Article

High Throughput Expression Screening of Arabinofuranosyltransferases from Mycobacteria

José Rodrigues ^{1,†} , Vanessa T. Almeida ^{1,†} , Ana L. Rosário ¹, Yong Zi Tan ^{2,3,‡} , Brian Kloss ⁴ ,
Filippo Mancia ^{2,*} and Margarida Archer ^{1,*}

¹ Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa (ITQB NOVA), 2780-157 Oeiras, Portugal; jose.rodrigues@itqb.unl.pt (J.R.); vcalmeida@itqb.unl.pt (V.T.A.); anarosar@gmail.com (A.L.R.)

² Department of Physiology and Cellular Biophysics, Columbia University Irving Medical Center, New York, NY 10032, USA; yongzi.tan@sickkids.ca

³ National Resource for Automated Molecular Microscopy, Simons Electron Microscopy Center, New York Structural Biology Center, New York, NY 10027, USA

⁴ Center on Membrane Protein Production and Analysis, New York Structural Biology Center, New York, NY 10027, USA; bkloss@nysbc.org

* Correspondence: fm123@cumc.columbia.edu (F.M.); archer@itqb.unl.pt (M.A.)

† These authors contributed equally to this publication.

‡ Present address: Molecular Medicine Program, The Hospital for Sick Children, Toronto, ON M5G 1X8, Canada

Abstract: Studies on membrane proteins can help to develop new drug targets and treatments for a variety of diseases. However, membrane proteins continue to be among the most challenging targets in structural biology. This uphill endeavor can be even harder for membrane proteins from *Mycobacterium* species, which are notoriously difficult to express in heterologous systems. Arabinofuranosyltransferases are involved in mycobacterial cell wall synthesis and thus potential targets for antituberculosis drugs. A set of 96 mycobacterial genes coding for Arabinofuranosyltransferases was selected, of which 17 were successfully expressed in *E. coli* and purified by metal-affinity chromatography. We herein present an efficient high-throughput strategy to screen in microplates a large number of targets from *Mycobacteria* and select the best conditions for large-scale protein production to pursue functional and structural studies. This methodology can be applied to other targets, is cost and time effective and can be implemented in common laboratories.

Keywords: membrane proteins; overexpression in *E. coli*; protein purification; high-throughput protocol; Arabinofuranosyltransferases; *Mycobacteria*

Citation: Rodrigues, J.; Almeida, V.T.; Rosário, A.L.; Tan, Y.Z.; Kloss, B.; Mancia, F.; Archer, M. High Throughput Expression Screening of Arabinofuranosyltransferases from Mycobacteria. *Processes* **2021**, *9*, 629. <https://doi.org/10.3390/pr9040629>

Academic Editor: Florian M. Wurm

Received: 4 March 2021

Accepted: 26 March 2021

Published: 2 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Membrane proteins represent 20 to 30% of open-reading frames of all genomes sequenced [1,2] and perform essential functions in cells, such as transportation, signal transduction and energy production [3]. They also play important roles in several diseases and, as a result, are attractive therapeutic targets, estimated to represent more than 30% of all marketed drugs [4–6]. However, biochemical and structural characterization of membrane proteins have several bottlenecks, namely toxicity by excess of mRNA levels of the target protein [7], toxicity caused by heterologous expression [8], membrane lipid composition [9,10], detergent extraction and solubility [11,12], which ultimately results in low amounts of membrane protein produced.

Many efforts have been devoted on the development of protocols to efficiently produce membrane proteins in *Escherichia coli*. An elegant approach to accelerate this process involves the fusion of green fluorescent protein (GFP) to monitor the expression and purification processes [13,14]. A commonly used strategy consists of varying different parameters simultaneously, such as expression vectors with different tags and promoters,

host strains, homologues or solubilizing detergents [15,16]. High-throughput (HTP) protein production platforms have also been developed by Structural Genomics Consortia, such as JCSG [17], Northeast SGC [18] or the New York Consortium on Membrane Protein Structure (NYCOMPS, New York, NY, USA) [19,20].

Based on the HTP strategy to express prokaryotic membrane proteins previously developed at NYCOMPS, we have devised an accessible protocol to screen mycobacterial membrane proteins, which are difficult to express in heterologous systems [21]. An initial set of 96 target genes was assembled from the genomic sequences coding for Arabinofuranosyltransferases (AraTs) from 14 different *Mycobacterium* species. Even though *Mycobacterium* (*Mb*) proteins display low yield of production in *E. coli*, it remains one of preferred hosts for heterologous expression of *Mb* proteins and is compatible with HTP strategies.

The AraT targets are integral membrane enzymes that play a pivotal role in the synthesis of arabinan, an important component of *Mycobacterium* cell envelope [22,23]. AraTs transfer arabinose in the furanose conformation (Araf) from a single donor decaprenylphosphoryl- β -D-arabinose (DPA) to the arabinan domain of arabinogalactan (AG) of mycobacterial cell wall [24]. The cell envelope is crucial for growth and virulence of pathogenic *Mycobacterium* [25] and is a major contributor to resistance against common antibiotics [26]. AraT family comprises 7 sub-families: Embs (EmbA, EmbB and EmbC), which are inhibited by the first-line antitubercular drug Ethambutol (EMB), and Afts (AftA, AftB, AftC and AftD), which are potential novel therapeutic targets against tuberculosis.

In this study, we present a simple and cost-effective methodology to screen AraTs from different *Mycobacterium* species and chose the most promising targets to proceed biochemical and structural studies.

2. Materials and Methods

2.1. High-Throughput Cloning of Arabinofuranosyltransferase Genes

96 genes of AftA, AftB, AftC, AftD, EmbA, EmbB and EmbC were identified from 14 *Mycobacterium* genomes using a bioinformatics approach [19] (Table A1). Ligation Independent Cloning (LIC) was used to clone all selected targets, using the protocols previously described by Bruni and Kloss [27], with the following modifications: (1) target sequences were amplified by Polymerase Chain Reaction (PCR) using oligonucleotides that were compatible with LIC-adapted expression vectors (pNYCOMPS-N23 and pNYCOMPS-C23) containing appropriate overhangs with the start codon ATG, instead of the endogenous GTG start codon for some of the target genes; (2) XL10 *E. coli* was used for cloning purposes. All liquid handling was performed by hand, using multichannel pipettes. The resulting constructs were used to transform *E. coli* C41, C43 and BL21 (DE3) pLysS strains. Transformants were selected on LB [10 g/L Tryptone, 5 g/L Yeast Extract and 10 g/L NaCl] plates containing appropriate antibiotics: 100 μ g/mL of ampicillin for *E. coli* C41 and C43 cells, 100 μ g/mL of ampicillin and 34 μ g/mL of chloramphenicol for *E. coli* BL21 (DE3) pLysS cells.

2.2. High-Throughput Expression Screening and Purification

Pre-cultures were grown overnight at 37 °C, 200 rpm in 600 μ L of LB medium supplemented with the appropriate antibiotics. For this purpose, 96-well plates (VWR) were used. The overnight cultures were used to inoculate 2.5 mL of 2xYT medium [16 g/L Tryptone, 10 g/L Yeast Extract and 5 g/L NaCl], supplemented with antibiotics, in 24-well plates (UNIPLATE Collection and Analysis Microplate), at an initial optical density (OD₆₀₀) of 0.03–0.08. Cells were grown at 37 °C, 200 rpm, until the cultures reached OD₆₀₀ of 0.4–1.2 (2 to 2.5 h) (see Table A2), then cultures were cooled to 22 °C and gene expression was induced overnight (~16 h) with 0.25 mM isopropyl- β -D-thiogalactoside (IPTG). Cells were harvested the next day by centrifugation at 3200 $\times$ g, for 20 min at 4 °C. OD₆₀₀ measurements were done in TECAN Spark 10 M. Cell pellets were re-suspended in 300 μ L of lysis buffer (BugBuster supplemented with 0.1 mg/mL Lysozyme, 3 U/mL Benzonase, 2 mM MgCl₂ and 0.5 mM PMSF) through 10 min of vigorous shaking, using a plate shaker, at

room temperature. Detergent n-Dodecyl β -D-maltoside (DDM) was added to the lysate at 1% (*w/v*) final concentration and the plate containing the samples was incubated for 2 h at 4 °C, with gentle agitation. For the separation of the insoluble cell debris, plates were centrifuged at $3200\times g$ for 20 min, 4 °C and 250 μ L of the supernatants were transferred to a 96-well filter plate containing a bed of 50 μ L Ni-NTA agarose resin (HisPur™ Ni-NTA Spin plate (Thermo Scientific™, Waltham, MA, USA)), previously washed with double distilled water and equilibrated with buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 10 mM Imidazole, 0.1% DDM). Imidazole at 10 mM final concentration was added to each sample, to avoid unspecific binding of contaminants to the Ni-NTA resin. Plates were incubated for 15 min in a plate-shaker at 4 °C. The plates were then centrifuged, the flowthrough fractions collected and reloaded to the resin bed, repeating the 15 min incubation with the Ni-NTA resin bed. After the second incubation step, plates were centrifuged to remove unbound proteins. The resin was washed three times with 250 μ L washing buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 60 mM Imidazole and 0.1% DDM) and finally eluted with 250 μ L of elution buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 300 mM Imidazole and 0.05% DDM). Eluted samples were run on SDS-PAGE: 10% polyacrylamide gels were used for targets with molecular weights between 73–149 kDa (AftD, EmbA, EmbB and EmbC) and 12% polyacrylamide gels for 47–75 kDa targets (AftA, AftB and AftC).

2.3. Large Scale Protein Expression and Purification

50 mL cultures of each target in *E. coli* C41 cells were grown overnight in 250 mL flasks at 37 °C, 200 rpm, in LB medium supplemented with 100 μ g/mL of ampicillin. The overnight cultures were used to inoculate 4×500 mL of 2xYT medium, supplemented with 100 μ g/mL of ampicillin, in 2.5 L Thomson's Ultra Yield™ Flasks (Oceanside, CA, USA), at an initial OD₆₀₀ around 0.05. Cells were grown at 37 °C, 200 rpm, until the cultures reached OD₆₀₀ of 0.8 (2 to 2.5 h), then cultures were cooled to 22 °C and gene expression was induced overnight (~16 h) with 0.25 mM IPTG. Cells were harvested in the next day by centrifugation at $4472\times g$, for 15 min at 4 °C. OD₆₀₀ measurements were done in Ultrospec 10 Cell Density Meter. Cell pellets were re-suspended and homogenized in lysis buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 20 mM MgSO₄, 1 mM TCEP), protease inhibitor EDTA-free cocktail (Thermo Scientific™, Waltham, USA; Catalog number: 88266) and 25 U/mL of Benzonase nuclease (Santa Cruz Biotechnology, Dallas, USA; Catalog number: sc-391121). Cell suspension was passed twice at 15,000 psi on a cell disruptor (Constant Systems Ltd., Daventry, UK). Membranes were collected by ultracentrifugation at $197,215\times g$, for 30 min at 4 °C. Membranes were manually homogenized using a Wheaton® glass homogenizer (DWK Life Sciences Limited, Stoke-on-Trent, UK) in 20 mM HEPES pH 7.5 and 200 mM NaCl, to which DDM was added to a final concentration of 1% (*w/v*). Membranes were solubilized for 2 h, with gentle agitation, at 4 °C. Soluble membrane fraction was collected by ultracentrifugation at $203,756\times g$ for 30 min, at 4 °C. The supernatants were collected and incubated with 2 mL of equilibrated Ni-NTA agarose resin for 1.5 h at 4 °C, with gentle agitation. Imidazole was added to each sample to a final concentration of 10 mM, to prevent unspecific binding of contaminants. After incubation, the sample was loaded into a column for elution by gravity flow. The resin bed was washed with 10 column volumes (CV) with washing buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 0.1% DDM, 60 mM Imidazole), and the proteins were eluted with 4 CV of elution buffer (20 mM HEPES pH 7.5, 200 mM NaCl, 0.05% DDM and 300 mM Imidazole). Eluted samples were concentrated and injected into a Superdex 200 column (Cytiva Europe GmbH, Freiburg, Germany) to assess protein dispersity. The collected fractions were run on SDS-PAGE: 10% polyacrylamide for 73–149 kDa targets (AftD, EmbA, EmbB and EmbC) and 12% polyacrylamide for those around 47–75 kDa (AftA, AftB and AftC).

3. Results

3.1. Genomic Expansion and High-Throughput Cloning of Arabinofuranosyltransferases

A set of 96 target genes was assembled from the genomic sequences coding for seven AraTs from *Mtb* (AftA, AftB, AftC, AftD, EmbA, EmbB and EmbC). To each “seed” sequence, a “cluster” of homolog sequences, from 14 different *Mycobacterium* genomes was expanded, coding for proteins likely to have similar structure as the seed protein [28] (Table A1). LIC was performed as described previously by Bruni and Kloss [27]. Briefly, all sequences were amplified by PCR, using genomic DNA available from ATCC® (Manassas, VA, USA) [<https://www.lgcstandards-atcc.org> (accessed on 26 March 2021)] and primer pairs compatible with LIC-adapted expression vectors (pNYCOMPS-N23 and pNYCOMPS-C23) that contained decahistidine affinity tag and Tobacco Etch Virus (TEV) protease cleavage site (ENLYFQS). 56 targets were successfully cloned into pNYCOMPS-N23 and 40 targets into pNYCOMPS-C23. Previous screening experiments had shown no expression for all constructs in pNYCOMPS-C23 vector (data not shown), therefore only the clones in pNYCOMPS-N23 were used for the HTP expression screening approach.

3.2. Small Scale High-Throughput Expression of Arabinofuranosyltransferases

All 56 positive clones in pNYCOMPS-N23 were transformed into C41, C43 and BL21 (DE3) pLysS *E. coli* strains. 24 deep-well plates were used to grow the positive clones simultaneously. Growth conditions, 2xYT rich medium, 0.25 mM IPTG and overnight post-induction at 22 °C, were established based on the results obtained in previous experiments. This allowed a fast and reliable comparison between different *E. coli* strains, also leaving room for optimization after target selection.

Cell harvesting by centrifugation and lysis were performed in 24 deep-well plates, maintaining a HTP downstream processing of the samples. Extraction of membrane proteins was achieved by adding detergent directly to each well, after cell lysis, incubating the plate at low temperature. The 24 deep-well plate is centrifuged again to clear the solubilized lysate from the cell debris. The solubilized lysate was transferred to a HisPur™ Ni-NTA Spin 96 well plate (Thermo Scientific™, Waltham, MA, USA) for affinity chromatography purification. In this step, the use of adjustable multichannel pipettes to transfer solutions from 24-well plate to 96-well plate was important for sake of speed and reproducibility/reliability, however, standard multichannel pipettes can also be used although not in an optimal manner. After a single Ni-NTA purification step, the amount of eluted target protein was too low to be detected by SDS-PAGE. Since the sample solution slowly flows from the filter plate by gravity during the incubation period, a second passage was deemed necessary to increase the contact time between the sample and Ni-NTA resin, after which the eluted AraTs could be visualized on the gel. The full pipeline is summarized in Figure 1.

In total, 17 out of 96 distinct proteins were produced and purified, resulting in 18% success rate of protein production (Table 1). All three different *E. coli* host strains were able to produce target proteins: 16 in C41, 6 in C43 and 8 in BL21 (DE3) pLysS (Table A2). AftB and EmbC proteins were not detected in any *E. coli* strain using this HTP method, suggesting that different, perhaps more tailored conditions may be needed to successfully produce these proteins. We found that a single His-tag purification step was not very efficient, considering that persistent contaminants from the host cell are present across all targets (Figure 2; see Figures A1 and A2). Moreover, the production yields for the target proteins herein studied were low in all *E. coli* host strains. Nevertheless, we were still able to successfully identify bands in the SDS-PAGE that could correspond to our target proteins, based on their predicted molecular weight (MW) and considering the gel shifting for membrane proteins in denaturing protein gels [29]. Due to this anomalous migration pattern, bands related to membrane proteins in SDS-PAGE most often appear ~20–30% below their predicted MW.

Figure 1. Schematic representation of the high-throughput screening for membrane protein production and purification.

Table 1. Summary of high-throughput screening of membrane protein production.

	Number	Success Rate (%)
Targets	96	-
Positive Clones (pNYCOMPS-N23)	56	58
¹ Positive Clones (pNYCOMPS-C23)	40	42
Proteins purified (total)	17	18
<i>E. coli</i> C41::pNYCOMPS-N23	16	17
<i>E. coli</i> C43::pNYCOMPS-N23	6	6
<i>E. coli</i> BL21 (DE3) pLysS::pNYCOMPS-N23	8	8

¹ Expression of these constructs was not determined in this study.

Figure 2. Protein expression results from the HTP screening of 56 AraTs from *Mycobacteria* overexpressed in *E. coli* C41 cells. **A**—AftA proteins: 1—A1; 2—A8; 3—C2; 4—C9; 5—F1; 6—F3; 7—F7; 8—F8. **B**—AftB proteins: 1—A2; 2—A9; 3—C7; 4—D9; 5—D10; 6—D11; 7—E2; 8—E5. **C**—AftC proteins: 1—A3; 2—A10; 3—C11; 4—C12; 5—D1; 6—D2; 7—E6; 8—E7; 9—E8; 10—E9; 11—F9. **D**—AftD and EmbA proteins: 1—A4; 2—D4; 3—H11; 4—A5; 5—A11; 6—B4; 7—B8; 8—C5; 9—G5; 10—G11; 11—H1; 12—H2. **E**—EmbB proteins: 1—A6; 2—A12; 3—B3; 4—B7; 5—C4; 6—G3; 7—G6; 8—G9; 9—H7; 10—H9. **F**—EmbC proteins: 1—B1; 2—C3; 3—G4; 4—G7; 5—G10; 6—H4; 7—H5. Asterisks (*) indicate protein bands corresponding to the predicted MW of the corresponding target.

3.3. Validation of HTP Target Selection by Large-Scale Protein Production

Based on SDS-PAGE analysis, we selected one target from each cluster for large-scale production: AftA and AftC from *M. neoaurum*, AftD from *M. abscessus* 1948 F5/8, EmbA from *M. marinum* M. and EmbB from *M. vanbaalenii* PYR-1, all produced in *E. coli* C41. Growth conditions were similar to the ones used in the HTP screening, although cell lysis and membrane extraction steps were modified according to the cell mass. Most importantly, the incubation time of solubilized membranes with Ni-NTA resin was increased to improve protein binding and purification yield. All chosen targets were successfully produced in large scale, thus validating the selection made from the HTP screening. Size exclusion chromatography (SEC) was performed after affinity chromatography to further purify the protein and as tool for preliminary biophysical characterization of each protein (Figure 3). Although all targets show some aggregation in the presence of DDM, it was still possible to identify heterogeneous protein populations in most target samples. Upon SDS-PAGE analysis of the SEC elution fractions, we observed that the dominant protein bands correspond to the desired targets, however there were still contaminants present. AftA (Figure 3A), EmbA (Figure 3D) and EmbB (Figure 3E) showed the least amount of contaminant proteins.

Figure 3. Size-exclusion chromatography (SEC) elution profiles of large-scale expression experiments of AraTs and respective SDS-PAGE. Vertical arrows indicate void volume; asterisks (*) and (**) indicate different populations observed in the SEC elution profiles. Ni: Ni-NTA elution samples; HTP: Small-scale HTP results; horizontal arrows indicate the bands corresponding to the target protein of interest. (A) AftA from *M. neoaurum*, (B) AftC from *M. neoaurum*, (C) AftD from *M. abscessus* 1948 F5/8, (D) EmbA from *M. marinum* M. and (E) EmbB from *M. vanbaalenii* PYR-1.

For EmbB, a second SEC step was performed (Figure 4), running each population separately. We could observe that the high molecular weight EmbB population behaves as a stable monodisperse population (Figure 4A,B), while the low molecular weight EmbB population splits into the same two populations observed in the first SEC run (Figure 3E), suggesting that EmbB monomers are prone to form an equilibrium with stable EmbB dimers.

Figure 4. Size-exclusion chromatography elution profiles of EmbB from *M. vanbaalenii* PYR-1. (A) SEC elution profiles of EmbB: after Ni-NTA (full black line), peak (*) after 1st SEC (red dashed lines), peak (*) after 1st SEC (spotted blue line). (B) Normalized SEC elution profiles of EmbB: after Ni-NTA (full black line), peak (*) after 1st SEC (red dashed lines), peak (*) after 1st SEC (spotted blue line). (C) SDS-PAGE analysis of peak (*) and (*) SEC. Vertical arrows indicate void volume. Colored asterisks correspond to the peaks observed in each SEC run, respective to different EmbB populations. Arrow indicates the corresponding band of EmbB (115 kDa).

4. Discussion

The need to screen the expression of a large number of membrane protein targets, as well as the selection of optimal conditions for production and purification of desired targets, led to the development of several HTP strategies. The strategy used in this study is not novel and was intended to setup a protocol to search for the best candidates to pursue functional and structural studies on AraTs from *Mycobacteria*. Embs are targets of ethambutol, whereas Afts are potential targets to develop new drugs to treat tuberculosis. Nevertheless, the protocol herein described can also be applied to evaluate the expression and purification of other membrane proteins.

The methodology involved the selection of 13–14 orthologue genes of each AraT sub-family (EmbA–C, AftA–D) from a variety of host genomes, gene expression with vectors harboring a poly-histidine affinity tag at either N- or C-terminus, transformation into three different *E. coli* strains, membrane extraction and protein solubilization by DDM detergent, and purification by Ni-NTA chromatography. By using this simple combinatorial approach, we were able to clone 56 genes at pNYCOMPS-N23 and 40 at pNYCOMPS-C23, and produce 17 proteins out of 96 chosen targets, corresponding to a success rate of 18%. Such rate is not surprising, considering that membrane proteins are often difficult to express and purify [7,30]. Heterologous expression of mycobacterial proteins in *E. coli* has previously been reported not to exceed 40% [30–32].

No expression of AraTs cloned into pNYCOMPS-C23 was observed (data not shown). It is well known that type and location of the fused affinity tag has a significant effect

at all stages of protein production [33], however it is not possible to know a priori the impact caused by tag addition. This unpredictability is somehow the foundation of HTP approaches—try as many conditions as is reasonably possible and assess what works to proceed with further studies.

Concerning the host organism, *M. smegmatis* could be a viable alternative for the heterologous expression of *Mb* proteins [31], yet we considered it not appropriate for a HTP approach due to its slower growth rate compared to *E. coli*, and mostly due to its waxy surface [34], which promotes clumping, film formation and cell adhesion to surfaces, especially plastic, preventing an optimal use of 96- or 24-well plates for cell growth. Instead, we used *E. coli* C41 and C43 suited for overexpression of toxic and membrane proteins [35], and BL21 (DE3) pLysS for controlled expression [36]. The different expression levels observed among *E. coli* hosts suggests that the type of strain plays a pivotal role in the number of well-expressed AraTs, as also reported for other target proteins [37]. Indeed, regulation of T7 RNA polymerase expression either by mutations in its promoter (C41 and C43 Walker strains) or by its natural inhibitor T7 lysozyme (T7Lys, pLysS strain) can significantly influence membrane protein overexpression yields [38].

Large scale production (from 2 L of culture) of 5 targets, chosen based on the results of small-scale experiments, yielded purified proteins in milligram amounts, although differences are observed on the intensity of their respective bands (SDS-PAGE) in small and large-scale experiments. The shorter incubation time with the Ni-NTA agarose in the small-scale screening (2×15 min vs. 1.5 h) could account for this discrepancy. Moreover, the aeration rate related with the size and shape of the growth vessel (24-well plate vs. 2.5 L flasks) may also affect the overexpression levels. In addition, switching to a cobalt spin plate, instead of nickel, may increase binding specificity of the target protein and thus further improve the results for the small-scale screening. We expect similar results will be obtained on the scale-up production of other targets that showed expression on the HTP screening. Fusions with GFP tag could be advantageous to monitor the various steps of protein production by measuring fluorescence, a very sensitive detection method [13]. However, this methodology is not suitable for membrane proteins with periplasmic C-terminus [13,20,39], which is the case for most of the AraTs herein studied, so it was not considered.

Detergents are required to extract and purify target proteins and their choice is a key parameter on the entire process. We chose to use DDM since it is a mild detergent and one of the most commonly used for this purpose [20,27,33,40]. The aggregation detected in the large-scale production experiments suggests further detergent screens may be needed to select the best detergent formula for each individual target. We were able to separate two different populations of EmbB from *M. vanbaalenii* PYR-1, likely constituted by monomer and dimer, respectively. Despite the aggregation, all targets showed soluble populations in the SEC elution profiles, which represents a good starting point for optimization towards structural studies. Interestingly, 3D structures of both oligomeric states have been already characterized by single particle cryo-electron microscopy (cryo-EM) for EmbB from *M. smegmatis* [41,42]. Noteworthy, different detergents or solubilizing agents may be needed for structural studies. The cryo-EM structures of several AraTs, namely EmbA-EmbB complex, *M. tuberculosis* EmbB [43], *M. smegmatis* EmbB [42] and AftD [44], have been recently characterized and different solubilization agents were used, namely glyco-diosgenin (GDN) detergent, amphipols or nanodiscs. Interestingly, the structure of EmbC solubilized in DDM has been determined by X-ray crystallography [43].

On one hand, the production of AftB and EmbC targets was not achieved using the HTP workflow with “standard” conditions. Therefore, other parameters must be explored, such as growth media, temperature, incubation time and type of detergent, host strains or expression vectors, which will likely lead to better success rates. On the other hand, AftA and AftC from *M. neoaurum* were expressed and are attractive targets for drug development [45–47] and structural elucidation, since their structures are not yet known.

The presented protocol stands as a simplified approach based in previous HTP strategies developed at NYCOMPS [19,20] and by others [15], to identify the best candidates for further biochemical and structural studies in a fast and affordable manner for most laboratories. Most importantly, this methodology delivers similar results to conventional medium throughput approaches and, by reducing the variables throughout, it allows target optimization for large-scale protein production.

Author Contributions: Conceptualization, M.A., F.M., B.K. and J.R.; methodology, J.R., V.T.A., A.L.R. and Y.Z.T.; data analysis, all authors; original draft preparation, J.R. and V.T.A.; review and editing, all; supervision and funding acquisition, M.A. and F.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Fundação para a Ciência e Tecnologia (FCT), Lisbon, Portugal; grants (PTDC/BIA-BQM/30421/2017 and PTDC/BIA-BQM/4056/2020 to M.A. and PD/BD/128261/2016 to J.R.), European Union's Horizon 2020 research and innovation programme under grant agreements: No. 857203 (Twinning), No. 823780 (MSCA-RISE) and No. 731005, Instruct-ULTRA, a project to further develop the services of Instruct-ERIC (M.A, J.R. and V.T.A.). This work was also supported by National Institutes of Health (NIH), Bethesda, MD, USA; grants GM132120 (to F.M.) and GM116799 (to Wayne A. Hendrickson).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Alessio Bortoluzzi, Catarina Paiva and Sandra Santos for advice and help with equipment for high throughput protein expression.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A.

Table A1. Protein targets used in the high-throughput screening and expression results for each *E. coli* strain tested.

ID	GenBank ID	Organism	Predicted Protein	C41	C43	BL21 (DE3) pLysS
A1	SIU02450.1	<i>Mycobacterium bovis</i> AF2122/97	AftA	—	—	—
A8	EUA63955.1	<i>Mycobacterium abscessus</i> 1948	AftA	—	—	+
C2	ABP43658.1	<i>Mycobacterium gilvum</i> PYR-GCK	AftA	+	—	—
C9	AFC41461.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftA	—	—	—
F1	ABM16394.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftA	+	—	+
F3	AGP61782.1	<i>Mycobacterium yongonense</i> 05-1390	AftA	—	—	—
F7	CDQ43571.1	<i>Mycobacterium neoaurum</i>	AftA	+	—	+
F8	AAS02550.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	AftA	—	—	—
A2	SIU02464.1	<i>Mycobacterium bovis</i> AF2122/97	AftB	—	—	—
A9	EUA63936.1	<i>Mycobacterium abscessus</i> 1948	AftB	—	—	—
C7	CCP46634.1	<i>Mycobacterium tuberculosis</i> H37Rv	AftB	—	—	—
D9	AGP61763.1	<i>Mycobacterium yongonense</i> 05-1390	AftB	—	—	—
D10	AFC41442.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftB	—	—	—
D11	ABM16411.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftB	—	—	—
E2	AIR19061.1 *	<i>Mycobacterium kansasii</i> 662	AftB	—	—	—
E5	CDQ43590.1	<i>Mycobacterium neoaurum</i>	AftB	—	—	—

Table A1. Cont.

ID	GenBank ID	Organism	Predicted Protein	C41	C43	BL21 (DE3) pLysS
A3	AMC65006.1	<i>Mycobacterium bovis</i> AF2122/97	AftC	+	—	+
A10	EUA61591.1	<i>Mycobacterium abscessus</i> 1948	AftC	+	—	—
C11	AFC44620.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftC	+	+	—
C12	AGZ53302.1	<i>Mycobacterium kansasii</i> ATCC 12478	AftC	+	+	—
D1	ABP46386.1	<i>Mycobacterium gilvum</i> PYR-GCK	AftC	+	+	+
D2	CDQ43952.1	<i>Mycobacterium neoaurum</i>	AftC	+	+	+
E6	EH50241.1	<i>Mycobacterium rhodesiae</i> JS60	AftC	—	—	—
E7	AFM16967.1	<i>Mycobacterium chubuense</i> NBB4	AftC	—	—	—
E8	AAS05110.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	AftC	+	+	—
E9	AGP64972.1	<i>Mycobacterium yongonense</i> 05-1390	AftC	+	+	—
F9	ABM13300.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftC	—	—	—
A4	CAB5247947.1	<i>Mycobacterium bovis</i> AF2122/97	AftD	—	—	—
D4	AGZ51741.1	<i>Mycobacterium kansasii</i> ATCC 12478	AftD	—	—	—
H11	EUA63217.1	<i>Mycobacterium abscessus</i> 1948 F5/8	AftD	+	—	+
A5	SIU02452.1	<i>Mycobacterium bovis</i> AF2122/97	EmbA	—	—	—
A11	EUA63951.1	<i>Mycobacterium abscessus</i> 1948	EmbA	+	—	—
B4	AAC45280.1	<i>Mycobacterium tuberculosis</i> H37Rv	EmbA	—	—	—
B8	ACC43760.1	<i>Mycobacterium marinum</i> M	EmbA	+	—	—
C5	ABP43656.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbA	—	—	—
G5	AAS02546.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbA	—	—	—
G11	CDQ43576.1	<i>Mycobacterium neoaurum</i>	EmbA	—	—	—
H1	ABM16396.1	<i>Mycobacterium vanbaalenii</i> PYR-1	EmbA	—	—	—
H2	AGZ51276.1	<i>Mycobacterium kansasii</i> ATCC 12478	EmbA	—	—	—
A6	SIU02453.1	<i>Mycobacterium bovis</i> AF2122/97	EmbB	—	—	—
A12	EUA63949.1	<i>Mycobacterium abscessus</i> 1948	EmbB	+	—	—
B3	AAC45281.1	<i>Mycobacterium tuberculosis</i> H37Rv	EmbB	—	—	—
B7	ACC43761.1	<i>Mycobacterium marinum</i> M	EmbB	—	—	—
C4	ABP43655.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbB	—	—	—
G3	AFC41455.1	<i>Mycobacterium intracellulare</i> ATCC 13950	EmbB	—	—	—
G6	AAS02545.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbB	—	—	—
G9	AGP61776.1	<i>Mycobacterium yongonense</i> 05-1390	EmbB	—	—	—
H7	KEP38884.1	<i>Mycobacterium kansasii</i>	EmbB	—	—	—
H9	ABM16397.1	<i>Mycobacterium vanbaalenii</i> PYR-1	EmbB	+	—	+
B1	EUA63954.1	<i>Mycobacterium abscessus</i> 1948	EmbC	—	—	—
C3	ABP43657.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbC	—	—	—
G4	AFC41460.1	<i>Mycobacterium intracellulare</i> ATCC 13950	EmbC	—	—	—
G7	AAS02549.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbC	—	—	—
G10	AGP61781.1	<i>Mycobacterium yongonense</i> 05-1390	EmbC	—	—	—
H4	CDQ43572.1	<i>Mycobacterium neoaurum</i>	EmbC	—	—	—
H5	AGZ51274.1	<i>Mycobacterium kansasii</i> ATCC 12478	EmbC	—	—	—

* Record removed.

Table A2. List of genes coding for AraTs from several *Mycobacterium* species selected for cloning and expression screening.

ID	GenBank ID	Organism	Predicted Protein	% Identity (<i>M. tuberculosis</i>)	Predicted Molecular Weight (kDa)	Predicted Transmembrane Helixes
A1	SIU02450.1	<i>Mycobacterium bovis</i> AF2122/97	AftA	100	70	13
A2	SIU02464.1	<i>Mycobacterium bovis</i> AF2122/97	AftB	99	69	9
A3	AMC65006.1	<i>Mycobacterium bovis</i> AF2122/97	AftC	99	49	8
A4	CAB5247947.1	<i>Mycobacterium bovis</i> AF2122/97	AftD	99	146	9
A5	SIU02452.1	<i>Mycobacterium bovis</i> AF2122/97	EmbA	99	116	13
A6	SIU02453.1	<i>Mycobacterium bovis</i> AF2122/97	EmbB	99	118	13
A7	SIU02451.1	<i>Mycobacterium bovis</i> AF2122/97	EmbC	99	118	13
A8	EUA63955.1	<i>Mycobacterium abscessus</i> 1948	AftA	65	68	13
A9	EUA63936.1	<i>Mycobacterium abscessus</i> 1948	AftB	67	71	10
A10	EUA61591.1	<i>Mycobacterium abscessus</i> 1948	AftC	64	47	8
A11	EUA63951.1	<i>Mycobacterium abscessus</i> 1948	EmbA	65	114	12
A12	EUA63949.1	<i>Mycobacterium abscessus</i> 1948	EmbB	68	73	8
B1	EUA63954.1	<i>Mycobacterium abscessus</i> 1948	EmbC	68	117	11
B2	AAC45279.1	<i>Mycobacterium tuberculosis</i> H37Rv	EmbC	100	117	13
B3	AAC45281.1	<i>Mycobacterium tuberculosis</i> H37Rv	EmbB	100	118	12
B4	AAC45280.1	<i>Mycobacterium tuberculosis</i> H37Rv	EmbA	100	116	13
B5	CCP42964.1	<i>Mycobacterium tuberculosis</i> H37Rv	AftD	100	146	9
B6	ACC43759.1	<i>Mycobacterium marinum</i> M	EmbC	86	117	14
B7	ACC43761.1	<i>Mycobacterium marinum</i> M	EmbB	89	116	12
B8	ACC43760.1	<i>Mycobacterium marinum</i> M	EmbA	87	118	13
B9	AFM19671.1	<i>Mycobacterium chubuense</i> NBB4	EmbB	72	115	13
B10	AFM19669.1	<i>Mycobacterium chubuense</i> NBB4	EmbC	75	116	12
B11	AFM19670.1	<i>Mycobacterium chubuense</i> NBB4	EmbA	69	115	13
B12	AFM19668.1	<i>Mycobacterium chubuense</i> NBB4	AftA	67	71	10
C1	ACC43758.1	<i>Mycobacterium marinum</i> M	AftA	83	70	13
C2	ABP43658.1	<i>Mycobacterium gilvum</i> PYR-GCK	AftA	68	67	13
C3	ABP43657.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbC	74	115	14
C4	ABP43655.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbB	70	115	13
C5	ABP43656.1	<i>Mycobacterium gilvum</i> PYR-GCK	EmbA	69	114	12
C6	AIU11367.1	<i>Mycobacterium smegmatis</i> str. MC2 155	AftA	68	67	12
C7	CCP46634.1	<i>Mycobacterium tuberculosis</i> H37Rv	AftB	100	69	9
C8	AAS02532.1	<i>Mycobacterium avium</i> subsp. Paratuberculosis	AftB	82	70	9
C9	AFC41461.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftA	77	68	13
C10	ABK72123.1	<i>Mycobacterium smegmatis</i> str. MC2 155	AftC	70	49	8
C11	AFC44620.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftC	84	50	8
C12	AGZ53302.1	<i>Mycobacterium kansasii</i> ATCC 12478	AftC	89	49	8

Table A2. Cont.

ID	GenBank ID	Organism	Predicted Protein	% Identity (<i>M. tuberculosis</i>)	Predicted Molecular Weight (kDa)	Predicted Transmembrane Helices
D1	ABP46386.1	<i>Mycobacterium gilvum</i> PYR-GCK	AftC	70	48	9
D2	CDQ43952.1	<i>Mycobacterium neoaurum</i>	AftC	70	48	8
D3	ABK71542.1	<i>Mycobacterium smegmatis</i> str. MC2 155	AftD	71	148	12
D4	AGZ51741.1	<i>Mycobacterium kansasii</i> ATCC 12478	AftD	82	148	13
D5	ACC38960.1	<i>Mycobacterium marinum</i>	AftD	80	146	7
D6	AFM15049.1	<i>Mycobacterium chubuense</i> NBB4	AftD	70	145	12
D7	ADT97050.1	<i>Mycobacterium gilvum</i> Spyr1	AftD	70	148	13
D8	ABP43645.1	<i>Mycobacterium gilvum</i> PYR-GCK	AftB	71	70	9
D9	AGP61763.1	<i>Mycobacterium yongonense</i> 05-1390	AftB	82	72	10
D10	AFC41442.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftB	82	72	10
D11	ABM16411.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftB	70	72	9
D12	EHB54870.1	<i>Mycobacterium rhodesiae</i> JS60	AftB	68	73	10
E1	CDM79377.1	<i>Mycobacterium marinum</i> E11	AftB	85	73	10
E2	AIR19061.1 *	<i>Mycobacterium kansasii</i> 662	AftB	86	73	10
E3	ABK75671.1	<i>Mycobacterium smegmatis</i> str. MC2 155	AftB	73	70	9
E4	AFM19681.1	<i>Mycobacterium chubuense</i> NBB4	AftB	68	72	11
E5	CDQ43590.1	<i>Mycobacterium neoaurum</i>	AftB	67	67	11
E6	EHB50241.1	<i>Mycobacterium rhodesiae</i> JS60	AftC	71	49	7
E7	AFM16967.1	<i>Mycobacterium chubuense</i> NBB4	AftC	71	49	9
E8	AAS05110.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	AftC	83	49	8
E9	AGP64972.1	<i>Mycobacterium yongonense</i> 05-1390	AftC	83	50	8
E10	ACC40492.1	<i>Mycobacterium marinum</i>	AftC	87	50	8
E11	AAS06236.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	AftD	79	145	9
E12	EHB55421.1	<i>Mycobacterium rhodesiae</i> JS60	AftD	71	149	13
F1	ABM16394.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftA	67	68	13
F2	CDQ42439.1	<i>Mycobacterium neoaurum</i> F5/8	AftD	67	148	13
F3	AGP61782.1	<i>Mycobacterium yongonense</i> 05-1390	AftA	77	68	13
F4	EHB54850.1	<i>Mycobacterium rhodesiae</i> JS60	AftA	71	66	13
F5	AGZ51273.1	<i>Mycobacterium kansasii</i> ATCC 12478	AftA	84	70	13
F6	AGP66385.1	<i>Mycobacterium yongonense</i> 05-1390	AftD	80	146	13
F7	CDQ43571.1	<i>Mycobacterium neoaurum</i>	AftA	67	67	11
F8	AAS02550.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	AftA	79	75	11
F9	ABM13300.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftC	69	48	9

Table A2. Cont.

ID	GenBank ID	Organism	Predicted Protein	% Identity (<i>M. tuberculosis</i>)	Predicted Molecular Weight (kDa)	Predicted Transmembrane Helices
F10	ABM11102.1	<i>Mycobacterium vanbaalenii</i> PYR-1	AftD	70	147	10
F11	AFP42646.1	<i>Mycobacterium smegmatis</i> str. MC2 155	EmbA	69	117	13
F12	ABK72840.1	<i>Mycobacterium smegmatis</i> str. MC2 155	EmbB	69	117	13
G1	ABK72375.1	<i>Mycobacterium smegmatis</i> str. MC2 155	EmbC	75	115	10
G2	AFC41456.1	<i>Mycobacterium intracellulare</i> ATCC 13950	EmbA	83	115	14
G3	AFC41455.1	<i>Mycobacterium intracellulare</i> ATCC 13950	EmbB	85	115	12
G4	AFC41460.1	<i>Mycobacterium intracellulare</i> ATCC 13950	EmbC	85	114	13
G5	AAS02546.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbA	83	117	14
G6	AAS02545.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbB	84	115	12
G7	AAS02549.1	<i>Mycobacterium avium</i> subsp. <i>paratuberculosis</i> K-10	EmbC	85	117	13
G8	AGP61777.1	<i>Mycobacterium yongonense</i> 05-1390	EmbA	83	116	14
G9	AGP61776.1	<i>Mycobacterium yongonense</i> 05-1390	EmbB	85	115	12
G10	AGP61781.1	<i>Mycobacterium yongonense</i> 05-1390	EmbC	85	114	13
G11	CDQ43576.1	<i>Mycobacterium neoaurum</i>	EmbA	69	116	13
G12	EHB54852.1	<i>Mycobacterium rhodesiae</i> JS60	EmbA	72	116	13
H1	ABM16396.1	<i>Mycobacterium vanbaalenii</i> PYR-1	EmbA	70	116	13
H2	AGZ51276.1	<i>Mycobacterium kansasii</i> ATCC 12478	EmbA	88	115	13
H3	AEV72559.1	<i>Mycobacterium rhodesiae</i> NBB3	EmbC	76	114	14
H4	CDQ43572.1	<i>Mycobacterium neoaurum</i>	EmbC	72	115	13
H5	AGZ51274.1	<i>Mycobacterium kansasii</i> ATCC 12478	EmbC	88	117	14
H6	ABM16395.1	<i>Mycobacterium vanbaalenii</i> PYR-1	EmbC	73	116	10
H7	KEP38884.1	<i>Mycobacterium kansasii</i>	EmbB	90	117	11
H8	AEV72557.1	<i>Mycobacterium rhodesiae</i> NBB3	EmbB	76	114	13
H9	ABM16397.1	<i>Mycobacterium vanbaalenii</i> PYR-1	EmbB	70	115	13
H10	AHC23140.2	<i>Mycobacterium neoaurum</i> VKM Ac-1815D	EmbB	69	115	13
H11	EUA63217.1	<i>Mycobacterium abscessus</i> 1948 F5/8	AftD	62	149	12
H12	AFC46048.1	<i>Mycobacterium intracellulare</i> ATCC 13950	AftD	79	146	13

* Record removed.

Table A3. Optical Density at 600 nm (OD₆₀₀) of small-scale cultures. In-plate target coordinates.

AftA	A1	A8	C2	C9	F1	F3	F7	F8			
AftB	A2	A9	C7	D9	D10	D11	E2	E5			
AftC	A3	A10	C11	C12	D1	D2	E6	E7	E8	E9	F9
AftD	A4	D4	H11								
EmbA	A5	A11	B4	B8	C5	G5	G11	H1	H2		
EmbB	A6	A12	B3	B7	C4	G3	G6	G9	H7	H9	
EmbC	B1	C3	G4	G7	G10	H4	H5				

Table A4. OD₆₀₀ upon induction of expression. Color scheme: green color—OD₆₀₀ interval (0.5 – 1.0), red color—OD₆₀₀ below 0.5 or above 1.0. The black contoured squares show the values obtained for the targets selected for large scale.

		OD of Induction									
BL21 (DE3) pLysS	AftA	0.92	0.99	1.04	0.85	1.03	1.05	0.80	1.10		
	AftB	0.82	0.87	1.00	0.75	0.78	0.82	0.58	0.65		
	AftC	1.02	0.64	0.72	0.80	1.05	0.84	0.88	1.07	0.86	0.75 1.23
	AftD	0.93	1.00	0.61							
	EmbA	0.87	0.72	0.79	0.74	0.93	0.93	1.16	1.11	0.89	
	EmbB	1.06	0.91	0.89	0.88	0.97	0.82	0.56	0.90	0.30	0.70
	EmbC	1.03	0.47	1.12	0.96	1.06	0.46	0.40			
C41	AftA	1.19	1.00	0.77	0.57	0.68	0.63	0.76	0.60		
	AftB	0.43	0.46	0.78	0.75	0.81	0.79	0.72	0.60		
	AftC	1.01	0.55	0.87	0.77	1.14	0.84	0.85	1.06	0.66	0.59 0.86
	AftD	0.55	0.75	0.62							
	EmbA	0.51	0.72	0.64	0.74	0.51	0.52	0.62	0.53	0.58	
	EmbB	0.58	0.56	0.37	0.67	0.40	0.43	0.50	0.53	0.74	0.68
	EmbC	0.76	0.97	0.70	0.63	0.81	0.63	0.40			
C43	AftA	0.73	0.80	0.88	0.71	0.84	0.81	0.92	0.81		
	AftB	0.81	0.75	0.52	0.83	0.78	0.85	0.79	0.76		
	AftC	0.79	0.76	0.75	0.72	0.82	1.04	0.83	0.87	0.80	0.78 0.83
	AftD	0.66	0.52	0.74							
	EmbA	0.71	0.65	0.62	0.70	0.67	0.61	0.57	0.60	0.56	
	EmbB	0.74	0.66	0.68	0.70	0.48	0.67	0.68	0.71	0.60	0.82
	EmbC	0.88	0.78	0.72	0.61	0.48	0.63	0.65			

Table A5. Variation of OD₆₀₀ between the induction time and cell harvesting. A graduated scale of three colors was applied to these values, indicating average values in white, the lower values in red and higher values in green. The black contoured squares show the values obtained for the targets selected for large scale.

		OD Variation									
BL21 (DE3) pLysS	AftA	9.36	6.43	8.44	7.87	3.82	4.13	1.47	8.71		
	AftB	7.94	8.33	8.26	6.76	5.51	7.11	7.80	5.70		
	AftC	8.94	6.73	7.39	1.55	7.17	6.97	6.96	6.82	7.20	7.44 8.25
	AftD	5.15	6.75	5.44							
	EmbA	8.54	6.51	7.85	8.08	7.78	8.30	7.71	7.60	7.58	
	EmbB	6.92	8.33	5.19	5.35	8.20	7.70	7.18	8.37	5.23	1.58
	EmbC	6.76	7.65	6.41	7.38	7.13	8.14	7.94			
C41	AftA	9.14	8.19	6.40	9.16	8.97	9.07	7.70	8.41		
	AftB	7.43	9.65	10.67	14.87	7.48	9.07	6.12	6.45		
	AftC	9.15	8.89	10.06	9.60	8.45	10.17	7.93	7.38	9.27	9.06 7.52
	AftD	5.62	7.52	5.03							
	EmbA	7.09	6.39	6.57	5.75	8.32	9.60	6.29	3.40	6.45	
	EmbB	6.67	7.67	4.15	5.01	5.28	5.41	4.00	5.22	7.18	6.98
	EmbC	6.56	8.90	7.96	8.69	9.38	9.45	7.72			
C43	AftA	6.97	7.06	6.96	6.82	6.66	6.76	7.70	7.25		
	AftB	7.31	6.85	12.09	8.64	7.66	7.27	7.22	7.09		
	AftC	7.24	9.82	7.95	11.34	7.62	12.73	7.83	8.02	8.80	7.64 7.38
	AftD	6.61	10.16	7.21							
	EmbA	6.70	10.83	7.38	7.06	7.56	7.82	6.82	7.30	6.60	
	EmbB	7.06	8.08	8.86	7.99	7.43	7.32	7.43	7.62	6.90	8.50
	EmbC	7.21	7.12	6.87	6.89	6.68	7.46	7.01

Figure A1. Protein expression results from the HTP screening of 56 AraTs from Mycobacteria, overexpressed in *E. coli* C43 cells. **A**—AftA proteins: 1—A1; 2—A8; 3—C2; 4—C9; 5—F1; 6—F3; 7—F7; 8—F8. **B**—AftB proteins: 1—A2; 2—A9; 3—C7; 4—D9; 5—D10; 6—D11; 7—E2; 8—E5. **C**—AftC proteins: 1—A3; 2—A10; 3—C11; 4—C12; 5—D1; 6—D2; 7—E6; 8—E7; 9—E8; 10—E9; 11—F9. **D**—AftD and EmbA proteins: 1—A4; 2—D4; 3—H11; 4—A5; 5—A11; 6—B4; 7—B8; 8—C5; 9—G5; 10—G11; 11—H1; 12—H2. **E**—EmbB proteins: 1—A6; 2—A12; 3—B3; 4—B7; 5—C4; 6—G3; 7—G6; 8—G9; 9—H7; 10—H9. **F**—EmbC proteins: 1—B1; 2—C3; 3—G4; 4—G7; 5—G10; 6—H4; 7—H5. Asterisks (*) indicate protein bands corresponding to the predicted MW of the corresponding target.

Figure A2. Protein expression results from the HTP screening of 56 AraTs from Mycobacteria, overexpressed in *E. coli* BL21 (DE3) pLysS cells. **A**—AftA proteins: 1—A1; 2—A8; 3—C2; 4—C9; 5—F1; 6—F3; 7—F7; 8—F8. **B**—AftB proteins: 1—A2; 2—A9; 3—C7; 4—D9; 5—D10; 6—D11; 7—E2; 8—E5. **C**—AftC proteins: 1—A3; 2—A10; 3—C11; 4—C12; 5—D1; 6—D2; 7—E6; 8—E7; 9—E8; 10—E9; 11—F9. **D**—AftD and EmbA proteins: 1—A4; 2—D4; 3—H11; 4—A5; 5—A11; 6—B4; 7—B8; 8—C5; 9—G5; 10—G11; 11—H1; 12—H2. **E**—EmbB proteins: 1—A6; 2—A12; 3—B3; 4—B7; 5—C4; 6—G3; 7—G6; 8—G9; 9—H7; 10—H9. **F**—EmbC proteins: 1—B1; 2—C3; 3—G4; 4—G7; 5—G10; 6—H4; 7—H5. Asterisks (*) indicate protein bands corresponding to the predicted MW of the corresponding target.

References

- Wallin, E.; Von Heijne, G. Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms. *Protein Sci.* **1998**, *7*, 1029–1038. [\[CrossRef\]](#) [\[PubMed\]](#)
- Krogh, A.; Larsson, B.; von Heijne, G.; Sonnhammer, E.L. Predicting transmembrane protein topology with a hidden markov model: Application to complete genomes. *J. Mol. Biol.* **2001**, *305*, 567–580. [\[CrossRef\]](#) [\[PubMed\]](#)
- Von Heijne, G. The membrane protein universe: What's out there and why bother? *J. Intern. Med.* **2007**, *261*, 543–557. [\[CrossRef\]](#)
- Yasi, E.A.; Kruyer, N.S.; Peralta-Yahya, P. Advances in G protein-coupled receptor high-throughput screening. *Curr. Opin. Biotechnol.* **2020**, *64*, 210–217. [\[CrossRef\]](#) [\[PubMed\]](#)
- Arinaminpathy, Y.; Khurana, E.; Engelman, D.M.; Gerstein, M.B. Computational analysis of membrane proteins: The largest class of drug targets. *Drug Discov. Today* **2009**, *14*, 1130–1135. [\[CrossRef\]](#) [\[PubMed\]](#)
- Davey, J. G-Protein-Coupled Receptors: New Approaches to Maximise the Impact of GPCRs in Drug Discovery. *Expert Opin. Ther. Targets* **2004**, *8*, 165–170. [\[CrossRef\]](#) [\[PubMed\]](#)
- Schlegel, S.; Löfblom, J.; Lee, C.; Hjelm, A.; Klepsch, M.; Strous, M.; Drew, D.; Slotboom, D.J.; de Gier, J.-W. Optimizing Membrane Protein Overexpression in the Escherichia coli strain Lemo21 (DE3). *J. Mol. Biol.* **2012**, *423*, 648–659. [\[CrossRef\]](#)
- Gubellini, F.; Verdon, G.; Karpowich, N.K.; Luff, J.D.; Boël, G.; Gauthier, N.; Handelsman, S.K.; Ades, S.E.; Hunt, J.F. Physiological Response to Membrane Protein Overexpression in *E. coli*. *Mol. Cell. Proteom.* **2011**, *10*. [\[CrossRef\]](#)
- Hunte, C. Specific protein–lipid interactions in membrane proteins. *Biochem. Soc. Trans.* **2005**, *33*, 938–942. [\[CrossRef\]](#)
- Lee, A.G. How lipids affect the activities of integral membrane proteins. *Biochim. Biophys. Acta Biomembr.* **2004**, *1666*, 62–87. [\[CrossRef\]](#)
- Hardy, D.; Mandon, E.D.; Rothnie, A.J.; Jawhari, A. The yin and yang of solubilization and stabilization for wild-type and full-length membrane protein. *Methods* **2018**, *147*, 118–125. [\[CrossRef\]](#)
- Smith, S.M. Strategies for the Purification of Membrane Proteins. *Methods Mol. Biol.* **2016**, *1485*, 389–400. [\[CrossRef\]](#)
- Drew, D.; Lerch, M.; Kunji, E.; Slotboom, D.-J.; De Gier, J.-W. Optimization of membrane protein overexpression and purification using GFP fusions. *Nat. Chem. Biol.* **2006**, *3*, 303–313. [\[CrossRef\]](#)
- Bird, L.E.; Rada, H.; Verma, A.; Gasper, R.; Birch, J.; Jennions, M.; Löwe, J.; Moraes, I.; Owens, R.J. Green fluorescent protein-based expression screening of membrane proteins in Escherichia coli. *J. Vis. Exp.* **2015**, *95*, e52357. [\[CrossRef\]](#)
- Eshaghi, S.; Hedrén, M.; Nasser, M.I.A.; Hammarberg, T.; Thornell, A.; Nordlund, P. An efficient strategy for high-throughput expression screening of recombinant integral membrane proteins. *Protein Sci.* **2005**, *14*, 676–683. [\[CrossRef\]](#) [\[PubMed\]](#)
- Ma, P.; Varela, F.; Magoch, M.; Silva, A.R.; Rosário, A.L.; Brito, J.; Oliveira, T.F.; Nogly, P.; Pessanha, M.; Stelter, M.; et al. An Efficient Strategy for Small-Scale Screening and Production of Archaeal Membrane Transport Proteins in Escherichia coli. *PLoS ONE* **2013**, *8*, e76913. [\[CrossRef\]](#)
- Elslinger, M.-A.; Deacon, A.M.; Godzik, A.; Lesley, S.A.; Wooley, J.; Wüthrich, K.; Wilson, I.A. The JCSG high-throughput structural biology pipeline. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **2010**, *66*, 1137–1142. [\[CrossRef\]](#) [\[PubMed\]](#)
- Xiao, R.; Anderson, S.; Aramini, J.; Belote, R.; Buchwald, W.A.; Ciccocanti, C.; Conover, K.; Everett, J.K.; Hamilton, K.; Huang, Y.J.; et al. The high-throughput protein sample production platform of the Northeast Structural Genomics Consortium. *J. Struct. Biol.* **2010**, *172*, 21–33. [\[CrossRef\]](#) [\[PubMed\]](#)
- Love, J.; Mancia, F.; Shapiro, L.; Punta, M.; Rost, B.; Girvin, M.; Wang, D.-N.; Zhou, M.; Hunt, J.F.; Szyperski, T.; et al. The New York Consortium on Membrane Protein Structure (NYCOMPS): A high-throughput platform for structural genomics of integral membrane proteins. *J. Struct. Funct. Genom.* **2010**, *11*, 191–199. [\[CrossRef\]](#) [\[PubMed\]](#)
- Mancia, F.; Love, J. High-throughput expression and purification of membrane proteins. *J. Struct. Biol.* **2010**, *172*, 85–93. [\[CrossRef\]](#)
- Bellinzoni, M.; Riccardi, G. Techniques and Applications: The heterologous expression of Mycobacterium tuberculosis genes is an uphill road. *Trends Microbiol.* **2003**, *11*, 351–358. [\[CrossRef\]](#)
- Jankute, M.; Grover, S.; Rana, A.K.; Besra, G.S. Arabinogalactan and lipoarabinomannan biosynthesis: Structure, biogenesis and their potential as drug targets. *Futur. Microbiol.* **2012**, *7*, 129–147. [\[CrossRef\]](#) [\[PubMed\]](#)
- Umesiri, F.E.; Sanki, A.K.; Boucau, J.; Ronning, D.R.; Sucheck, S.J. Recent advances toward the inhibition of mAG and LAM synthesis in Mycobacterium tuberculosis. *Med. Res. Rev.* **2010**, *30*, 290–326. [\[CrossRef\]](#) [\[PubMed\]](#)
- Wolucka, B.; McNeil, M.; De Hoffmann, E.; Chojnacki, T.; Brennan, P. Recognition of the lipid intermediate for arabinogalactan/arabinomannan biosynthesis and its relation to the mode of action of ethambutol on mycobacteria. *J. Biol. Chem.* **1994**, *269*, 23328–23335. [\[CrossRef\]](#)
- Jankute, M.; Cox, J.A.; Harrison, J.; Besra, G.S. Assembly of the Mycobacterial Cell Wall. *Annu. Rev. Microbiol.* **2015**, *69*, 405–423. [\[CrossRef\]](#)
- Abrahams, K.A.; Besra, G.S. Mycobacterial cell wall biosynthesis: A multifaceted antibiotic target. *Parasitology* **2018**, *145*, 116–133. [\[CrossRef\]](#)
- Bruni, R.; Kloss, B. High-Throughput Cloning and Expression of Integral Membrane Proteins in Escherichia coli. *Curr. Protoc. Protein Sci.* **2013**, *74*, 29.6.1–29.6.34. [\[CrossRef\]](#)
- Punta, M.; Love, J.; Handelsman, S.; Hunt, J.F.; Shapiro, L.; Hendrickson, W.A.; Rost, B. Structural genomics target selection for the New York consortium on membrane protein structure. *J. Struct. Funct. Genom.* **2009**, *10*, 255–268. [\[CrossRef\]](#)
- Rath, A.; Glibowicka, M.; Nadeau, V.G.; Chen, G.; Deber, C.M. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 1760–1765. [\[CrossRef\]](#)

30. Moreland, N.; Ashton, R.; Baker, H.M.; Ivanović, I.; Patterson, S.; Arcus, V.L.; Baker, E.N.; Lott, J.S. A flexible and economical medium-throughput strategy for protein production and crystallization. *Acta Crystallogr. Sect. D Biol. Crystallogr.* **2005**, *61*, 1378–1385. [[CrossRef](#)]
31. Bashiri, G.; Baker, E.N. Production of recombinant proteins in *Mycobacterium smegmatis* for structural and functional studies. *Protein Sci.* **2014**, *24*, 1–10. [[CrossRef](#)] [[PubMed](#)]
32. Goldstone, R.M.; Moreland, N.J.; Bashiri, G.; Baker, E.N.; Lott, J.S. A new Gateway® vector and expression protocol for fast and efficient recombinant protein expression in *Mycobacterium smegmatis*. *Protein Expr. Purif.* **2008**, *57*, 81–87. [[CrossRef](#)] [[PubMed](#)]
33. Lewinson, O.; Lee, A.T.; Rees, D.C. The Funnel Approach to the Precrystallization Production of Membrane Proteins. *J. Mol. Biol.* **2008**, *377*, 62–73. [[CrossRef](#)] [[PubMed](#)]
34. Kieser, K.J.; Rubin, E.J. How sisters grow apart: Mycobacterial growth and division. *Nat. Rev. Genet.* **2014**, *12*, 550–562. [[CrossRef](#)]
35. Miroux, B.; Walker, J.E. Over-production of Proteins in *Escherichia coli*: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels. *J. Mol. Biol.* **1996**, *260*, 289–298. [[CrossRef](#)]
36. Studier, F. Use of bacteriophage T7 lysozyme to improve an inducible T7 expression system. *J. Mol. Biol.* **1991**, *219*, 37–44. [[CrossRef](#)]
37. Mathieu, K.; Javed, W.; Vallet, S.; Lesterlin, C.; Candusso, M.-P.; Ding, F.; Xu, X.N.; Ebel, C.; Jault, J.-M.; Orelle, C. Functionality of membrane proteins overexpressed and purified from *E. coli* is highly dependent upon the strain. *Sci. Rep.* **2019**, *9*, 2654. [[CrossRef](#)]
38. Wagner, S.; Klepsch, M.M.; Schlegel, S.; Appel, A.; Draheim, R.; Tarry, M.; Högbom, M.; Van Wijk, K.J.; Slotboom, D.J.; Persson, J.O.; et al. Tuning *Escherichia coli* for membrane protein overexpression. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 14371–14376. [[CrossRef](#)]
39. Fan, J.; Heng, J.; Dai, S.; Shaw, N.; Zhou, B.; Huang, B.; He, Z.; Wang, Y.; Jiang, T.; Li, X.; et al. An efficient strategy for high throughput screening of recombinant integral membrane protein expression and stability. *Protein Expr. Purif.* **2011**, *78*, 6–13. [[CrossRef](#)]
40. Willis, M.S.; Koth, C.M. Structural Proteomics of Membrane Proteins: A Survey of Published Techniques and Design of a Rational High Throughput Strategy. In *Advanced Structural Safety Studies*; Springer: Berlin, Germany, 2008; Volume 426, pp. 277–295.
41. Zhang, L.; Zhao, Y.; Gao, R.; Li, J.; Yang, X.; Gao, Y.; Zhao, W.; Gurcha, S.S.; Veerapen, N.; Batt, S.M.; et al. Cryo-EM snapshots of mycobacterial arabinosyltransferase complex EmbB2-AcpM2. *Protein Cell* **2020**, *11*, 505–517. [[CrossRef](#)]
42. Tan, Y.Z.; Rodrigues, J.; Keener, J.E.; Zheng, R.B.; Brunton, R.; Kloss, B.; Giacometti, S.I.; Rosário, A.L.; Zhang, L.; Niederweis, M.; et al. Cryo-EM structure of arabinosyltransferase EmbB from *Mycobacterium smegmatis*. *Nat. Commun.* **2020**, *11*, 3396. [[CrossRef](#)]
43. Zhang, L.; Zhao, Y.; Gao, Y.; Wu, L.; Gao, R.; Zhang, Q.; Wang, Y.; Wu, C.; Wu, F.; Gurcha, S.S.; et al. Structures of cell wall arabinosyltransferases with the anti-tuberculosis drug ethambutol. *Science* **2020**, *368*, 1211–1219. [[CrossRef](#)]
44. Tan, Y.Z.; Zhang, L.; Rodrigues, J.; Zheng, R.B.; Giacometti, S.I.; Rosário, A.L.; Kloss, B.; Dandey, V.P.; Wei, H.; Brunton, R.; et al. Cryo-EM Structures and Regulation of Arabinofuranosyltransferase AftD from *Mycobacteria*. *Mol. Cell* **2020**, *78*, 683–699.e11. [[CrossRef](#)] [[PubMed](#)]
45. Alderwick, L.J.; Seidel, M.; Sahm, H.; Besra, G.S.; Eggeling, L. Identification of a Novel Arabinofuranosyltransferase (AftA) Involved in Cell Wall Arabinan Biosynthesis in *Mycobacterium tuberculosis*. *J. Biol. Chem.* **2006**, *281*, 15653–15661. [[CrossRef](#)]
46. Zhang, J.; Angala, S.K.; Pramanik, P.K.; Li, K.; Crick, D.C.; Liav, A.; Jozwiak, A.; Swiezewska, E.; Jackson, M.; Chatterjee, D.; et al. Reconstitution of Functional Mycobacterial Arabinosyltransferase AftC Proteoliposome and Assessment of Decaprenylphosphorylarabinose Analogues as Arabinofuranosyl Donors. *ACS Chem. Biol.* **2011**, *6*, 819–828. [[CrossRef](#)] [[PubMed](#)]
47. Favrot, L.; Ronning, D.R. Targeting the mycobacterial envelope for tuberculosis drug development. *Expert Rev. Anti-Infect. Ther.* **2012**, *10*, 1023–1036. [[CrossRef](#)] [[PubMed](#)]