2. Very little information is available concerning the function of this PTM but it has been clearly shown that mycoloylation of PorB, PorC and PorH (but not of PorA) is essential for their association with the outer membrane. In terms of activity, PorA mycoloylation (but not PorH) is essential for the pore-forming activity of PorAH in black lipid bilayers.¹⁰ Although only a few examples have been described so far, the existence of this pathway raises several important questions in the field of cell biology and protein trafficking. Indeed, the role of this modification, compared to the canonical *N*-acylation of bacterial lipoprotein is puzzling, given that both lipidation pathways coexist in *C. glutamicum* and are equally presumed to anchor proteins to a lipid bilayer.^{11,12} Because PorBC is found in the external medium when it is not mycoloylated, Carel *et al.*⁹ proposed that protein mycoloylation could be an anchoring process specifically restricted to the association of some lipidated proteins to the mycomembrane. Although appealing, this hypothesis is still speculative as mycoloylation of inner membrane-associated proteins cannot yet be excluded. Furthermore, several *N*-acylated proteins have been proposed to be anchored in the mycomembrane.¹³ In this context, a number of questions regarding the mechanism and specificity of protein *O*-mycoloylation remain unanswered. It is thus necessary to fully understand the significance of the evolutionary appearance of protein mycoloylation in Mycobacteriales and its function in cell physiology.

To address these important issues, a robust determination of the *O*-mycoloylome of *C. glutamicum* is necessary. It may shed light on the real occurrence of mycoloylated proteins (their number, nature, and variety). Classical protein-lipid analysis at the-proteome scale by liquid chromatography-tandem mass spectrometry (LC-MS/MS) is very challenging due to the difficulty of detecting low-abundant, highly hydrophobic peptides bearing the modification and has proved to be quite inefficient for protein lipidation PTMs. In this context, recent advances

in metabolic labeling combined with click chemistry and proteomics provide a method of choice for studying PTMs. The strategy involves using a PTM substrate analog that bears a bioorthogonal functional handle, such as an alkyne or an azide. This analog is supplied during cell growth and incorporated into proteins *via* endogenous metabolic pathways. The PTM-modified protein is then chemically derivatized with a detection tag through bioorthogonal chemistry to facilitate its analysis by several methods, including global proteomics.^{14,15}

To set up such an approach for the study of protein mycoloylation, we need to examine the mycolate donor for this unusual PTM. Mycolic acids are produced in the cytoplasm of Mycobacteriales by condensation of two fatty acid molecules that are immediately esterified on trehalose by a polyketide synthase (Pks13) (Figure 1A) giving rise to trehalose monomycolate (TMM). The trehalose monomycolate is then translocated across the inner membrane by a transporter (MmpL) and serves as a donor of mycolate chain for various envelope acceptors such as TMM itself to form trehalose dimycolate (TDM) or, alternatively, for some arabinosyl terminal ends of arabinogalactan (AG). TMM is assumed to be the mycolate donor involved in this PTM.⁵ The protein mycoloylation reaction is supposed to be catalyzed by MytC and proceeds through two distinct steps involving a double transesterification reaction at the catalytic site like all mycoloyltransferases.⁶ In the first step, the mycolate of TMM is transferred on the catalytic serine (S189) forming a covalent mycoloyl-enzyme intermediate, followed by a second transesterification onto the serine of the protein acceptor (Figure 1B).

In 2016, Swarts introduced the first bioorthogonal analog related to trehalose using commercially available 6-heptynoic acid as a simplified single chain mycolic acid mimic, facilitating its rapid and efficient linkage to trehalose (**O-Alk-Acyl-Tre**, Figure 1A). It has been proved to be effective for various applications, from imaging mycobacteria to proteomic studies.¹⁶ In particular it was employed to tentatively uncover the mycoloylome of *C. glutamicum*.¹⁷ This method, coupled to SDS PAGE and Western blot analysis allowed the authors to propose a list of about 50 suspected mycoloylated proteins without, however, providing direct evidence of actual mycoloylation by lipidic moiety of the probe by mass spectrometry on the identified proteins. Similarly, in 2017, Issa *et al.* used externally added 17-octadecynoic acid (**Alk-fatty acid**, Figure 1A), a commercial alkynyl palmitic acid analog to label PorA specifically onto Ser15.⁸ Interestingly, this labeling is dependent not only on MytC (the mycoloylation enzyme) but also on Pks13 (mycolic acid synthase), suggesting that an alkyne fatty acid can be metabolized by the cell and incorporated into mycolic acid. During the preparation of this manuscript, Banahene *et al.* revisited the usage of **O-Alk-Acyl-Tre** and were able to label around 30 candidates with this probe in a MytC-dependent manner.¹⁸ Interestingly, by chemical proteomics strategies they were able to identify the sites of modification by this probe. So far this is the most reliable list of putative mycoloylated proteins available in the literature, but the mycoloylation of these candidates remains to be verified by direct purification and analysis of the proteins in a natural context and, as mentioned by the authors, there is an interest in optimizing the specificity of the probe to more closely resemble native mycolates.

Recently, we reported the synthesis of a bioorthogonal TMM-based probe with the natural pattern of mycolic acid that is β -hydroxylated α -branched 6-*O*-acylated trehalose with two lipid chains mimicking the native meromycolic chain (C13) and the α -chain (C10) (**Alk₁₃ α ₁₀TMM**, Figure 1A).¹⁹ The chain lengths were chosen to obtain a more soluble analog than native TMM, while closely mimicking the natural compounds. In *C. glutamicum*, the predominant saturated form of TMM is the trehalose C32:0 corynomycolate [(2*R*, 3*R*) 3-

hydroxy-2-tetradecyloctadecanoate] (Figure 1A). The synthesis was performed in two main steps: *i*) preparation of the fatty acids parts based on Noyori enantioselective reduction of β -ketoester while retaining the terminal alkyne during reduction and diastereoselective alkylation of the resulting β -hydroxyester using Fráter-Seebach alkylation and *ii*) esterification on a selectively protected trehalose derivative (Figure 2A). As a proof of concept, we demonstrated its potential for labeling the mycomembrane of *C. glutamicum* even at low concentrations.¹⁹ Encouraged by these results, we anticipated that **Alkm₁₃ α ₁₀TMM** could provide additional information to that obtained with the **O-Alk-Acyl-Tre** for proteomic studies to gain insight into the *O*-mycoloylome of *C. glutamicum*. Indeed, **Alkm₁₃ α ₁₀TMM** is supposed to avoid the substantial acylhydrolase activity observed with unbranched lipid TMM analogs,^{20,21} thereby achieving two main goals: *i*) preventing the possible recycling of a free alkynoic acid in bacterial lipid metabolism, which could lead to nonspecific labeling, and *ii*) improving the native transfer activity of mycolate to its acceptors (Figure 1A).

In this study, we used **Alkm₁₃ α ₁₀TMM** to label and purify putative mycoloylated proteins, which were subsequently identified by mass spectrometry analysis. In addition, we combined these results with those obtained from a differential analysis of the cell envelope proteome of wild-type and Δ *mytC* strains to propose a list of 21 proteins that are very likely mycoloylated in the cell. Importantly, mycoloylation of several of these proteins was directly confirmed on purified native proteins. Indeed, after purification and mass characterization, these proteins unambiguously displayed the characteristic profile of proteins post-translationally modified with the main natural mycolates.

Results

Alkm₁₃ α ₁₀TMM is a valuable probe to label PorAH *in vivo* in a MytC-dependent manner.

In order to compare the properties of the two available bioorthogonal mycolate analogs (Figure 2A), we evaluated the labeling rate of the mycomembrane when bacteria were incubated in the presence of **Alkm₁₃ α ₁₀TMM** or **O-Alk-Acyl-Tre** that was used by Kavunja *et al.*¹⁷. WT *C. glutamicum* was cultured until mid-log phase, and then treated with **Alkm₁₃ α ₁₀TMM** or **O-Alk-Acyl-Tre** for 1 h at 5 and 50 μ M, then reacted with azide functionalized carboxyrhodamine 110 (CR110-Az) using Cu(I)-catalyzed Azide Alkyne Cycloaddition (CuAAC). Analysis by flow cytometry showed that both probes could label *C. glutamicum*. However, labeling with **Alkm₁₃ α ₁₀TMM** appears significantly more efficient, even at a concentration as low as 5 μ M (Figure 2B).

In order to show the direct transfer of the synthetic lipid moiety from the TMM bioorthogonal analog to its acceptors, we sought to analyze known mycoloylated membrane proteins extracted from bacteria after metabolic labeling. WT *C. glutamicum* was cultured either without any probe or in the presence of **Alkm₁₃ α ₁₀TMM** or **O-Alk-Acyl-Tre**. Then porins PorAH were extracted and analyzed using MALDI TOF mass spectrometry as originally described when these proteins were first found to be mycoloylated.⁴ Figure 2D shows the spectra obtained for PorH analysis. Different proteoforms of PorH were found: the non-mycoloylated and mycoloylated forms either with C32:0, C34:1 or C36:2 (Figure 2C and 2D).^{22,23} Consequently, in all three samples, we observe the characteristic mycolate signature with a mass centered around 6687 Da (Figure 2D). The mass spectrum of PorH extracted from bacteria cultured in

the presence of **Alkm₁₃α₁₀TMM** reveals an additional peak corresponding to the mass of PorH modified by the synthetic mycolate (expected mass: 6531 Da, Figure 2C), providing clear evidence of its transfer onto PorH. Conversely, under identical conditions, PorH modified with the acyl chain from **O-Alk-Acyl-Tre** (expected mass: 6290 Da) was not detected (Figure 2D). Similar results were obtained for PorA (Figure S1).

As presented in Figure 2E, we also extracted porins from the $\Delta mytC$ *C. glutamicum* strain grown in the presence of this bioorthogonal TMM. In this context, PorH is not mycoloylated, either by the natural TMM present in the bacteria or by the TMM analog. Similar results were observed for PorA (Figure S1). These results clearly confirm that the functionalization of these membrane proteins by the bioorthogonal chemical reporter **Alkm₁₃α₁₀TMM** *in vivo* is strictly dependent on MytC. Overall, these experiments provide a direct characterization of the lipidation of mycomembrane acceptors by bioorthogonal TMM processed by bacteria.

Identification of cell envelope proteins labeled with **Alkm₁₃α₁₀TMM**

As shown before, **Alkm₁₃α₁₀TMM** is likely metabolized in a manner similar to natural TMM. In particular, its mycolate moiety can be efficiently transferred to membrane proteins in a MytC dependent manner suitable for detection by mass spectrometry. To systematically identify all proteins labeled by **Alkm₁₃α₁₀TMM**, we devised a comprehensive strategy involving 4 steps: i) *in vivo* cell labeling with the TMM analog, ii) clicking biotin to the alkyne-mycolate transferred onto proteins, iii) purification of labeled proteins on a streptavidin column and finally, iv) identification of proteins by mass spectrometry fingerprint analysis.

In a preliminary experiment, the WT strain, its isogenic mutant $\Delta mytC$, and the complemented $\Delta mytC$ (*mytC*) strains, were grown at 30° C to mid-log phase before the addition of **Alkm₁₃α₁₀TMM** and further incubation for 1 h. A negative control without the TMM analog was performed in parallel. Cells were recovered, and cell envelope proteins were extracted according to a previously reported standard procedure.²⁴ Biotin-azide was added to this extract to react with the alkyne group of the transferred mycolate analog. Proteins were analyzed by SDS PAGE and Western blot using streptavidin-conjugated HRP. As shown in Figure S2, several proteins were detected in the WT strain protein extract but significantly fewer were observed in the $\Delta mytC$ strain. This specific labeling was restored in the complemented strain clearly indicating that the mycolate analog has been incorporated into some proteins in a MytC-dependent manner.

Encouraged by these positive results, we designed a metabolic labeling experiment combined with a proteomic study (Study 1) to identify proteins labeled by **Alkm₁₃α₁₀TMM** in the WT strain. For this, three independent cultures of *C. glutamicum* WT strain were grown in the presence or in the absence of **Alkm₁₃α₁₀TMM** as previously described. Cell envelope proteins were extracted, clicked with biotin-azide, and subsequently immobilized on streptavidin magnetic beads. Multiple washing steps, including a mild SDS treatment, were performed to remove non-specific binding. On-bead digestion with trypsin, followed by mass spectrometry analysis, were finally performed to identify the proteins tightly bound to the magnetic beads. Spectral counting, where the number of spectra matched to peptides from a protein was used as a surrogate measure of protein abundance. In total, 104 proteins were identified (Table S1, panel 1). Among these proteins, 61 were identified as enriched at least 2-fold in probe-treated versus

untreated samples with statistical significance ($p < 0.05$) (Figure 3). Notably, PorB whose mycoloylation has been previously described, was included in this list which also contains almost all cell mycoloyltransferases involved in mycomembrane biogenesis. Several hydrolases and cell surface proteins with unknown or not completely characterized function were also detected. Interestingly, a high proportion of proteins displays a fold change (FC) change greater than 100 highlighting the high specificity of the labeling. Despite this, we cannot rule out the possibility that the lipid surrogate may covalently label canonical lipoproteins (some of them are found in our list) or proteins with a high affinity for a hydrophobic chain.

Identification of cell envelope and secreted proteins whose localization is altered in a $\Delta mytC$ strain

To reinforce these results, we decided to compare the first proteomic study (Study 1) with a different approach. We reasoned that protein mycoloylation is expected to anchor a polypeptide chain to a membrane, and therefore, its absence may lead to mis-localization of the corresponding protein. Indeed, it was reported that PorB, PorC and PorH are no longer associated with the cell envelope but are instead secreted into the cell medium when they are not mycoloylated.⁸ Even if this particular behavior may not be general for all mycoloylated proteins, we thought to compare the proteomes of both cell envelope proteins and secreted proteins in a $\Delta mytC$ strain as compared to the WT strain. Cell envelope proteins were extracted as previously described (Study 2), and proteins secreted into the culture medium were precipitated with TCA (Study 3). This experiment was repeated three times from three independent cultures. In all cases, proteins were identified by mass spectrometry analysis. In total, 1329 and 697 proteins (Table S1, panels 2 and 3) were identified respectively in the cell envelope (study 2) and the culture supernatant (Study 3) of either the WT or the $\Delta mytC$ strains. As previously, spectral counting was used as a surrogate measure of protein abundance. Considering a criterion of at least 2 peptides detected per protein, this analysis revealed significant differences (p -value < 0.05) for 138 cell envelope proteins and 54 secreted proteins. Interestingly, several of these proteins exhibit distinct distributions between the two strains as depicted in Figure 4. In the cell envelope of the $\Delta mytC$ strain, 49 proteins were significantly less abundant than in the cell envelope of the WT strain ($FC > 2$). Conversely, in the culture supernatant of the $\Delta mytC$ strain, 37 proteins were significantly more abundant ($FC > 2$) as compared to the supernatant of the WT strain.

Proteins specifically secreted in the $\Delta mytC$ strain, as compared to the WT strain, could be attributed to the release of surface mycolate-anchored proteins. However, the presence of several typical cytosolic proteins (ribosomal proteins and metabolic enzymes) in this extract also suggest that the mutant strain may be slightly more susceptible to lysis than the WT strain, or that the permeability of its envelope has been slightly impaired. This observation raises concerns about the reliability of the list of proteins obtained from study 3 which may contain a high number of false positive candidates. In contrast, the identification of proteins whose abundance decreased significantly in the cell envelope of the $\Delta mytC$ strain seems to be a more robust parameter with less potential false positive results because the absence of a protein in the cell envelope extract cannot be explained by a higher susceptibility of the $\Delta mytC$ strain to lysis. This observation is further supported by the comparison of common proteins found in the different studies which indicated that only 7 proteins are found in common between study 1 and

3 while 22 proteins are in common between study 1 and 2. These 22 candidates represent as much as 36% of proteins labeled with **Alkm₁₃α₁₀TMM** (Table S1, panel 4). Although false positives are still expected due to indirect association with the cell envelope, we believe that crossing the list of proteins labeled with **Alkm₁₃α₁₀TMM** (61 proteins) and the list of proteins exhibiting decreased abundance in the cell envelope of the $\Delta mytC$ strain (49 proteins) may be relevant. The result of this crossing is listed in Table S1, panel 5. Interestingly, none of them is predicted to have a function in the cytosol, and they are all (except PorB) predicted to contain a signal sequence, suggesting they are localized in the cell envelope. Notably, PorB has already been experimentally shown to be exported and localized in the mycomembrane.^{25,26} The 21 remaining proteins may represent a list of high-confidence candidates that are putatively mycoloylated in *C. glutamicum*.

Biological validation of native mycoloylation for MytA, MytB and 4 other cell wall proteins.

To validate our list of 21 putative mycoloylated proteins, we decided to purify most of these proteins directly from the WT strain and determine their mass by MS analysis. We randomly selected 14 of them, and we cloned their respective genes under the control of the *ptac* promoter and a 6xHis-epitope fused to their 3' part to facilitate protein expression and subsequent purification. Upon introduction into *C. glutamicum*, 6 out of 14 proteins showed high production levels upon IPTG induction and were purified from the cell envelope (Figure S3). Interestingly, in some cases (Cgl0524, Cgl0628 and Cgl1822), two very close bands are detected, indicating a possible mass heterogeneity. In the case of MytA, it is noteworthy that the protein may be purified either from the cell envelope or from the culture supernatant without obvious difference in molecular mass. All 6 proteins were then analyzed, in a first approach, by LC-MS to rapidly assess their heterogeneity at the intact level (Figure 5). Since *O*-mycoloylation increases the hydrophobic behavior of proteins, isoforms of different stoichiometries were efficiently separated by reversed-phase chromatography and identified by intact mass measurements. *O*-mycoloylation was then evaluated at the peptide level after digestion to gain more insight into its localization on the peptide sequence (Figure 5, grey background). Figure 5, panel A shows the results obtained for MytA, which eluted in four chromatographically resolved peaks. The first one corresponded to the unmodified form at 34.28 min (marked with an asterisk), the second form eluted at 45.77 min and was assigned as a low abundant singly modified protein, and finally, two more abundant peaks were detected corresponding to the bi- and tri-*O*-mycoloylated forms at 58.97 min and 61.12 min, respectively. The deconvoluted MS spectrum of the most abundant tri-*O*-mycoloylated form shows mass heterogeneities due to the number of combinations between the three major mycolic chains produced by the cell, *i.e.* C32:0, C34:1 and C36:2. After digestion, two modified peptides were identified, an *N*-terminal Gln₁-Lys₆₁ peptide modified twice and another one with a single *O*-mycoloylation on the Glu₂₇₆-Lys₃₈₃ sequence. For the latter large peptide, the high-energy spectrum showed multicharged signals attributed to *y*-type *C*-terminal ions from cleavage after position Ile₂₈₁. These ions were unmodified, suggesting a mycoloylation at position Ser₂₇₈ (Figure S5). In addition, the high-energy spectrum of the di-mycoloylated Gln₁-Lys₆₁ peptide was also rich in *y*-type fragment ions, allowing us to confirm a mycoloylation at position Ser₆, while the second one was at either position Ser₂ or Ser₃. Panel B shows the results

of MytB, which was detected mainly as bi-*O*-mycoloylated at 59.07 min, and singly modified and unmodified as traces at 54.20 and 39.29 min, respectively. For this protein, a large *N*-terminal Ala₁-Lys₁₅₅ peptide bearing the two modifications was detected. The high-energy spectrum of this peptide showed a series of unmodified *N*-terminal b-ions, indicating the absence of modification up to position Met₈₆ (Figure S5). Panel C shows the results of Cgl1822, which was detected mainly as unmodified at 28.05 min and as bi-*O*-mycoloylated at 58.01 min. The single *O*-mycoloylated form was only detected as a trace at 42.76 min. A *C*-terminal peptide on the Ser₂₃₃-His₂₅₄ sequence was detected with the two modification sites but the associated high-energy mass spectrum gave no further information. Panel D shows the results for Cgl0628, which was detected as a double modified form at 59.22 min, while the unmodified and single modified forms eluted as traces at 39.39 min and 43.81 min, respectively. Digestion experiments of Cgl0628 revealed the position of the two modifications at the *N*-terminus between amino acids Gln₁-Lys₄₁. The high-energy mass spectrum allowed the unambiguous position of the two modifications at Ser₁₃ and Ser₂₀ (Figure S5). Panel E shows the results of Cgl0524, which was very close to Cgl1822, *i.e.* with two major forms, one is unmodified at 25.14 min and the other doubly modified at 57.10 min. The single modified form at 36.88 was still detected but was much less abundant. At the peptide level, the two modifications were detected on the *N*-terminal peptide Gln₁-Lys₃₄. The high-energy spectrum was not very informative as only one b-type ion, Gln₁-Gly₂₂, was detected with the two modifications. Finally, the last protein analyzed, Cgl0393, is shown in panel F. Cgl0393 was detected mainly as a single modification at 59.32 min and the unmodified form was also observed at low abundance at 48.43 min. Unfortunately, we were not able to specify the localization of the modification site for this protein.

Discussion

In this study, we used for the first time a mycolic acid-derived bioorthogonal probe to investigate protein mycoloylation, a new lipidation pathway in Mycobacteriales. We showed unambiguously that this probe is incorporated *in vivo* into porin PorAH. These proteins were the first protein reported to be mycoloylated, in a MytC-dependent manner, in *C. glutamicum*⁴. Under these conditions, it is reasonable to hypothesize that this probe is directly recognized, in the cell envelope, by the mycoloyltransferase MytC, that can transfer the mycolate moiety of the probe onto PorAH. These results provide a direct characterization of the mycoloylation of mycomembrane acceptors by TMM-based chemical reporters processed by bacteria.

Using our bioorthogonal TMM, we successfully enriched and purified a relatively limited number of proteins putatively mycoloylated in *C. glutamicum*. This list contains a high proportion of proteins (50 out of 61) for which the enrichment factors are very high: over 100 for 19 of them and over 10 for the other 31. These strong values likely indicate that these proteins have incorporated the bioorthogonal mycolate moiety and thus, do not represent non-specific binding which can occur during purification procedures. Focusing on these 50 proteins, it is noteworthy that very few characterized cytosolic proteins are contaminating our results. This is expected since we selectively extracted cell envelope proteins, nevertheless it demonstrates that our biochemical procedures for protein extraction are robust and reliable. Additionally, our list contains PorB, a porin previously shown to be mycoloylated and therefore validating the proof of concept of our method. Notably, PorB is the only known mycoloylated

porin identified in our study. The absence of the principal other porin of the cell, PorAH (45aa and 57 aa), is likely attributable to a technical limitation of the MS analysis, given that low molecular weight proteins generate only a limited number of peptides following digestion, thereby impeding their identification. This observation also indicates that probably several potential “small” proteins are missed by our approach as also reported by Banahene *et al.* in their study.¹⁸

While convincing, the metabolic labeling approach cannot discriminate between proteins that have directly incorporated the native bioorthogonal mycolate through the catalytic action of MytC and those that have incorporated a possible metabolically modified version of our probe. To overcome this issue, we used a $\Delta mytC$ strain that is viable, although exhibiting a slow growth. We did not use this strain directly for metabolic labeling experiments because its higher susceptibility to lysis would make the comparison with the WT strain quite irrelevant. Instead, we decided to perform a differential analysis of the cell envelope proteome in WT and $\Delta mytC$ strains. This analysis clearly showed that the abundance of several proteins (49) decreased in the mutant strain. This reduction may likely be correlated to the absence of mycoloylation which may impair (directly or indirectly) the localization of cell envelope proteins. Indeed, 36 % of these proteins are also metabolically labeled by our bioorthogonal TMM probe, suggesting they may constitute high-confidence candidates for the mycoloylome. This list is composed of 21 proteins including the major esterases of the cell, the mycoloyltransferases A, B and D, and several other cell envelope proteins with unknown or uncharacterized functions. Protein annotations suggest that at least some of them could be related to peptidoglycan metabolism. A careful observation of the protein sequences of these candidates reveals that they all contain stretches of serine residues located in unfolded regions as predicted by the AlphaFold algorithm, that could therefore correspond to putative mycoloylation sites.

Finally, to validate our approach, we decided to biologically confirm the mycoloylation of most proteins (14 out of 21) of our list, through direct analysis of native proteins. We cloned the corresponding recombinant genes (including a His-tag coding sequence) with the hope that some of them would be well expressed in *C. glutamicum* and subsequently easily purified with their native PTM. This was indeed the case for 6 of them, which were all analyzed by MS. All 6 purified proteins displayed a microheterogeneity at the molecular mass level. A fine MS analysis unambiguously showed that they were all mycoloylated, at least once, by natural mycolate chains of the cell (Table 1). This positive result clearly indicates that our list is reliable and validates the strategy of our studies. This work also definitively identifies and validates biologically, for the first time, 6 new mycoloylated non-porin proteins in their native form in *C. glutamicum*, suggesting that this atypical modification is not restricted to a subfamily of proteins but is more generally widespread among cell envelope proteins. Notably, three mycoloyltransferases are part of the mycoloylome. Mycoloyltransferases are major enzymes of the cell envelope that are essential for the biogenesis of the mycomembrane in all Mycobacteriales species including *M. tuberculosis*.^{27,28} Their mycoloylation raises several questions about the regulation of their activity or localization during cell division, given their potential role in synchronizing mycomembrane and peptidoglycan biogenesis during cell growth. Additionally, among the other mycoloylated proteins of our list, and whose function has not been yet characterized, it is interesting to note that several of them are predicted or reported to localize at the cell surface. This is the case for Cgl0559 and Cgl1391, which have both been described to be cell surface exposed,²⁹ for Cgl2353, which contains a PE-PPE

domain-containing protein involved in cell wall localization,³⁰ or for Cgl1836 and Cgl1822, which contain YPDG domains generally found in bacterial cell surface proteins (genome annotation NCBI HMM accession: NF038186.1).

For all proteins whose mycoloylation was biologically validated, it is interesting to observe that they are all mono- and bi-*O*-mycoloylated or, in the case of MytA, even tri- *O*-mycoloylated. The presence of several modification sites for a single protein has been previously reported^{8,9} for porins, but its significance remains elusive. Here, for the first time, we have determined the proportion of the different native isoforms (Table 1) and showed that multi-mycoloylated forms are generally the most abundant ones. The need for several mycolate chain additions could be related to the necessity of ensuring a stable anchor in the membrane or alternatively to fix different loops of the polypeptide chain together in a precise position. Notably, in the case of MytA, the three potential sites of mycoloylation are very close in the 3D structure of the protein, despite being connected to two very distant sequences of the protein (Figure S6). The presence of several mycoloylated and non-mycoloylated forms of a given protein also support the hypothesis previously raised⁹, that protein mycoloylation may be regulated in order to modulate the protein activity or its localization at specific subcellular sites.⁹

Identifying the mycoloylome is a crucial step toward understanding the function of this new lipidation pathway specific to Mycobacteriales. Here, we definitively proved the native mycoloylation of some cell envelope enzymes indicating that this modification is not restricted to the unique porins of the cell, which are composed of very small polypeptide chains and whose structure is probably very different from the canonical porins of Gram-negative bacteria.^{31,32} Our work suggests that this type of modification may affect several cell envelope proteins (about 21) and may be important for regulating or optimizing their function in the cell. Given that our bioorthogonal TMM has proved highly effective in identifying the mycoloylome of the model bacterium *C. glutamicum*, it would be now very interesting to use this molecular tool to determine the whole mycoloylome of other species, particularly relevant pathogenic species. In *C. glutamicum*, our work also opens up new perspectives at a fundamental level to understand the function of this new lipidation pathway. The identification of several candidates paves the way for further studies of the specific localization of all these proteins and their dedicated export machinery, in order to determine whether they share a common fate related to their *O*-mycoloylation. Indeed, as already suggested, protein mycoloylation could be restricted to proteins localized in the mycomembrane. We propose that the mycoloylation process is linked to a sorting machinery that selectively target the modified proteins to their final destination as soon as they emerge from the inner membrane translocation machinery. Although attractive, this hypothesis lacks supporting data beyond the observation that some porins are released into the culture supernatant when mycoloylation is disrupted.⁹ Here, we demonstrate that all newly identified mycoloylated proteins of this study are predicted to be part of the mycomembrane proteome.^{13,33} Therefore, to date, 100% of the 11 proteins shown to be mycoloylated (i.e. the porins PorAH and PorBC, ProtX and the 6 newly identified proteins in this study) are mycomembrane proteins. This interesting observation supports the hypothesis that all mycoloylated proteins share the same subcellular localization and, if confirmed, opens inspiring perspectives in terms of protein traffic in the cell envelope of Mycobacteriales.

As mentioned before, the team of Swarts published recently a study attempting to identify the mycoloylome of *C. glutamicum* using a bioorthogonal acyl trehalose probe, the *O*-**Alk-Acyl-**

Tre previously described.^{16,34} They proposed a list of about 27 new proteins that could be mycoloylated with high confidence (reported in the study 3 of Banahene *et al.*¹⁸) based on the characterization by MS of the corresponding peptides labeled with their probe. Interestingly, among the 21 candidates of our study, 11 are also present in their list, while 10 of them are new candidates not labeled by their probe. These ten proteins include Cgl0393, for which mycoloylation was definitively validated by our MS analysis on native protein.

Surprisingly, for some proteins common to both Swarts' study and the present study, different mycoloylation sites were found, which is probably explained by the different method used to characterize these sites. In Swarts' study, they directly identified peptides labeled with the alkyne fatty acid after enrichment with a biotin containing a cleavable linker. In our study, following proteomic studies to obtain the protein lists, we purified several candidate proteins in their native mycoloylated form and the mycoloylation sites were determined by direct analysis of native proteins. For example, the mycoloyltransferase MytA is a protein found in both studies. Banahene *et al.* identified 4 labeled sites for MytA at positions S103, S132, S517 and the catalytic S226 from their proteomic analysis. In our study, we purified the native mycoloylated MytA from *C. glutamicum* and determined that it is tri-mycoloylated at positions S2 or S3, S6 and S278. This discrepancy between the results of the two studies is intriguing but, may reflect a non-specific labeling of the *O-Alk-Acyl-Tre* probe, which could be hydrolyzed prior to incorporation in proteins, or may react at low levels with several accessible serines or threonines due to the use of a high concentration probe over an extended period of time (400 μ M for 16 h instead of 50 μ M for 1 h in our study). This latter hypothesis is further supported by the fact that the putative mycoloylation of S517 of MytA, described in Swarts's study, occurs in the PG-binding domain of the enzyme and therefore its biological significance is difficult to understand. Conversely, as shown in Figure S6, serines S2-3, 6 and 278 are all clustered in the 3D structure of MytA, making their mycoloylation consistent with the anchoring of this specific part of the enzyme in the mycomembrane. Similarly, Banahene *et al.* identified MytB as mycoloylated at five positions, including three localized after amino acid 155, while in our study, MytB was only found to be mycoloylated at two different serines between residues 1 and 155. Similar discrepancies were also observed for proteins Cgl0628 and Cgl0524. As our site determination is performed on a purified protein expressed and post-translationally modified in a native context, we are confident that it is likely to better reflect the physiological situation. Although the modification sites that we have determined for Mycoloyltransferases and other proteins differ from those proposed by Banahene *et al.*,¹⁸ we observed as they do, that according to AlphaFold 3D structures, they are localized in non-ordered regions of the proteins.

In conclusion, our study and the recently published study of Banahene *et al.*, both point to the potential existence of a large number of putative mycoloylated proteins in the cell envelope of *C. glutamicum*. A high portion of proteins are found in both studies. These studies demonstrate the advantage of using trehalose-derived probes to detect mycoloylated proteins, which will be very useful to evaluate the occurrence of this unusual PTM in other species and, in particular, in pathogenic species of interest. Although attractive and efficient, these probes only bring indirect information and may lead to the identification of a certain number of false positive candidates. Thus, a confirmation of candidates mycoloylation is needed by direct analysis of native proteins. This was partially done in our study and we definitively confirm the mycoloylation of 6 candidates by analyzing proteins purified from their native context, 5 of

which were found to be labeled by the probes used in both studies. These identifications include the mycolyltransferases MytA and MytB, for which we show that they are tri- and bi-mycoloylated in their native state. Although MytA and MytB were already proposed to be mycoloylated based on in silico analysis,⁹ and in the recent proteomic study of Banahene *et al.*,¹⁸ this is the first definitive evidence of their modification that now needs to be taken into account in future studies to better understand their function in the biogenesis of the mycomembrane. Here, the mycoloylation sites of MytA and MytB (and also of 3 other proteins) were also determined. Surprisingly, they clearly differ from the *O-Alk-Acyl-Tre* labeled sites in the same proteins. This may suggest, as mentioned by the authors, that non-enzymatic labeling cannot be excluded. Finally, both studies clearly pave the way for the elucidation of the role and importance of this new lipidation pathway in Mycobacteriales. In particular, the probes and methods described in both papers will certainly be very useful to study mycoloylation regulation and its impact on protein trafficking through the cell envelope.

Supporting information

Additional experimental details and materials and methods.

List of strains, oligonucleotides and plasmids.

Additional figures include the purification of six mycoloylated candidates and their 3D models constructed by Alphafold and some complementary LC-MS spectra.

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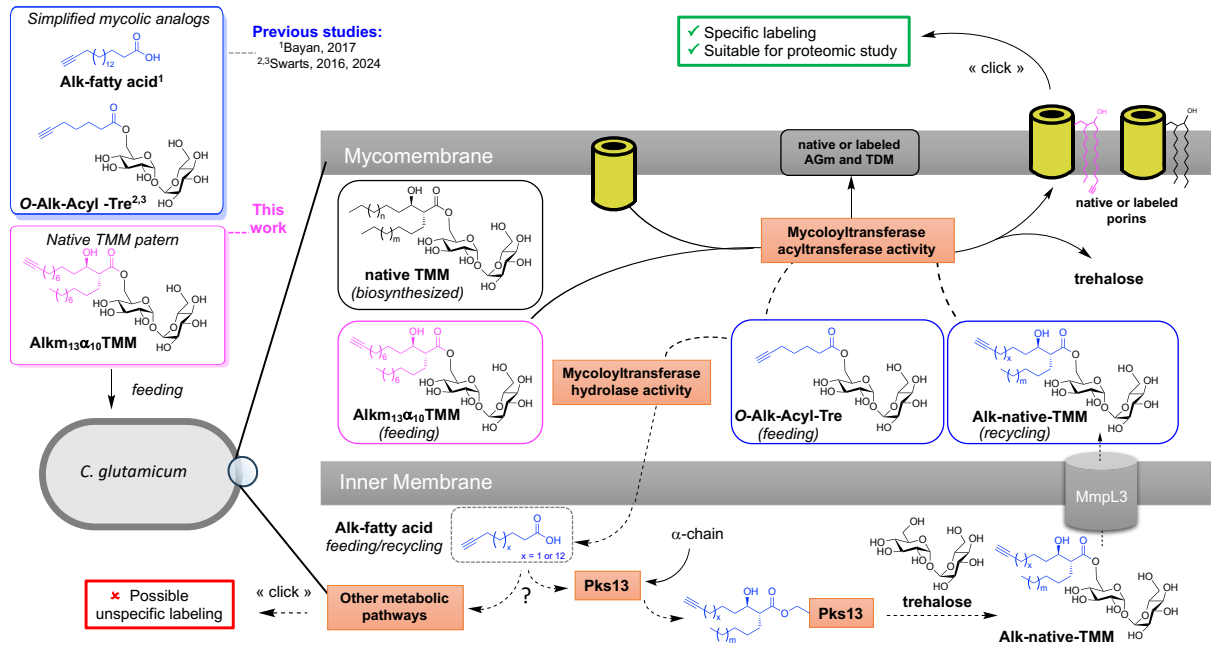
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Tables and Figures

Table 1: List of 14 tested candidates found in both study 1 and 2: Status for gene cloning, protein purification and MS analysis.

Protein Name	Protein description	Recombinant plasmid	Purification	MS analysis	
				Non mycoloylated form (0)	Mycoloylated forms (+1, +2 or +3)
MytA_{his}	Mycoloyltransferase	pCGL-2875	Yes	18% (0)	4% (+1) 28% (+2) 50% (+3)
MytB_{his}	Mycoloyltransferase	pXMJ-2879	Yes	20% (0)	13% (+1) 67% (+2)
Cgl1822_{his}	Long Rib domain-containing protein	pXMJ-1822	Yes	40% (0)	12% (+1) 32% (+2)
Cgl0628_{his}	Putative bacterial lipocalin	pXMJ-0628	Yes	7% (0)	11% (+1) 73% (+2)
Cgl0524_{his}	Putative secreted hydrolase	pXMJ-0524	Yes	35% (0)	18% (+1) 47% (+2)
Cgl0393_{his}	HtaA like (iron acquisition)	pXMJ-0393	Yes	11% (0)	89 % (+1)
Cgl0559_{his}	Probable L,D transpeptidase	pXMJ-0559	Very low expression	nd	nd
Cgl1093_{his}	Putative peptidase	pXMJ-1093	Low expression	nd	nd
Cgl1391_{his}	Putative secreted hydrolase	pXMJ-1391	Very low expression	nd	nd
Cgl1696_{his}	Secreted protein	pXMJ-1696	Very low expression	nd	nd
Cgl2353_{his}	PE-PPE domain-containing protein	pXMJ-2353	Low expression	nd	nd
Cgl2614_{his}	Glycosyl hydrolase	pXMJ-2614	Very low expression	nd	nd
Cgl2889_{his}	Putative N-acetylmuramoyl-L-alanine amidase	pXMJ-2889	Low expression	nd	nd
Cgl1836_{his}	Long Rib domain-containing protein	Not succesful	nd	nd	nd
PorB	Porin	Already shown to have two sites of mycoloylation ⁹ % of mycoloylation not determined			

A



B

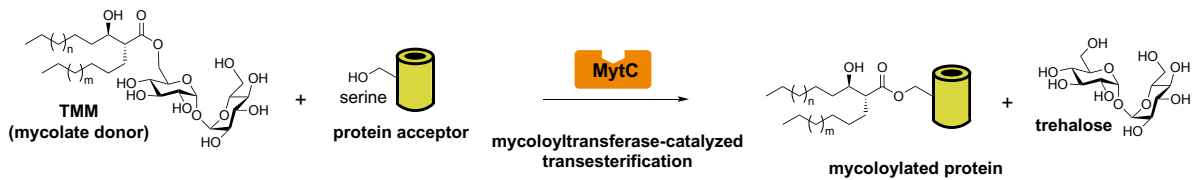


Figure 1. Protein mycoloylation. (A) Putative model for incorporation of TMM analogs in mycoloylated proteins in previous studies (blue) and in this study (pink). (B) MytC catalyzes the transfer of a mycolate moiety from TMM to proteins through a transesterification reaction.

AGm: Mycoloylated arabinogalactan. TDM: Trehalose dimycolate. TMM: Trehalose monomycolate.

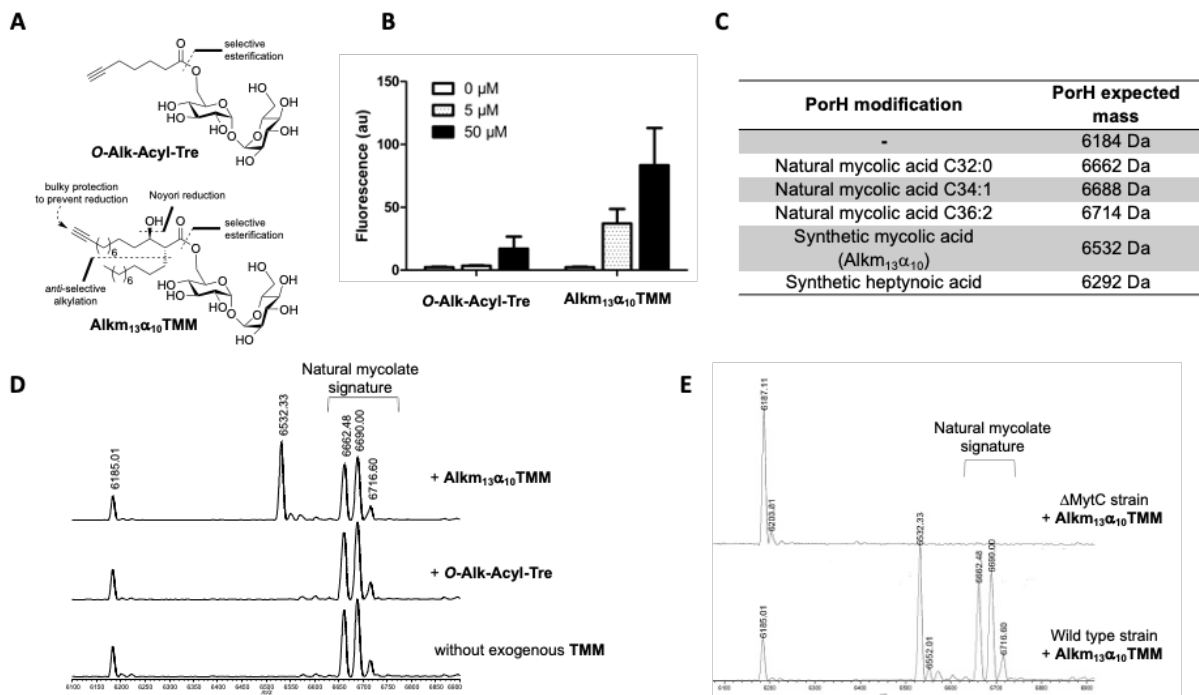


Figure 2. Comparative incorporation of bioorthogonal TMM analogs *O*-Alk-Acyl-Tre and Alkm₁₃α₁₀TMM in PorH. (A) Synthetic strategy to obtain the TMM analogs with an acyl chain and a mycolate pattern featuring the stereochemistry of the natural compounds. (B) *C. glutamicum* incubated with 5 or 50 μM of TMM analogs (or untreated) for 1 h, then reacted with CR110-Az and analyzed by flow cytometry (data are represented as a mean of at least 2 independent experiments +/- SD). (C) PorH expected mass after native or synthetic mycolate functionalization. (D) MALDI-TOF mass spectra obtained for the membrane protein PorH extracted from WT bacteria cultured for 2 hours in the presence of the TMM analogs. (E) MALDI-TOF mass spectra obtained for the membrane protein PorH extracted from WT and $\Delta mytC$ *C. glutamicum* strains cultured for 2 hours in the presence of the Alkm₁₃α₁₀TMM analog.

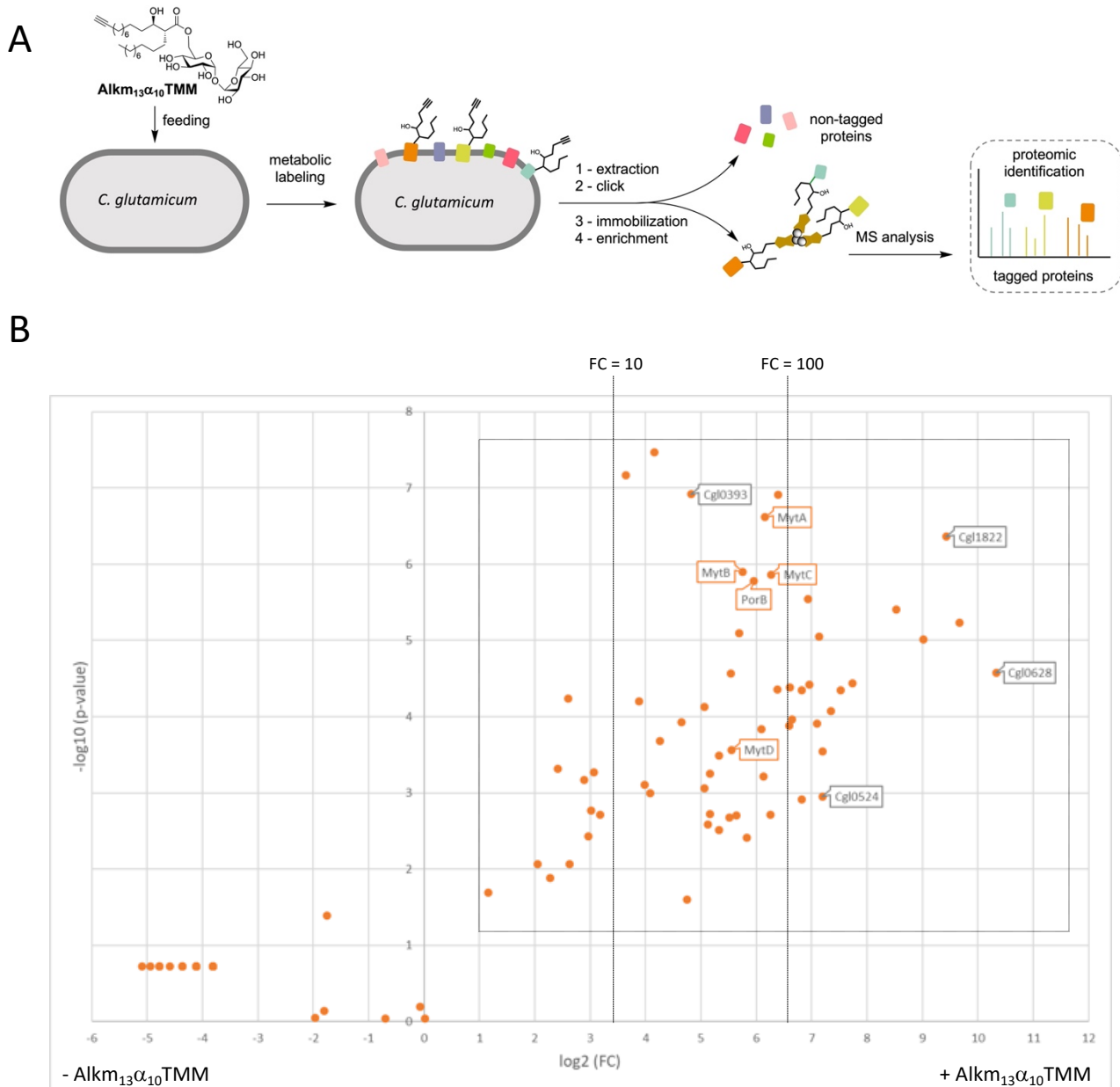


Figure 3. Identification of $Alkm_{13}\alpha_{10}$ TMM labeled cell envelope proteins of *C. glutamicum*. The WT *C. glutamicum* strain was grown in presence (+) or absence (-) of the alkyne-based TMM reporter $Alkm_{13}\alpha_{10}$ TMM. Cell envelope proteins were submitted to click chemistry with azido biotin and immobilized on streptavidin beads before further identification by MS analysis. (A) Schematic representation of the experiment. (B) Volcano plot of cell envelope proteins identified in the absence (- $Alkm_{13}\alpha_{10}$ -TMM) or presence (+ $Alkm_{13}\alpha_{10}$ -TMM) of $Alkm_{13}\alpha_{10}$ -TMM. The x axis represents the fold change and the y axis the p value calculated from the t-test. The dotted box includes the 61 proteins significantly enriched (FC threshold of 2, p-value <0.05). The position of the known proteins (i.e. mycoloyltransferases MytA, B, C and D and porin PorB) are indicated as well as the mycoloylated proteins purified in this study. The values are the results of 3 different experiments.

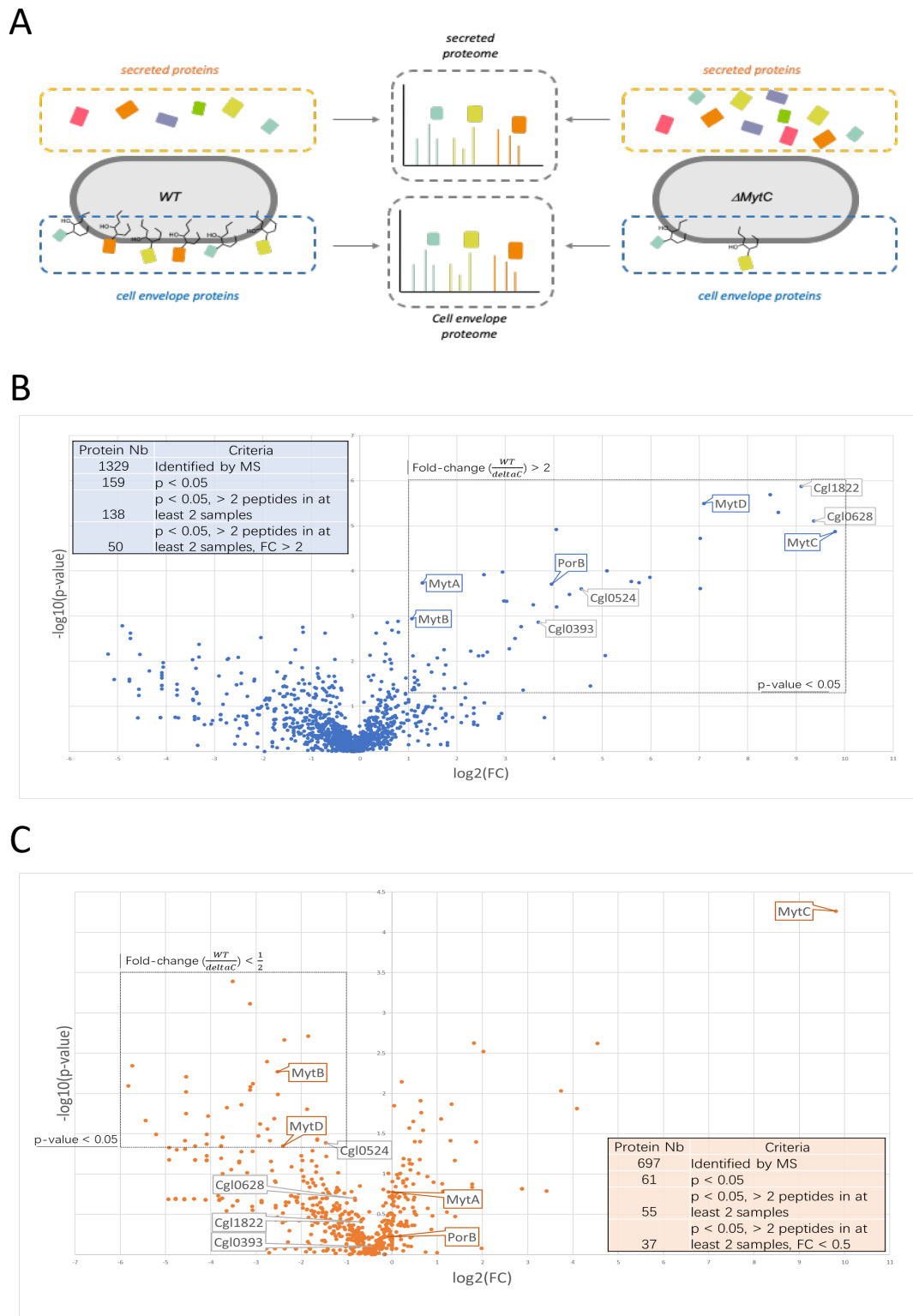


Figure 4. Differential analysis of cell envelope and secreted proteomes of *C. glutamicum* WT and $\Delta mytC$ strains. (A) Schematic representation of the experiment. (B) Volcano plot of cell envelope proteins and (C) secreted proteins. The x axis represents the fold change and the y axis the p value calculated from the t-test. The dotted box includes the proteins significantly enriched (FC threshold of 2, p-value <0.05). The position of the known proteins (i.e. mycoloyltransferases MytA, B, C and D and porin PorB) are indicated as well as the mycoloylated proteins purified in this study. The values are the results of 3 different experiments.

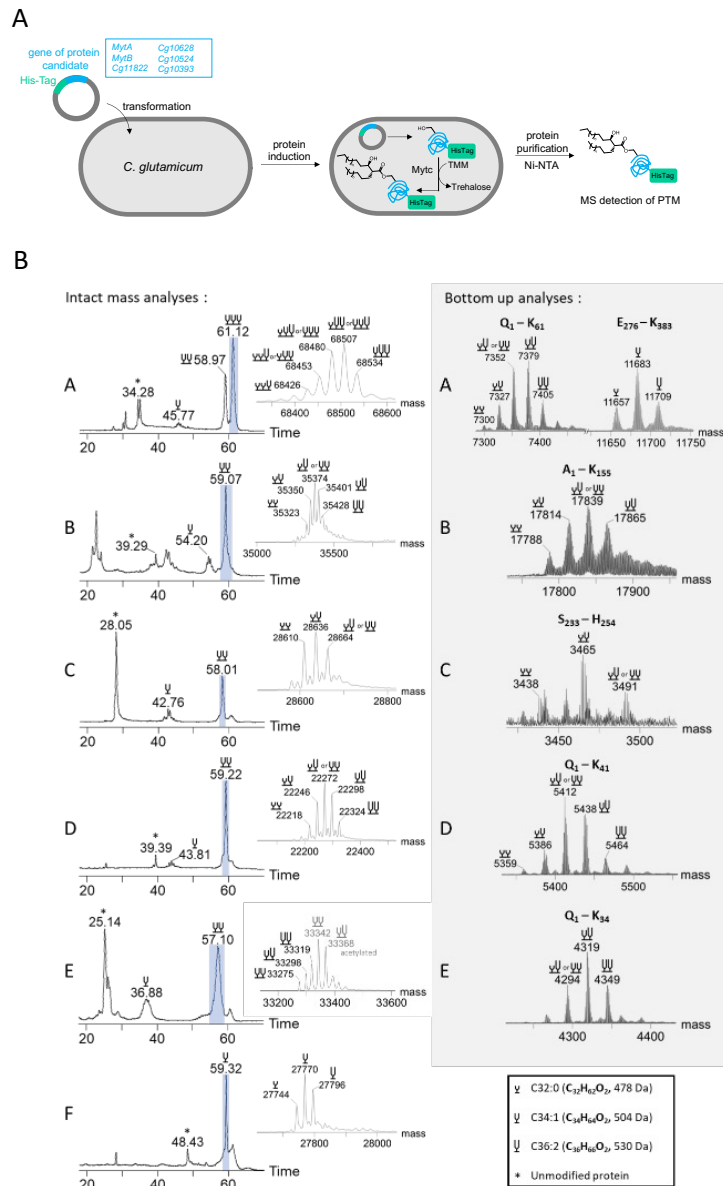


Figure 5. MS analysis of purified protein candidates. (A) Schematic representation of the experiment. (B) Chromatograms of intact mass analyses of the following proteins: MytA (A), MytB (B), Cgl1822 (C), Cgl0628 (D), Cgl0524 (E), Cgl0393 (F) and deconvoluted mass spectra of chromatographic peaks highlighted in blue. Deconvoluted mass spectra of mycoloylated peptides detected by bottom-up analyses are presented in grey background.