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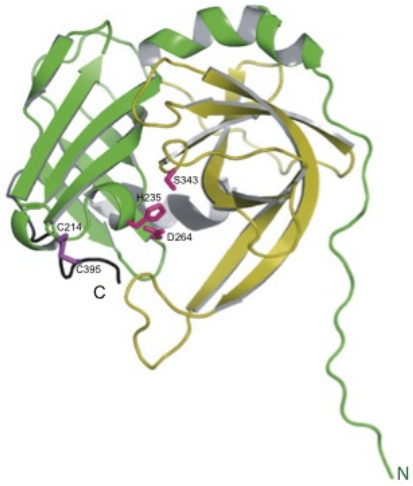
FUNCTIONAL CHARACTERIZATION OF THE FOUR TRANSMEMBRANE DOMAINS IN THE *MYCOBACTERIUM TUBERCULOSIS* SERINE PROTEASE RV3671C AND IDENTIFICATION OF PROTEASE SUBSTRATES



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1. ABSTRACT

Mycobacterium tuberculosis can survive in IFN-γ activated acidified macrophages by undefined means of intrabacterial pH control. Through transposon mutagenesis acid-sensitive mutants were found, that were unable to persist in acidified macrophages and maintain their intracellular pH. One of the genes disrupted was *Rv3671c*, coding for a membrane-bound serine protease. Structure analysis showed a high sequence homology to HtrA (high-temperature requirement) proteins, involved in stress control in Mtb. However, unlike HtrA proteins which only have one transmembrane (TM) domain, Rv3671c has four. This work tried to characterize the function of the four TM domains by creating five different TM constructs. The protease domain was either fused to only two, to three or to none of the Rv3671c TM domains or to the one TM domain of HtrA2. Thus, some constructs also changed the location of the protease domain from being in the native environment of the periplasm to being in the cytoplasm. One construct was made deleting the protease domain, which only left the four TM domains. *Rv3671c* knockout mutants of *M. smegmatis* and *M. tuberculosis* were transformed with the constructs and complementation was tested in Spotting and Nigericin Assays. None of the constructs could complement the *M. smegmatis* mutant in both assays, which might implicate the necessity of the four TM domains.

2. INTRODUCTION

2.1. Background

Tuberculosis (TB) is among the world's most common and most serious infectious diseases. Almost fallen into oblivion in industrialized countries, TB is spreading quickly not only - even though to a large extent - in developing countries and among HIV patients. [1] One third of the world's population is infected with *Mycobacterium tuberculosis*. In 2008 9.4 million new TB cases were reported and 1.8 million people died. [2] In addition, the emergence of multidrug- and extensively drug-resistant strains makes it difficult to cure patients, since these strains do not respond to the standard six month treatment with first-line anti-TB medication. [3, 4]

These facts make it necessary to gain further knowledge of the infectious process and consequently develop new therapies.

Tuberculosis is caused by the acid-fast bacillus *Mycobacterium tuberculosis*. The main mode of transmission is via respiratory droplets, thus the primary infection occurs in the lungs. However, hematogenous spread can carry the bacillus in any organ. [5, 6] Upon contact a significant proportion of individuals is able to clear infection through innate and adaptive immune responses . Only a small number of exposed and infected individuals (~ 5%) develop active TB within 2-5 years. The remaining infected individuals have latent infections without clinical symptoms and are not contagious. [5, 7]

Initial infection is assumed to occur in alveolar macrophages, which recognize specific patterns on the surface of the tubercle bacilli and consequently ingest them. [8, 9] Yet, unlike most other ingested bacteria, *M. tuberculosis* is able to survive and even replicate in resting macrophages by blocking the phagolysosomal fusion [8-12] and impeding acidification. [13] However, the cytokine IFN-γ was shown to be able to acidify also mycobacterium-containing macrophages [11] and activate reactive oxygen and nitrogen intermediates. [14, 15, 16]

In contrast to *M. tuberculosis* H37Rv (WT) which can survive even in activated acidified macrophages, five mutants were found by Vandal et al. through transposon mutagenesis that were unable to maintain intrabacterial pH in acid *in vitro* and in macrophages. [17] One of the five genes being disrupted was *Rv3671c*, coding for a membrane-bound serine protease of the chymotrypsin family, containing the canonical serine protease catalytic triad, with Ser343 as the catalytic residue. [17, 18, 19] Furthermore and more importantly, it was found that *Rv3671c* knockout mutants were severely attenuated in mice, [17] making Rv3671c an interesting target for new therapies. Little is known about the mechanism of action of Rv3671c and how it protects Mtb from acid, but it might be involved in cell wall modification or in signaling pathways that respond to extracellular stress. [17]

Protein topology of Rv3671c was predicted by the TMHMM server, which uses the hidden Markov model. [20] Rv3671c was predicted to have four transmembrane domains with the N-

terminus and the C-terminal protease domain being in the periplasm (Fig. 1). Location of the protease domain was confirmed by Jennifer Small (Department of Microbiology and Immunology at Weill Cornell Medical College) using a specific PhoA-fusion (unpublished data). Additionally, the protease shows autoproteolytic activity and cleaves itself near the N-terminus. No cleavage was seen in Ser343Ala mutants. [18]

Furthermore, Rv3671c was found to have a sequence homology to HtrA (high-temperature requirement) proteins, which three exist of in *M tuberculosis*. [17, 18] HtrA-proteins are involved in environmental and cellular stress damage control and showed attenuation of virulence in mice when deleted in Mtb. [21] Unlike HtrA-proteins, Rv3671c does not have any PDZ domains, where it might interact with other proteins, and it has four compared to one transmembrane domains. [18, 21]

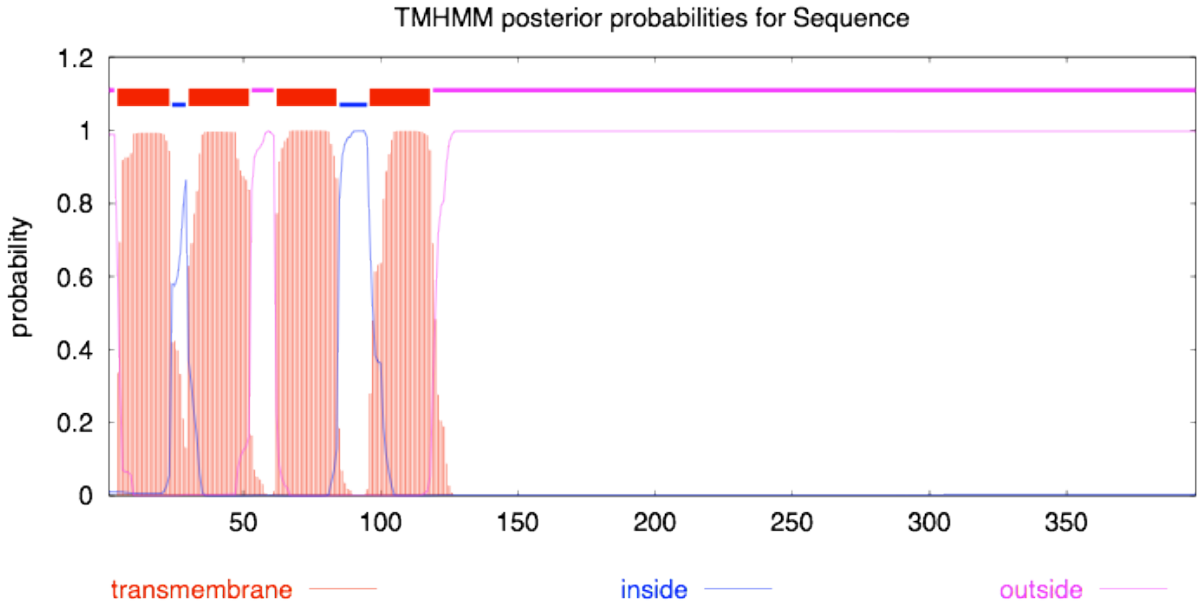

Fig 1: TMHMM topology prediction of Rv3671c, with the C-terminal protease domain and N-terminus both located in the periplasm and showing four transmembrane domains.

2.2. Aim of this work

In this work, we tried to characterize the function of the four transmembrane (TM) domains of Rv3671c by determining if all four of them are needed for the protein's activity and if the four

TM domains alone without protease are able to complement a *Rv3671c* knockout mutant, respectively.

First, two constructs should be made using Multisite Gateway® Pro 3-Fragment Recombination Technology (see chapter 2.3.) to test if the protease domain is required for complementation and moreover, if the protein with only two out of four TM domains can complement the knockout mutant. The first question was addressed by the creation of a construct containing the four TM domains without the protease (4 TM), the second question by making a fusion of the first two TM domains (including the secretion signal) and the protease domain (TM 1&2) (see Fig. 2). *M. smegmatis*, a non-pathogenic mycobacterium [22] with a doubling time of 3-4 hours in contrast to 18-22 hours of *M. tuberculosis*, *mc²155* (WT) and Δ*3671c* should be transformed with the constructs.

Possible complementation should be tested with Spotting and Nigericin Assays, two assays where knockout mutants were found to have growth deficiencies.

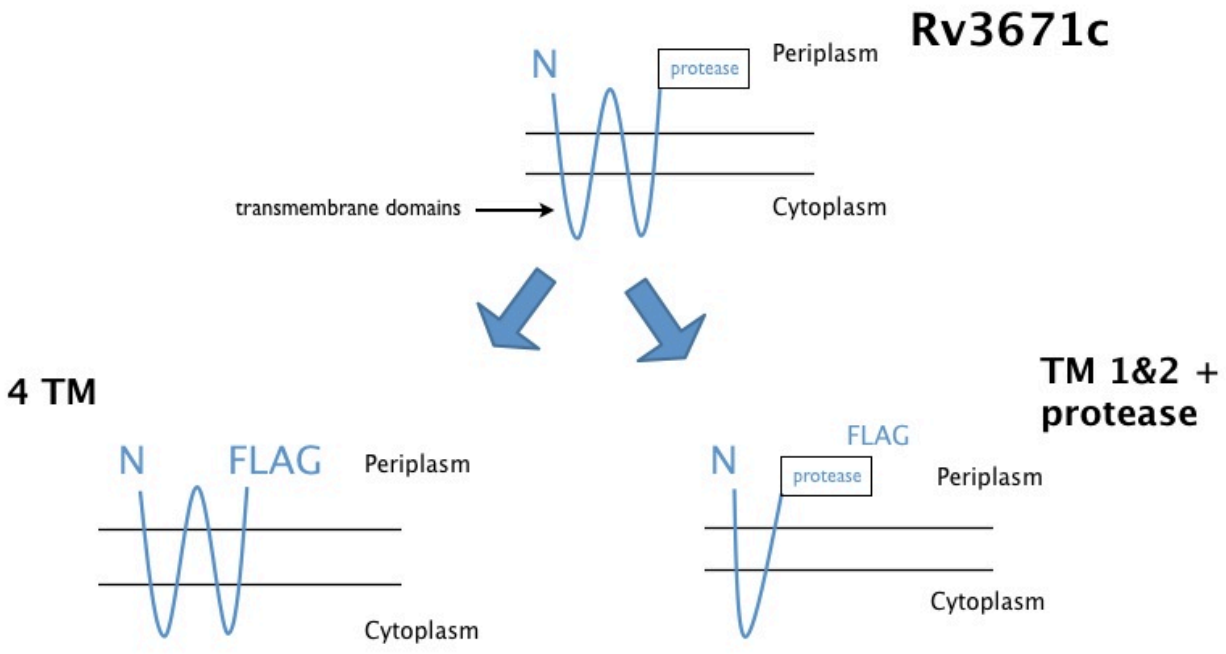

Fig 2: **Constructs created in the first run of this work to characterize the function of the four transmembrane domains of Rv3671c.** Top: topology of Rv3671c. Bottom left: 4 TM-construct, containing the four TM domains without protease. Bottom right: TM 1&2-construct, containing the first two TM domains and the protease domain

Following the results of the first two constructs, three more constructs were made (see Fig. 3). In TM 123 the protease domain should be fused to the first three TM domains, so that the protease is in the cytoplasm. This should give information about if the membrane-bound protease can also work in the cytoplasm.

In dTM all four TM domains were deleted in order to see if the protease can work freely moving in the cytoplasm.

As a third construct of the second run the one TM domain of HtrA2 should be fused to the protease domain of Rv3671c. This should show if one TM domain is sufficient for activity.

M. smegmatis mc²155 and Δ*3671c* should again be transformed with the constructs.

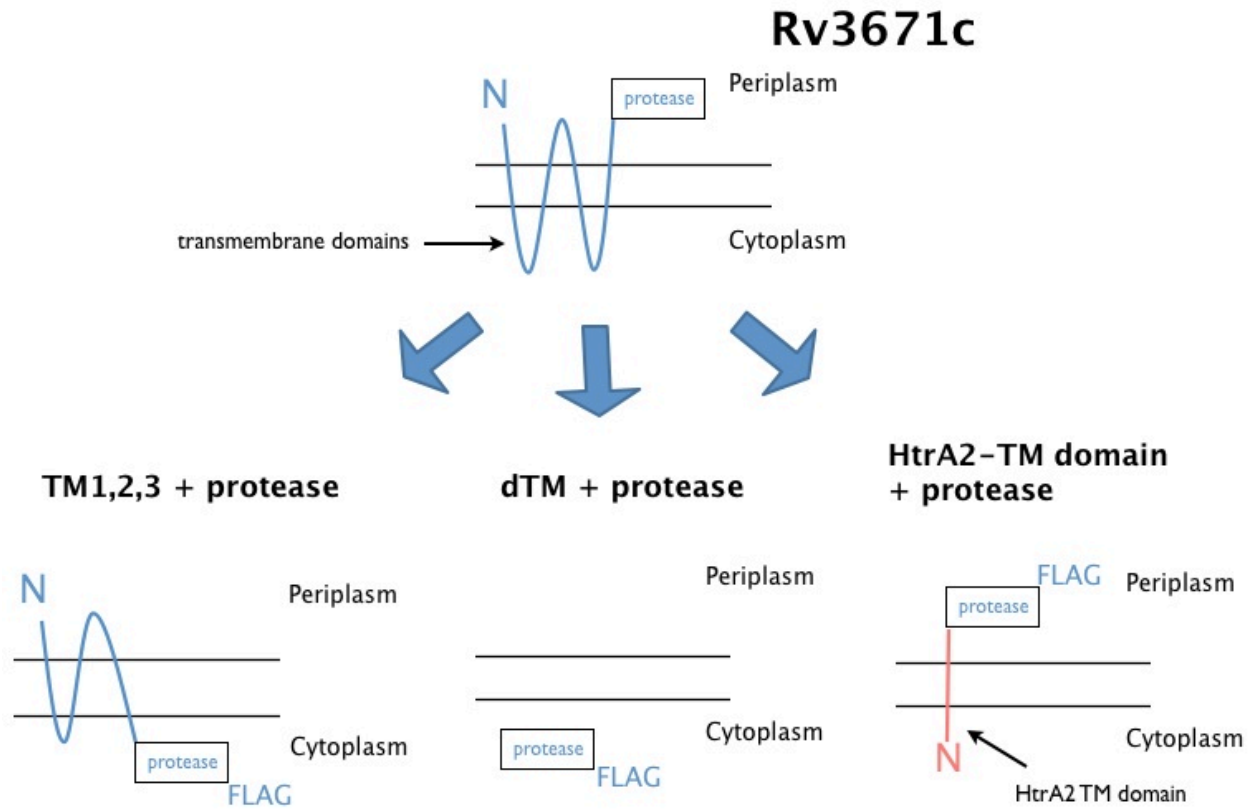

Fig 3: **Constructs created in the second run of this work to characterize the function of the four transmembrane domains of Rv3671c.** Top: topology of Rv3671c. Bottom left: TM 123-construct, containing the first three TM domains and the protease in the cytoplasm. Bottom centre: dTM-construct, with deleted TM domains and the protease staying in the cytoplasm. Bottom right: HtrA2-TM-construct, containing the TM domain of HtrA2 and the protease domain of Rv3671c

Also, *M. tuberculosis* H37Rv (WT) and Δ*3671c* should be transformed with all five constructs and assays should be done. Work with Mtb in BSL3 should be done by Jennifer Small (Department of Microbiology and Immunology at Weill Cornell Medical College), work outside BSL3 should be done by me.

2.3. Multisite Gateway® Pro 3- Fragment Recombination Technology

All the cloning in this work was done using the Multisite Gateway® Pro 3-Fragment Recombination Technology, which is based on the site-specific recombination of bacteriophage lamda. DNA fragments (genes) of interest can be directionally transferred into various vector backgrounds, allowing to change promoter and regulator elements easily and efficiently. Since the recombination reaction is highly specific and conservative, the DNA fragments maintain their orientation and reading frame. [23]

Recombination occurs at specific attachment sites (*att* sites) and is carried out in two reactions by a mixture of enzymes (Clonase™ II enzyme mixes). *att*B sites recombine with *att*P sites, which is catalyzed by the BP Clonase™ II enzyme mix. In this BP reaction, donor vectors recombine with DNA fragments that are flanked by modified *att*B-sites. This creates a so-called entry clone. The entry clone now shows specific *att*L and *att*R sites. [24] In this work the recombination occurs between the donor vector pDO23A and the amplified PCR fragments (see tables 6 and 7) with an *att*B2 site on the one end and an *att*B3 site on the other end of the DNA sequence and thus creates ‘pEN23’ entry clones.

Subsequently, LR Clonase™ II enzyme mix recombines the specific *att*L and *att*R sites of the entry clones with each other and a destination vector. In this LR reaction *att*L1 sites, for instance, react only with *att*R1 sites. This specific recombination of the *att* sites creates an expression clone, containing the PCR product with the desired promoter (and regulator) in the desired order of the fragments. [24]

In this work the following vectors were used for the LR reaction:

Tab 1: vectors used for LR reaction

vector	description	*att* sites
pEN41A-TO-2 #2[1]	no insert (dummy), replacement for e.g. regulator	*att*L1 & *att*L4
pEN21A hsp60[1]	contains the hsp60 promoter	*att*R1 & *att*R2
pEN23 [transmembrane construct]	contains the transmembrane constructs (PCR fragments)	*att*L2 & *att*L3
pDE43 mck[1]	destination vector for chromosomal expression of constructs in host	*att*R4 & *att*R3
pDE43 mek[1]	destination vector for episomal expression of constructs in host	*att*R4 & *att*R3

[1]in stock of Department of Microbiology and Immunology at Weill Cornell Medical College

An expression clone would be named as follows:

e.g.: pGMEK-0x-hsp60-Rv3671c-TM1&2-protease-FLAG

meaning: episomal expression (pGMEK)
dummy (0x)
hsp60 promoter (hsp60)
Rv3671c-TM1&2 + protease-FLAG construct

or

e.g. : pGMCK -0x-hsp60-Rv3671c-4TM-FLAG

meaning: chromosomal expression (pGMCK)
dummy (0x)
hsp60 promoter (hsp60)
Rv3671c-4TM-FLAG construct

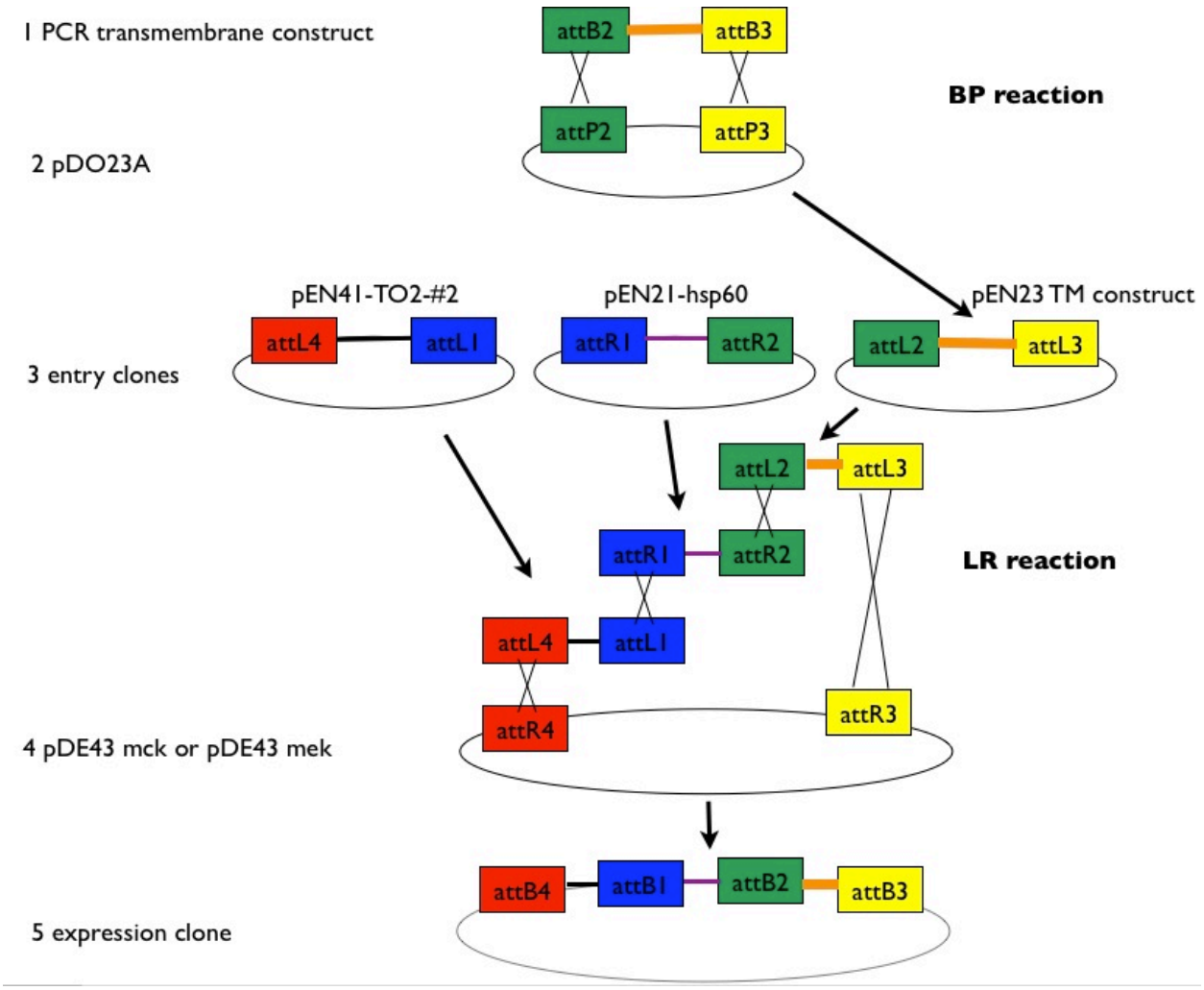

Fig 4: **Multisite Gateway® Pro 3-Fragment Recombination scheme used in this work to create expression clones containing the TM constructs.** TM constructs were gained by PCR reactions and transferred into entry clones (pEN23) in BP reactions with the donor vector pDO23A. Three entry clones (pEN41-TO2-#2, pEN21-hsp60 and pEN23 TM construct) were combined with a destination vector (either pDE43 mck for chromosomal expression or pDE43 mek for episomal expression) in LR reactions to create the final expression clone. The expression clones were composed of the dummy fragment (replacing e.g. a regulator), the hsp60-promoter and the TM-construct. att = attachment site.

3. Materials and Methods

3.1. Strains and Media

Bacterial strains used in this work are listed in Table 2, media that was used in Table 3.

Tab 2: Bacterial strains used in this work

	Strain	Description
M. smegmatis	mc^2155	wild type strain (avirulent)
	*Δ6183**	knockout mutant (avirulent) equivalent gene to *Rv3671c* in Mtb
M. tuberculosis	H37Rv	virulent wild type strain
	ΔRv3671c (in the background of H37Rv)	knockout mutant
E. coli	*Mach1*	fast growing chemically competent cloning strain

*for easier understanding the name '*Δ3671c*' will also be used for the *M. smegmatis* knockout mutant in this report

Tab 3: Media used in this work

Difco™ LB Broth/ Agar, Miller	Difco™ Middlebrook 7H9 Broth	Difco™ Middlebrook 7H10 Agar	Difco™ Middlebrook 7H11 Agar
Tryptone 10 g/l Yeast Extract 5 g/l NaCl 10 g/l	7H9 powder 4.7 g/l glycerol 2 ml/l	7H10 powder 19 g/l glycerol 5 ml/l	7H11 powder 21 g/l glycerol 5 ml/l
Agar 15 g/l	for Mtb: add 100 ml Middlebrook ADC Enrichment after autoclaving	for Mtb: add 100 ml Middlebrook ADC Enrichment after autoclaving	for Mtb: add 100 ml Middlebrook ADC Enrichment after autoclaving

Difco™ LB Broth/ Agar, Miller	Difco™ Middlebrook 7H9 Broth	Difco™ Middlebrook 7H10 Agar	Difco™ Middlebrook 7H11 Agar
for *Mach1* cells	media for cultivation of mycobacteria	• agar for cultivation of mycobacteria •contains 250 µg/l malachite green • *Rv3671c*-knockout mutant grows slower on 7H10 than WT	• agar for cultivation of fastidious mycobacteria • same as 7H10, but contains 1 mg/l malachite green and digested casein • *Rv3671c*-knockout mutant grows slower on 7H11 than WT

Antibiotics were used for assays or added to broth or agar plates for selection in following concentrations:

Carbenicillin	100 µg/ml (*E. coli*)
Kanamycin	50 µg/ml (*E. coli*) 20 µg/ml (*Mycobacterium spp.*)
Nigericin	different concentrations used for assay

3.2. Construction of Rv3671c-transmembrane constructs by Gateway® Cloning

Cloning was done using the Multisite Gateway® Pro 3-Fragment Recombination Technology as described in chapter 2.3.

First only two constructs pGMEK/pGMCK-0x-hsp60-Rv3671c-4TM-FLAG and pGMEK/ pGMCK-0x-hsp60-Rv3671c-TM1&2-protease-FLAG were made (see table 6). Results of the assays (see chapters 4.2. and 4.3.) with those two constructs made it necessary to create three more constructs pGMEK-0x-hsp60-Rv3671c-htrA2-protease-FLAG, pGMEK-0x-hsp60-Rv3671c-TM1&2&3-protease-FLAG and pGMEK-0x-hsp60-Rv3671c-DTM-protease-FLAG (see table 7).

3.2.1. Polymerase Chain Reactions (PCR)

Fragments that were flanked by attB2 and attB3 sites for subsequent creation of pEN23 entry clones were made by PCR. For TM1&2, TM123 and HtrA2 Overlap PCRs followed normal PCRs in order to fuse the protease domain to the TM domains, which were all amplified in separate PCR reactions. A Flag-tag for antibody detection was added to all five constructs.

Tab 6: Construction of attB2-Rv3671c-4TM-FLAG-attB3 (for pGMEK/pGMCK-0x-hsp60-Rv3671c-4TM-FLAG) and attB2-Rv3671c-TM1&2-protease-FLAG-attB3 (for pGMEK/pGMCK-0x-hsp60-Rv3671c-TM1&2-protease-FLAG) by PCR. Final PCR constructs were used to create pEN23 entry clones.

#	Fragment Name	Primers used[2]	template used	size of fragment	Final PCR Construct
1	attB2-Rv3671c-4TM-FLAG	fw: clo-3671-attB2-2 rev: clo-3671-4TM-FLAG	pgmck-hsp60-3671-flag[1]	444 bp	attB2-Rv3671c-4TM-FLAG-attB3
2	attB2-Rv3671c-4TM-FLAG-attB3	fw: clo-3671-attB2-2 rev: clo-FLAG-attB3	PCR product #1	469 bp	
3	attB2-Rv3671c-TM12	fw: clo-3671-attB2-2 rev: clo-3671-TM12-rev	pgmck-hsp60-3671-flag[1]	218 bp	attB2-Rv3671c-TM1&2-protease-FLAG-attB3
4	Rv3671c-protease-FLAG-attB3	fw: clo-3671-TM12-fw rev: clo-flag-attB3	pgmck-hsp60-3671-flag[1]	900 bp	
Overlap PCR of PCR products #3 and #4					
5	attB2-Rv3671c-TM12-protease-FLAG-attB3	fw: clo-3671-attB2-2 rev: clo-FLAG-attB3	PCR products #3 and #4	1093 bp	

[1]in stock of Department of Microbiology and Immunology at Weill Cornell Medical College

[2] Primers were ordered from Operon

Tab 7: Construction of attB2-Rv3671c-DTM-protease-FLAG-attB3 (for pGMEK-0x-hsp60-Rv3671c-DTM-FLAG), attB2-Rv3671c-TM123-protease-FLAG-attB3 (for pGMEK-0x-hsp60-Rv3671c-TM123-protease-FLAG) and attB2-Rv3671c-htrA2-protease-FLAG attB3 (for pGMEK-0x-hsp60-Rv3671c-htrA2-protease-FLAG) by PCR. Final PCR constructs were used to create pEN23 entry clones.

#	Fragment Name	Primers used[2]	template used	size of fragment	Final PCR Construct
6	attB2-Rv3671c-DTM-protease-FLAG-attB3	fw: clo-3671-att2-DTM-fw rev: clo-Flag-attB3	pEN23-Rv3671-TM12-FLAG[1]	940 bp	attB2-Rv3671c-DTM-protease-FLAG-attB3
7	attB2-Rv3671c-TM123	fw: clo-3671-att2-2 rev: clo-3671-TM123-rev	pEN23-Rv3671-FLAG[1]	370 bp	attB2-Rv3671c-TM123-protease-FLAG-attB3
8	Rv3671-protease-FLAG-attB3	fw: clo-Rv3671-TM123-fw rev: clo-flag-attB3	PEN23-Rv3671-FLAG[1]	893 bp	
Overlap PCR of PCR products #7 and #8					
9	attB2-Rv3671c-TM123-protease-FLAG-attB3	fw: clo-3671-att2-2 rev: clo-flag-attB3	PCR products #7 and #8	1243 bp	
10	attB2-Rv3671c-htrA2	fw: clo-htrA2-attB2 rev:clo-htrA2-rev	Mtb chromosomal DNA[1]	430 bp	attB2-Rv3671c-htrA2-protease-FLAG-attB3
11	Rv3671-protease-FLAG-attB3	fw: clo-htrA2-Rv3671-fw rev: clo-Flag-attB3	pEN23-Rv3671-TM12-FLAG[1]	976 bp	
Overlap PCR of PCR products #10 and #11					
12	attB2-Rv3671c-htrA2-protease-FLAG-attB3	fw: clo-htrA2-attB2 rev:clo-flag-attB3	PCR products #10 and #11	1309 bp	

[1]in stock of Department of Microbiology and Immunology at Weill Cornell Medical College

[2] Primers were ordered from Operon

For normal PCR and Overlap PCR the following amounts of reagents and the following temperature program was used:

Tab 4: Amounts of reagents used for normal and Overlap PCR

reagent	**amount (*µ*l)**
DMSO (10x)	2.5
5x Phusion Buffer	5
Primer 1 forward (100 *µ*M)	1.25
Primer 2 revers (100 *µ*M)	1.25
template (100 ng) (**normal PCR**)	...
PCR product A and B (1:10 dilution of each) (**Overlap PCR**)	1 each
dNTPs (10 mM)	0.625
Phusion polymerase	0.25
dH_20	25-x

Tab 5: PCR temperature program used

Program		
Denaturation	96ºC x 60s	
Cycles	**96ºC x 30s** **70ºC x 30s** **72ºC x 2 min**	x 35 cycles
Extension	72ºC x 7 min	
	4ºC x infinity	

Primers that were used were all diluted in sterile dH_20 to a final concentration of 100 µM.

Correct sizes of PCR products were checked on 1.2% Agarose gels. Those PCR products that were used as templates for another PCR reaction were purified using the Quiagen - QIAquick PCR Purification Kit. The final PCR constructs (see tables 6 and 7) were cut out of the 1.2% Agarose gels and purified using the Qiagen- QIAquick Gel Extraction Kit. DNA concentrations were taken using the Thermo Scientific NanoDrop™ 1000 Spectrophotometer.

3.2.2. BP reactions

Entry clones were made using the Gateway® Cloning BP reaction following the scheme:

Tab 8: Reagents and amounts for Gateway® Cloning BP reaction

reagents	amounts (µl)
pDO23A[1] (4825 bp; 100ng/µl)	1
PCR product (x bp; y ng/µl)	...
TE buffer (pH 8.0)	10-z
BP Clonase™ II (Invitrogen)	2

[1]in stock of Department of Microbiology and Immunology at Weill Cornell Medical College

Calculations for needed amount of PCR product for BP reaction:

$$\frac{4825 \text{ bp}}{x \text{ bp}} = \text{fold}$$

$$\frac{100 \text{ ng of pDO23A}}{\text{fold}} = \text{needed ng of PCR product}$$

y ng/µl of PCR product x **a** = needed ng of PCR product

a = µl of PCR product for BP reaction

Reactions were left overnight at room temperature (RT) and finished by addition of 1 µl Proteinase K (Invitrogen) and incubation at 37ºC for 10 min.

3.2.3. Transformation of *E.coli Mach1* cells with entry clones

Thawing of competent *E.coli Mach1* cells (in stock from Department of Microbiology and Immunology at Weill Cornell Medical College,) and addition of 5 µl BP reaction to 100 µl *Mach1* cells. Cells were left on ice for 30 min and then heat shocked at 42ºC for 90 seconds in a heat block. Addition of 1 ml sterile LB broth and shaking at 37ºC for 1 h. Harvesting cells by centrifugation at 5000 rpm for 5 min and removal of supernatant. Resuspension of pellet in 100 µl sterile LB broth and plating on LB Carb100 plate. Incubation at 37ºC overnight (10-12 h).

3.2.4. Streaking for single colonies

Six colonies were picked and restreaked on another LB Carb100 plate. Incubation at 37ºC overnight (10-12 h).

3.2.5. Plasmid Minipreps

Overnight cultures of transformed *Mach1* single colonies were set up in 5 ml LB broth with 100 µg/ml Carbenicillin. Entry clones were isolated using the Qiagen QIAprep® Miniprep Kit following the Protocol 'Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge'.

3.2.6. Digestion of Entry clones with restriction enzymes

Entry clones were digested depending on the restriction sites in the plasmids by restriction enzymes. Appropriate restriction sites were determined using Clone Manager Professional 9 (Sci-Ed Software). Optimal working conditions (including buffers, temperature, hindrance of methylation sites...) were determined using New England BioLabs handbook and website (www.neb.com).

Digested samples were checked on 1.2% agarose gels and those that showed the correct sizes were sent for sequencing to MACROGEN USA (www.macrogenusa.net).

3.2.7. LR Reactions

Expression clones were made using the Gateway® Cloning LR reaction following the scheme:

Tab 9: Reagents and amounts for Gateway® Cloning BP reaction

reagents	amounts (µl)
pEN41A-TO-2 #2[1] (100 ng/µl)	0.5
pEN21A hsp60[1] (100 ng/µl)	0.5
pEN23 [transmembrane construct] (100 ng/µl)	0.5
pDE43 mck[1,2] **OR** pDE43 mek[1,3] (100 ng/µl)	0.5
TE buffer (pH 8.0)	6
LR Clonase™ II (Invitrogen)	2

[1]in stock from Department of Microbiology and Immunology at Weill Cornell Medical College
[2] Destination vector for chromosomal expression in host
[3] Destination vector for episomal expression in host

Reactions were left overnight at RT and finished by addition of 1 µl Proteinase K (Invitrogen) and incubation at 37ºC for 10 min.

3.2.8. Transformation of *E.coli Mach1* cells with expression clones

Transformation of *Mach1* cells (One Shot® *Mach1*™ - T1 Phage Resistant Chemically Competent *E.coli,* Invitrogen) with expression clones follows the same protocol as transformation with entry clones (see 3.2.3.). Selection occurs on LB Kan50 plates.

3.2.9. Streaking for single colonies

Four colonies were picked and restreaked on another LB Kan50 plate. Incubation at 37ºC overnight (10-12 h).

3.2.10. Plasmid Minipreps

Overnight cultures of transformed *Mach1* single colonies were set up in 5 ml LB broth with 50 µg/ml Kanamycin. Expression clones were isolated using the Qiagen QIAprep® Miniprep Kit following the Protocol 'Plasmid DNA Purification Using the QIAprep Spin Miniprep Kit and a Microcentrifuge' and directly sent for sequencing to MACROGEN USA (www.macrogenusa.net). Correct expression vectors were used for transformation of *M. smegmatis* (see 3.4.).

3.3. Making of electrocompetent *M. smegmatis* cells

Inoculation of 400 ml 7H9 + glycerol (2 ml/l) + 0.05% Tween80 + appropriate antibiotic for selection with *M. smegmatis* culture. Incubation at 37ºC on shaker until OD_{580} reaches 1 (takes about 18-24 hrs).

Cells were split in eight 50 ml flasks and spun down at 4 500 rpm for 15 min at 4ºC and washed twice with ice cold sterile dH_2O (1x with same volume, 1x with 0.5 volume). Washing with ice cold sterile 15% glycerol (with 0.5 volume) and resuspension in 10 ml 15% glycerol. The suspensions of the eight flasks were transferred to two 50 ml flasks, spun down and again resuspended in 2 ml 15 % ice cold sterile glycerol. 100 and 200 µl aliquots were made and frozen at -70ºC.

3.4. Electroporation of *M. smegmatis*

Thawing of electrocompetent *M. smegmatis* cells (WT and Δ*3671c*) and spinning at 4000 rpm for 8 min at 4ºC. Resuspension of pellet in 1 ml sterile 15% glycerol and again spinning at 4000 rpm for 8 min at 4ºC. Resuspension of pellet in ~240 µl 15% glycerol.

40 µl of cells and 100 ng of expression vector (see 3.2.10.) were added to a cuvette and electroporated at 700W x 25 mF x 2.5kV. Immediately after electroporation 1 ml 7H9 media was added to cuvette and cells were incubated for 3 hrs at 37ºC. 100 µl of cells were plated on 7H10 Kan20 plates and incubated for 3-4 days at 37ºC.

3.5. Immunoblotting

Cell cultures were grown in 10 ml 7H9 + 0.05% Tween20 + appropriate antibiotic to mid-log phase (OD_{580} 0.8-1.0). Cultures were spun at 4000 rpm for 10 min, washed with same volume ice-cold PBS + 0.05% Tween (PBST) and spun again at 4000 rpm for 10 min at 4ºC. Resuspension of pellets in 600 µl ice cold PBST + Protease inhibitor (PI) mix and spinning at 13000 rpm for 5 min at 4ºC. Resuspension in 600 µl PBST + PI and bead beating (3x) to lyse cells. Spinning of tube for 1 min to settle beads and transfer of supernatant into a clean eppendorf tube. Centrifugation of lysates at 13 000 rpm for 1h at 4ºC to pellet cell wall fraction. Centrifugation of supernatants at 45 000 rpm for 1h at 4ºC to pellet cell membrane fraction. The supernatant is the cytosolic fraction. Washing of cell wall fraction with PBS containing PI mix and centrifugation at 13 000 rpm for 20 min at 4 ºC. Resuspension of cell wall and cell membrane in 6x SDS dye and 6 M urea. Prior usage heating of samples at 95ºC for 10 min.

For SDS-PAGE a 15% polyacrylamide gel was used. The same amount of cytosolic fraction (between 30-45 µg of protein) and an equal amount of cell wall and cell membrane fractions (based on total volume of the fractions) were loaded on the gels. Precision Plus Protein Standard (Biorad) was used as a size reference for the proteins.

After separation of proteins they were transferred onto PVDF membranes (Millipore) at 300mA for 2h at 4ºC. Non-specific binding sites were blocked by incubation in Odyssey Blocking Buffer (LI-COR) either for 45 min at RT or over night at 4ºC.

Membranes were incubated with primary antibody diluted in 1:1 Odyssey Blocking solution/ PBS + 0.05% Tween20 for 1h at RT. Flag-specific mouse antibody (Sigma) in a 1:2000 dilution was used to detect constructs without the Rv3671c-protease domain and Rv3671c-(protease domain)-specific rabbit antibody either purified in a 1:1000 or crude in a 1:2000 dilution were used to detect constructs containing the protease domain.

Membranes were washed in PBS + 0.05 % Tween20. Bound antibodies were detected with either goat anti-mouse800 (Invitrogen) or goat anti-rabbit800 (Invitrogen) in a 1:5000 dilution with Odyssey Blocking Buffer/ PBS + 0.05% Tween20. Membranes were incubated with fluorescent

secondary antibody for 45 min at RT and washed again in PBS + 0.05 % Tween20 to eliminate unbound antibody.

Bound secondary antibodies were visualized with a LI-COR-Odyssey Infrared Imager (LI-COR Biosciences).

3.6. Spotting Assay

Overnight cultures of *M. smegmatis* Δ*3671* were set up in 2 ml 7H9 + 0.05% Tween80 + Kan20 and grown to an OD_{580} of 0.7 - 1. Single cell suspension were made by spinning the cells at 800 rpm for 12 min at 4ºC. The supernatant is the single cell suspension.

10^{-1} to 10^{-4} dilutions of each cell culture were made in 7H9 using microtiter plates and 10 µl of each dilution + 10 µl of undiluted culture were spotted on square 7H10 Kan20 and 7H11 Kan20 plates with grids. Plates were incubated at 37ºC for 3-4 days.

3.7. Nigericin Assay

Overnight cultures of *M. smegmatis* Δ*3671* were set up in 2 ml 7H9 + 0.05% Tween80 + Kan20 and grown to an OD_{580} of 0.2 - 0.4. Cultures were diluted to an OD_{580} of 0.05 (2.77 x 10^7 bacteria/ml, which is equal to 1.11 x the desired initial density).

100 µl 7H9 + 0.05% Tween were added into each well of a 96 well plate with deep wells, except for the first three rows. To the first three rows 200 µl nigericin (100 µM) at 10 x the initial desired concentration was added (final concentration 10 µM). Using a multichannel pipettor, 100 µl were transferred to each third row (triplicates) (5 µM and 2.5 µM). No nigericin was added to the last three rows.

900 µl of the diluted cultures were added to each well, except for the last lane (control lane). Plate was sealed with Air Pore Tape Sheet and incubated on plate shaker at 37ºC for 4 days at 600 rpm. OD_{580} was measured every day, starting on Day 2, with a plate reader by sampling 100 µl of each well on a regular 96 well plate.

4. RESULTS AND DISCUSSION

4.1. Expression of pGMEK/pGMCK TM1&2 and pGMCK/pGMEK 4 TM

WT *M. smegmatis* and *M. smegmatis* Δ*3671c* were transformed with the constructs pGMEK and pGMCK-0x-hsp60-Rv3671c-TM1&2-protease-FLAG (abbreviated by pGMEK and pGMCK TM 1&2) and pGMCK and pGMEK-0x-hsp60-Rv3671c-4TM-FLAG (abbreviated by pGMEK and pGMCK 4TM). Comparison of their growth phenotypes on 7H10 Kan20 plates after transformation (Fig. 5) already allowed to draw first careful conclusions concerning the complementation of the mutants due to the constructs. It was commonly observed in several previous experiments with mycobacterial Δ*3671c* mutants (in Department of Microbiology and Immunology at Weill Cornell Medical College) that single colonies of the mutant were smaller in comparison to single colonies of the WT (in both, *M. smegmatis* and *M. tuberculosis*).

According to the small colonies of Δ*3671c* + pGMCK TM 1&2 (Fig. 5) compared to WT + pGMCK TM 1&2, complementation was not given with pGMCK TM 1&2. However, Δ*3671c* + pGMEK TM 1&2 colonies showed the same size as WT + pGMEK TM 1&2 and thus pGMEK TM 1&2 could be proposed to complement the mutants growth phenotype. An explanation for the different results of the same transmembrane construct could be that in the pGMCK vector the chromosomal expression of the construct was so low, that not enough construct was made in order to complement the mutant's phenotype, whereas episomal expression was sufficient.

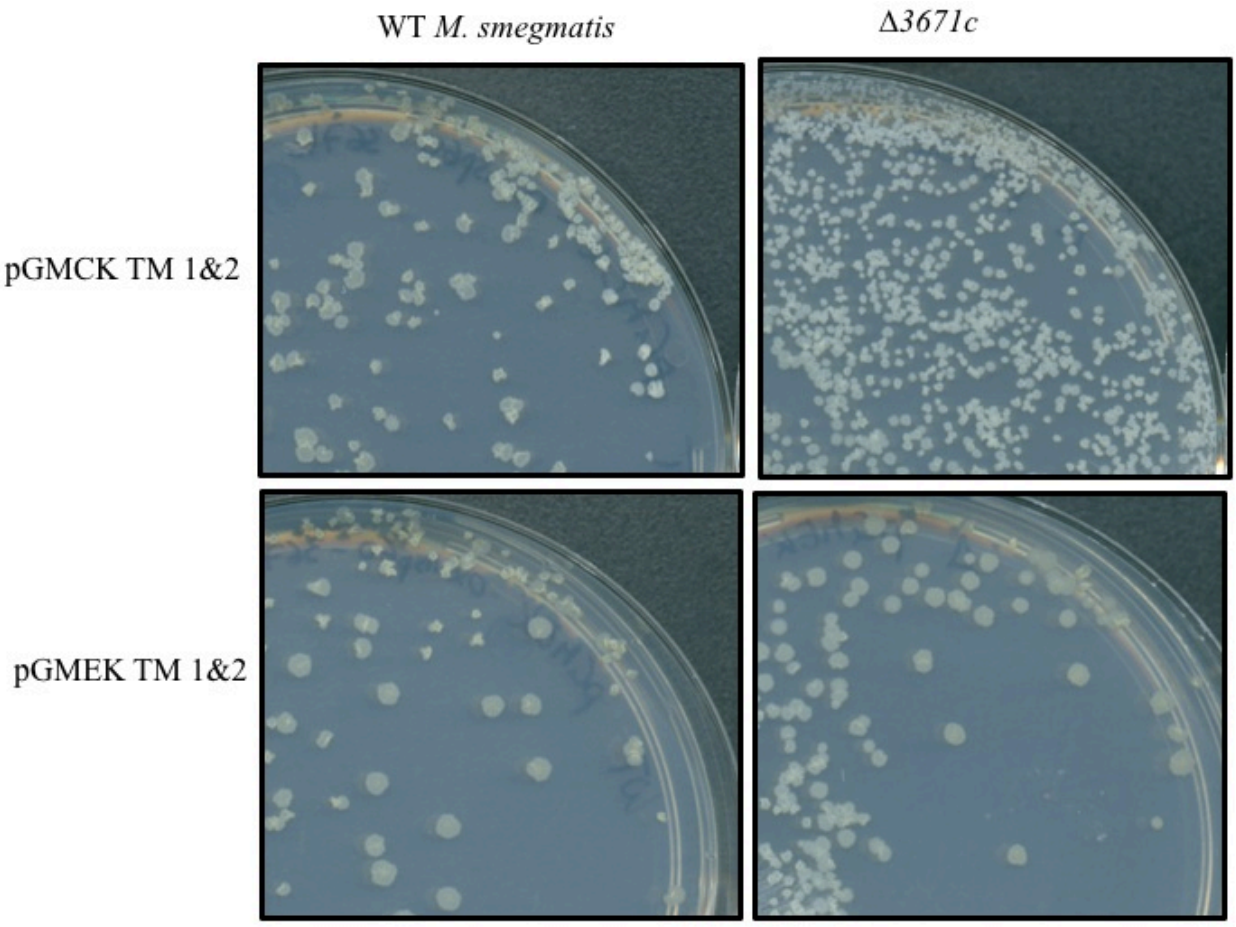

Fig 5: Growth phenotypes of WT *M. smegmatis* and Δ*3671c M. smegmatis* on 7H10 Kan20, both transformed with pGMCK TM 1&2 and pGMEK TM 1&2, respectively.

Before doing any further assays, expression of the constructs had to be tested. This was done through Western Blots with α-3671c purified antibody (Fig. 6).

The Western Blot of the TM 1&2 constructs was rather consistent with the conclusions regarding the growth phenotypes of the cells. However, α-3671c purified and crude antibodies have a lot of background noise in *M. smegmatis,* which results in a lot of unspecific detection.

Since Rv3671c is a transmembrane protein, we expect the 34.8 kD construct to be found in the insoluble fraction (membrane and cell wall fraction). No expression could be detected with the pGMCK vector and a low expression could be seen with the pGMEK vector. As a negative control WT *M. smegmatis* was used, since the α-3671c purified antibody does not bind the *M. smegmatis* homologue of Rv3671c. The complemented *M. smegmatis* knockout mutant (transformed with Rv3671c-Flag) was used as a positive control, with a protein size of 41.7 kD.

Unexpectedly, we found an intensive band of about 30 kD in the soluble fraction of pGMEK TM 1&2 (green arrow in Fig. 6), which could also slightly be seen in the soluble fraction of the positive control. This band could be a proteolytic product of Rv3671c or the degradation of the Rv3671c fusion construct by another protease due to the fusion construct being unstable. This band was also detected with two α-FLAG (Sigma and Millipore) and the α-3671c crude antibody (data not shown), which proves that it is a construct-specific product.

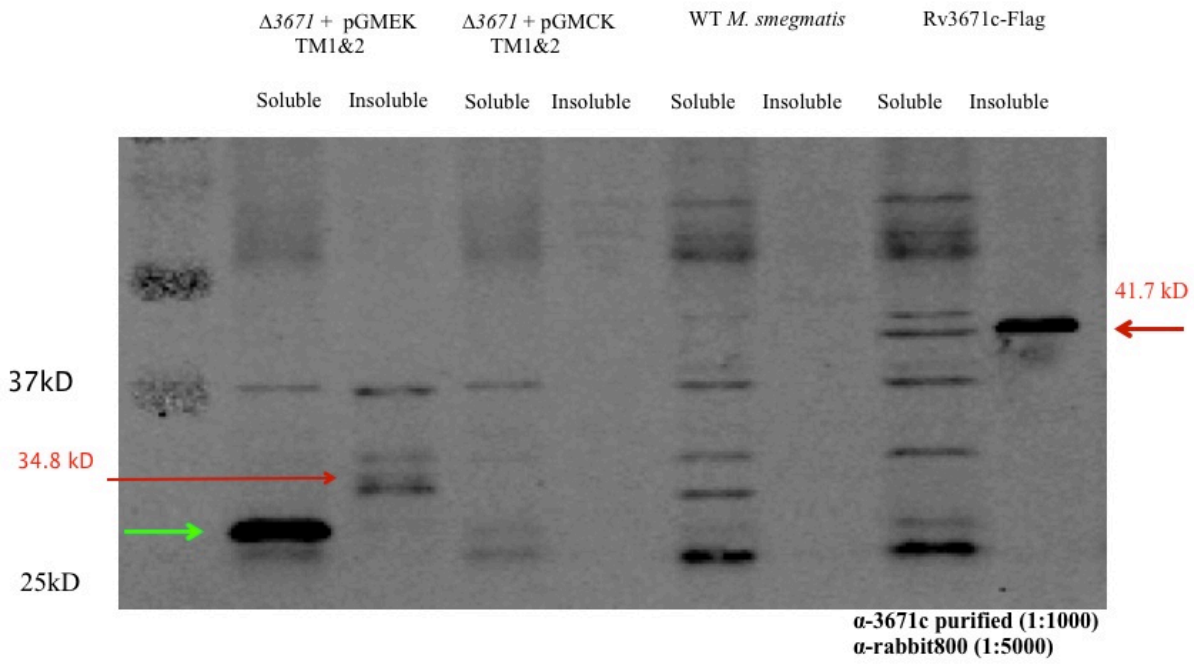

Fig 6: Detection of episomally (pGMEK) and chromosomally (pGMCK) expressed Rv3671c-TM1&2 (34.8 kD) in the lysates of transformed Δ*3671c M. smegmatis* by Western Blot. The protein is expected to be in the insoluble fraction. The green arrow shows a construct-specific undefined fragment in the soluble fraction. WT *M. smegmatis* was used as negative control, Δ*3671c* + Rv3671c-Flag as positive control (41.7 kD). The protein was detected using α-3671c purified (1:1000) and α-rabbit800 (1:5000) antibodies.

The same analysis was done with the pGMEK and pGMCK 4TM constructs, which lack the protease domain. Comparison of the growth phenotype of WT and Δ*3671c* after transformation showed that the colonies of the mutant with the 4TM constructs in both vectors did not have the same size as WT (Fig. 7). This could either be due to a lack of expression or to a lack of complementation. Expression of the construct was tested by doing Western Blots with α-FLAG antibody (Sigma and Millipore - Millipore data not shown). Detecting the 13.4 kD small construct with an antibody that showed even more background noise than α-3671c antibody

proved to be a challenge. Fig. 8 shows that even the positive control (Δ*3671c* + Rv3671c-Flag) at 41.7 kD was hard to detect. However, three different lysate preparations of the same transformation with pGMEK 4TM [(1) first lysates prepared from colonies after transformation, (2) lysates prepared about two weeks after transformation from cells that were in culture and had been split several times, (3) lysates prepared from cells in glycerol stock from colonies that were frozen after transformation] showed fragments at about the size of the constructs, which could not be detected in any of the controls.

Thus, we conclude that the 4 TM construct was expressed.

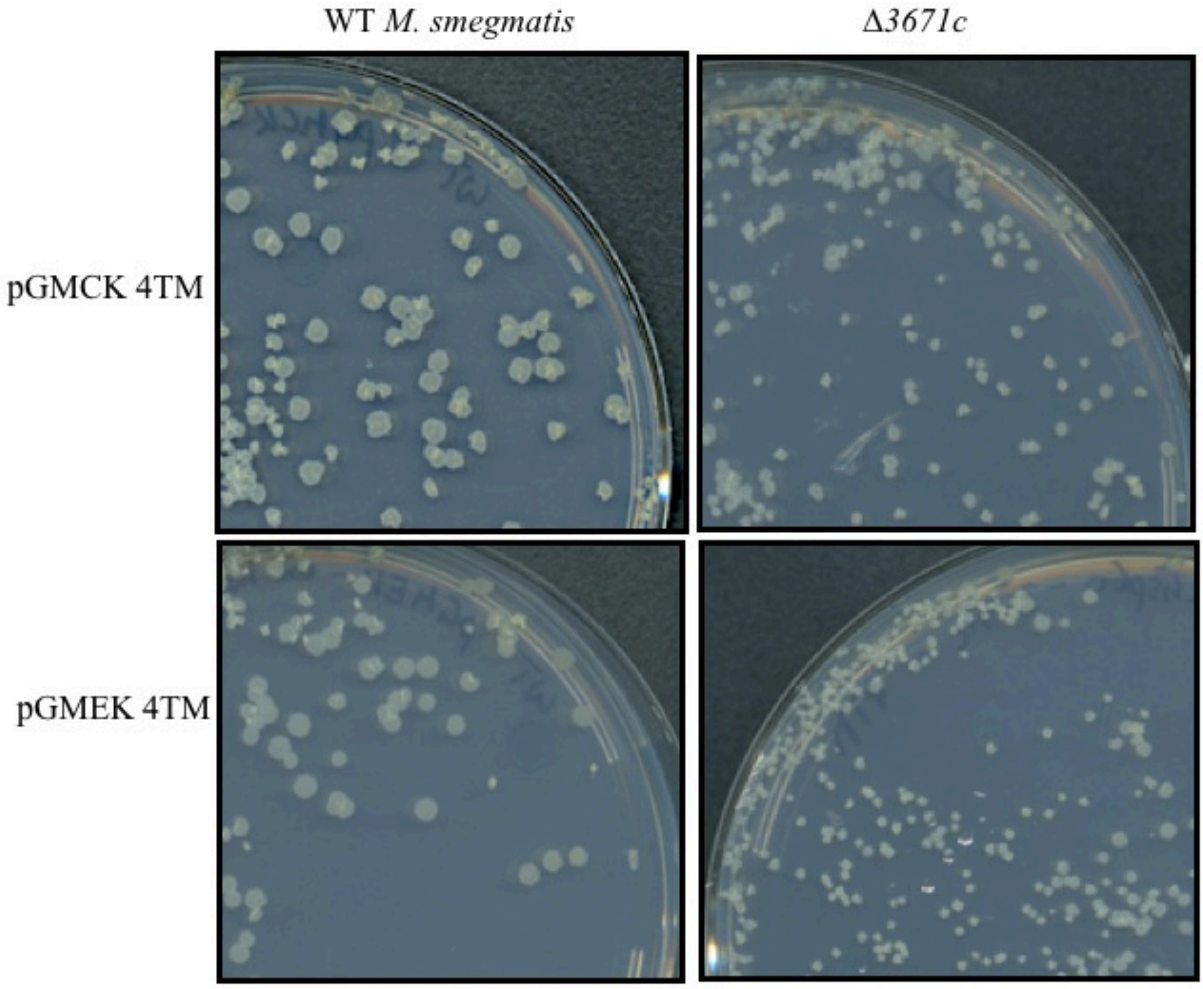

Fig 7: Growth phenotypes of WT *M. smegmatis* and Δ*3671c M. smegmatis* on 7H10 Kan20, both transformed with pGMCK 4TM and pGMEK 4TM, respectively.

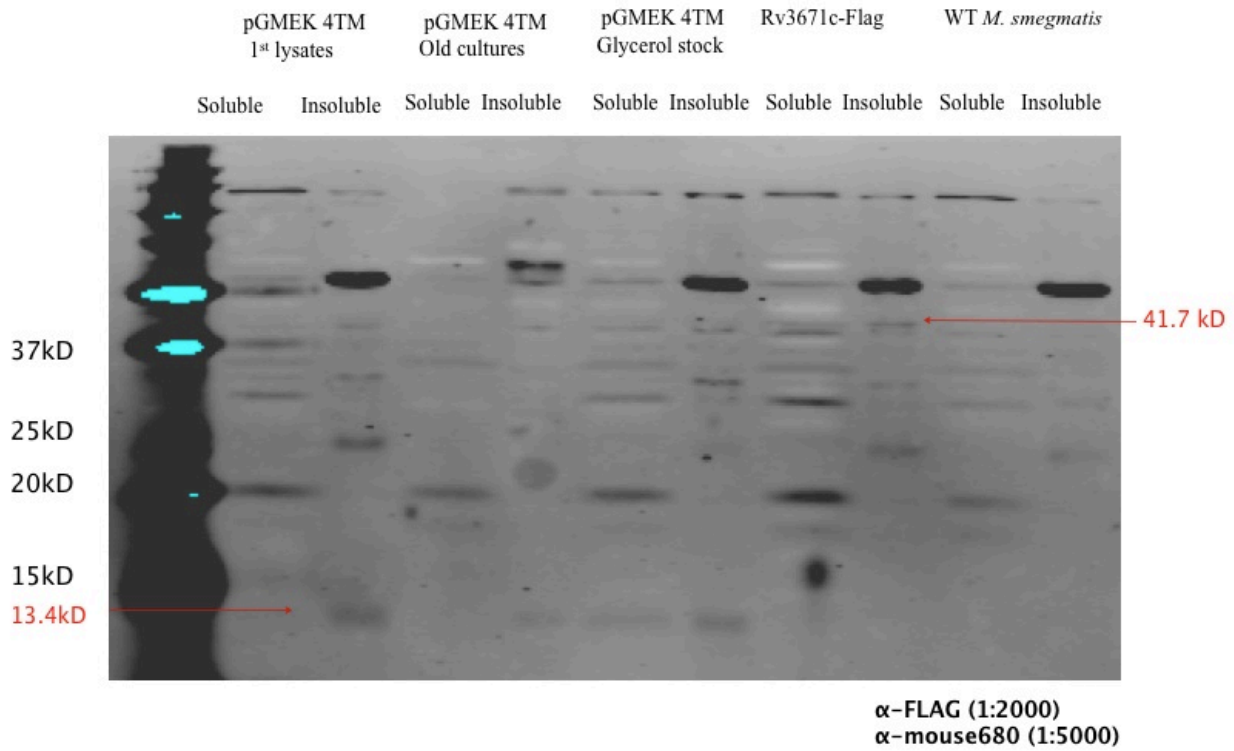

Fig 8: Detection of episomally (pGMEK) expressed Rv3671c-4TM (13.4 kD) in the lysates of transformed Δ*3671c M. smegmatis* by Western Blot. Three different lysate preparations of the same transformation were used: (1st lysates) first lysates prepared from colonies after transformation, (old cultures) lysates prepared about two weeks after transformation from cells that were in culture and had been split several times, (glycerol stock) lysates prepared from cells in glycerol stock from colonies that were frozen after transformation. The protein is expected to be in the insoluble fraction. WT *M. smegmatis* was used as negative control, Δ*3671c* + Rv3671c-Flag as positive control (41.7 kD). The protein was detected using α-FLAG(1:2000) and α-mouse680 (1:5000) antibodies.

Due to the lack of expression, the pGMCK constructs were not used for the following complementation assays.

4.2. Spotting Assay with pGMEK TM 1&2 and pGMEK 4TM in *M. smegmatis*

Complementation of the mutants with the transmembrane constructs was first tested using the Spotting Assay on 7H10 and 7H11 Kan20 plates. The difference between the two media is a higher concentration of the dye malachite green in 7H11, which obviously has a negative effect on the knockout mutant. This results in slower to no growth of the mutant, which is more obvious in *M. tuberculosis* than in *M. smegmatis* (compare Fig. 9 and Fig. 14). Growth of *M. tuberculosis* WT and *M. smegmatis* WT is not affected. Malachite green is used as an antibacterial, antifungal and antiparasitic agent and acts through poisoning of proteins of the

respiratory chain. This results in an uncoupling of the oxidative phosphorylation. Malachite green is only active in its oxidized form, reduction and decolorization inactivates its activities. Previous studies postulate that permeability of the cell wall among other things might be responsible for susceptibility to malachite green in *M. tuberculosis.* [25, 26]

WT *M. smegmatis* and Δ*3671c* + Rv3671c-Flag were used as positive controls, Δ*3671c* as a negative control. WT and Δ*3671c* had been transformed with a vector containing a Kanamycin resistance gene in order to be able to grow all cells on one plate.
Δ*3671c* was transformed with pGMEK TM 1&2 again in order to see if the results are reproducible. Both, the cells from the first transformation and the newly transformed cells, together with the mutants containing pGMEK 4TM were tested for complementation.

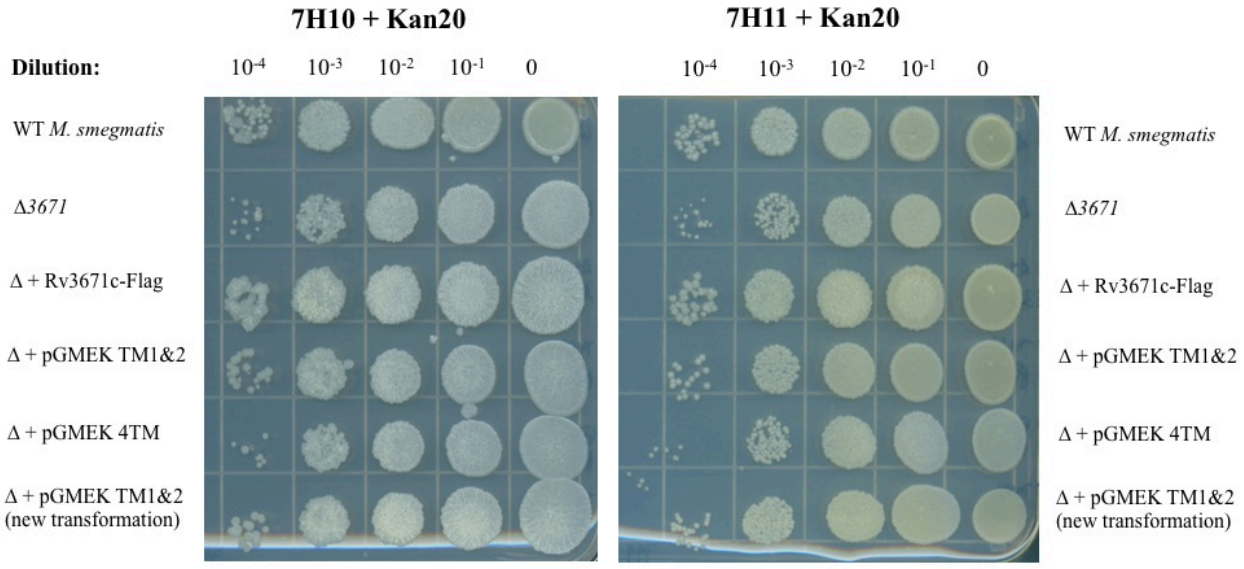

Fig 9: Spotting Assay used to determine if pGMEK TM 1&2 and pGMEK 4TM are able to complement the *M. smegmatis Rv3671c*-knockout mutant's growth deficiency on 7H10 and especially 7H11 (containing more malachite green) plates. WT *M. smegmatis* and Δ*3671* + Rv3671c-Flag were used as positive controls, Δ*3671* as negative control. Δ*3671* was transformed twice with pGMEK TM1&2 and both cultures were tested in the assay. Dilutions (10^{-1} to 10^{-4}) of each cultures were made in 7H9-Media.

Growth of the cells on the plates showed that mutants containing pGMEK TM 1&2 rather behaved like WT and complemented mutant, whereas the mutants containing pGMEK 4TM had a similar growth phenotype as the knockout mutant (Fig. 9).

Thus, we claim that the construct with only two transmembrane domains including the protease domain can complement the mutants phenotype in this assay, whereas the construct without protease domain (4TM) cannot. Hence, the protease domain is necessary for complementation.

Since permeability of the cell wall is postulated to be responsible for susceptibility of cells to malachite green, the growth deficiency of the mutant supports the assumption that Rv3671c is somehow involved in cell wall integrity. [17]

4.3. Nigericin Assay with pGMEK TM 1&2 and pGMEK 4TM in *M. smegmatis*

As a second complementation assay the Nigericin Assay was used. This assay was tested and optimized before by Uday Ganapathy from Department of Microbiology and Immunology at Weill Cornell Medical College.

The antibiotic nigericin is a K^+ ionophore, which was also shown to effectively transport Pb^{2+}. [27] It negatively affects ion transport in cells by causing efflux of K^+ and influx of H^+ (K^+/H^+ - antiporter) [28] and was also found to induce a potassium-dependent ATPase activity in mitochondria. [29, 30, 31]

WT *M. smegmatis,* Δ*3671c* and the complemented mutant were again used as controls and cells from both transformations with pGMEK TM 1&2 and mutants containing pGMEK 4TM were tested for complementation. Absorbance at 580 nm of each culture was measured at day 2, 3 and 4 after after adding the different concentrations of nigericin (10 µM, 5 µM, 2.5 µM and 0 µM) to the cells.

Results of the mutants containing pGMEK 4TM were consistent with the results seen in the spotting assay. The cells showed a similar absorbance as the knockout mutant of Δ*3671c*. This upholds our claim that the protease domain is required for complementation.

However, unexpectedly, the pGMEK TM 1&2 construct was not able to complement the knockout mutant in this assay. Mutants of both transformations with pGMEK TM 1&2 were

unable to reach the absorbance rates of WT and complemented mutant and instead were found to behave like Δ*3671c* and cells with pGMEK 4TM (Fig. 10).

A repeat of the assay brought the same results (data not shown).

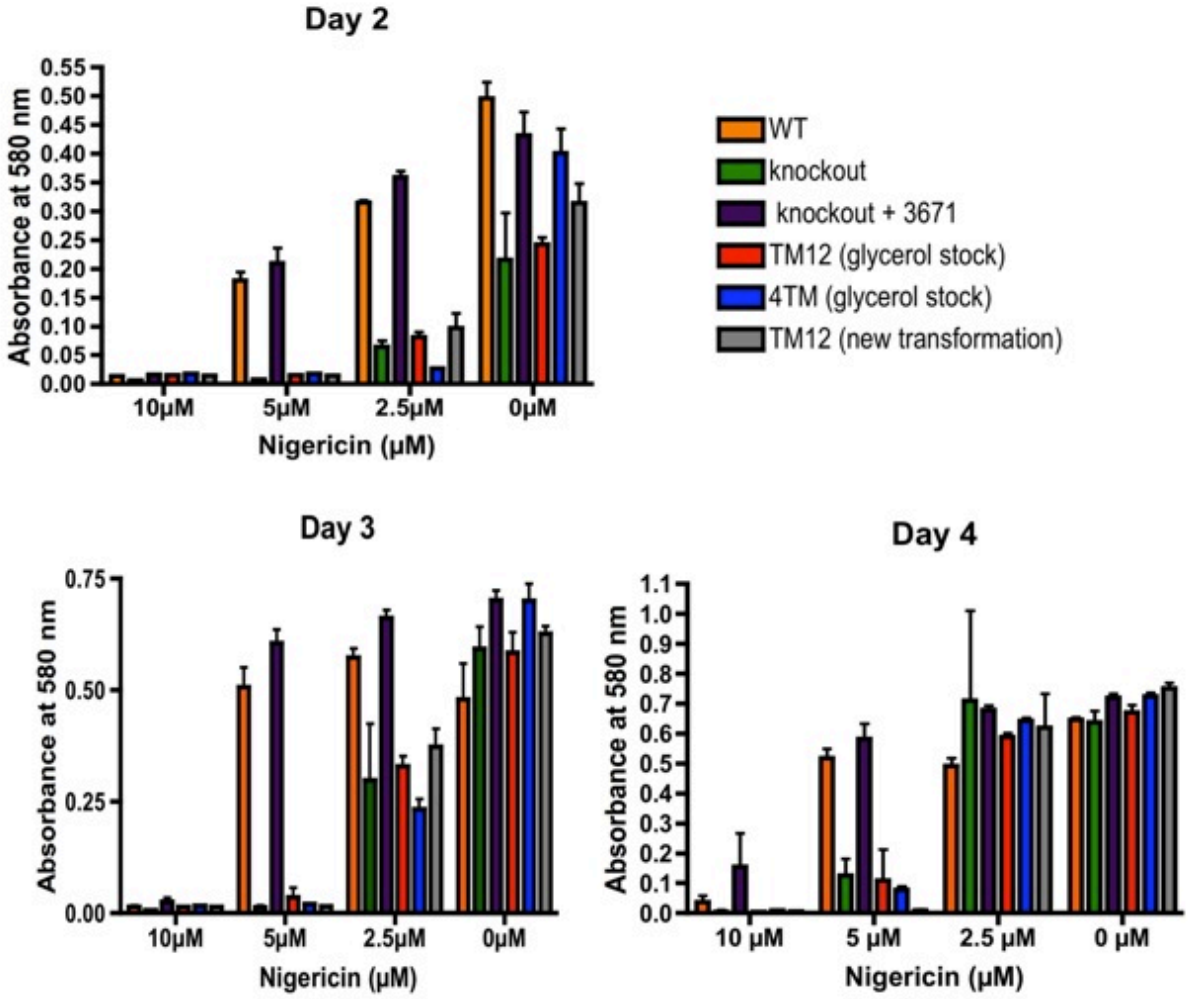

Fig 10: **Sensitivity of mutants containing pGMEK TM 1&2 and pGMEK 4TM to nigericin**. Cultures were exposed to different concentrations of nigericin (0 µM, 2.5 µM, 5 µM and 10 µM) and grown in 7H9 + 0.05% Tween in a final volume of 1 ml (Starting OD_{580} 0.05). Aliquots of each culture were taken at Day 2, Day 3 and Day 4 and OD_{580} was measured. WT *M. smegmatis* and knockout mutant + Rv3671c-Flag were used as a positive control, *Rv3671c* knockout mutant as a negative control. Δ*3671* was transformed twice with pGMEK TM1&2 and both cultures were tested in the assay

Since nigericin induces influx of H^+ in cells which - without counteractions - decreases the intracellular pH, the mutant's growth deficiency might be due to a defect in intracellular pH control. Thus, Rv3671c might also be involved in intracellular pH control.

4.4. Implications after comparison of the pGMEK TM 1&2 and pGMEK 4TM results of Spotting and Nigericin Assay

An explanation for the inconsistent results of the two assays could be that the (membrane/ cell wall?) stress exposure in the spotting assay might be comparatively low and can still be handled by the relatively low amounts of transmembrane construct 1&2 + protease that were made in the cells. The exposure to nigericin in a liquid culture might be a higher stress level and can not be coped with anymore. This might either result from a low expression of the construct in the first place or from the lack of functionality of TM 1&2-Rv3671c. The latter could be explained by the relatively small amount of protein that was found in the insoluble fraction (membrane). This could be due to a failure of integration in the membrane, which leads to degradation of the construct in the cytoplasm (or periplasm?) either by other proteins or by autocleavage. Rv3671c might need the four transmembrane domains for a stable integration into the membrane.
Another possibility is that Rv3671c dimerizes in the transmembrane region or is stabilized by other proteins and lack of two of the transmembrane domains impairs interaction. However, crystal structure analysis did not show any obvious binding and interaction sites for other proteins, which makes this option less likely. Nevertheless, the protease might interact with other proteins and immunoprecipitation assays could be used to further investigate this.
It should also be considered that the experiments were made in *M. smegmatis* and that results might look different in *M. tuberculosis*.

These results made it necessary to create three more constructs in order to see if the four transmembrane domains could be replaced by a single transmembrane domain of HtrA2 and to see if the protease domain could function in the cytoplasm, either moving freely (deletedTM) or fixed to the membrane (TM123).

4.5. Making of pGMEK TM123, pGMEK dTM and pGMEK HtrA2

The three new transmembrane constructs were also made by using the Multisite Gateway® Pro 3-Fragment Recombination Technology, which this time proved to be more difficult than with the first two constructs. A lot of error analysis had to be done with the BP and LR reactions. Finally WT *M. smegmatis* and Δ*3671c* were transformed with the three constructs (see Fig. 11). Growth phenotypes of the *M. smegmatis* knockout mutants after transformation with the three constructs give reason to the assumption that pGMEK TM123 and pGMEK dTM are not able to complement the knockout's phenotype, whereas pGMEK HtrA2 can. Before drawing further conclusions, expression of the constructs needs to be tested by Western Blots, however, it was decided that further tests and assays were only done in *M. tuberculosis*. Work in the BSL3 was done by Jennifer Small (from Department of Microbiology and Immunology at Weill Cornell Medical College), Western Blots and work with the constructs outside BSL3 was further done by me.

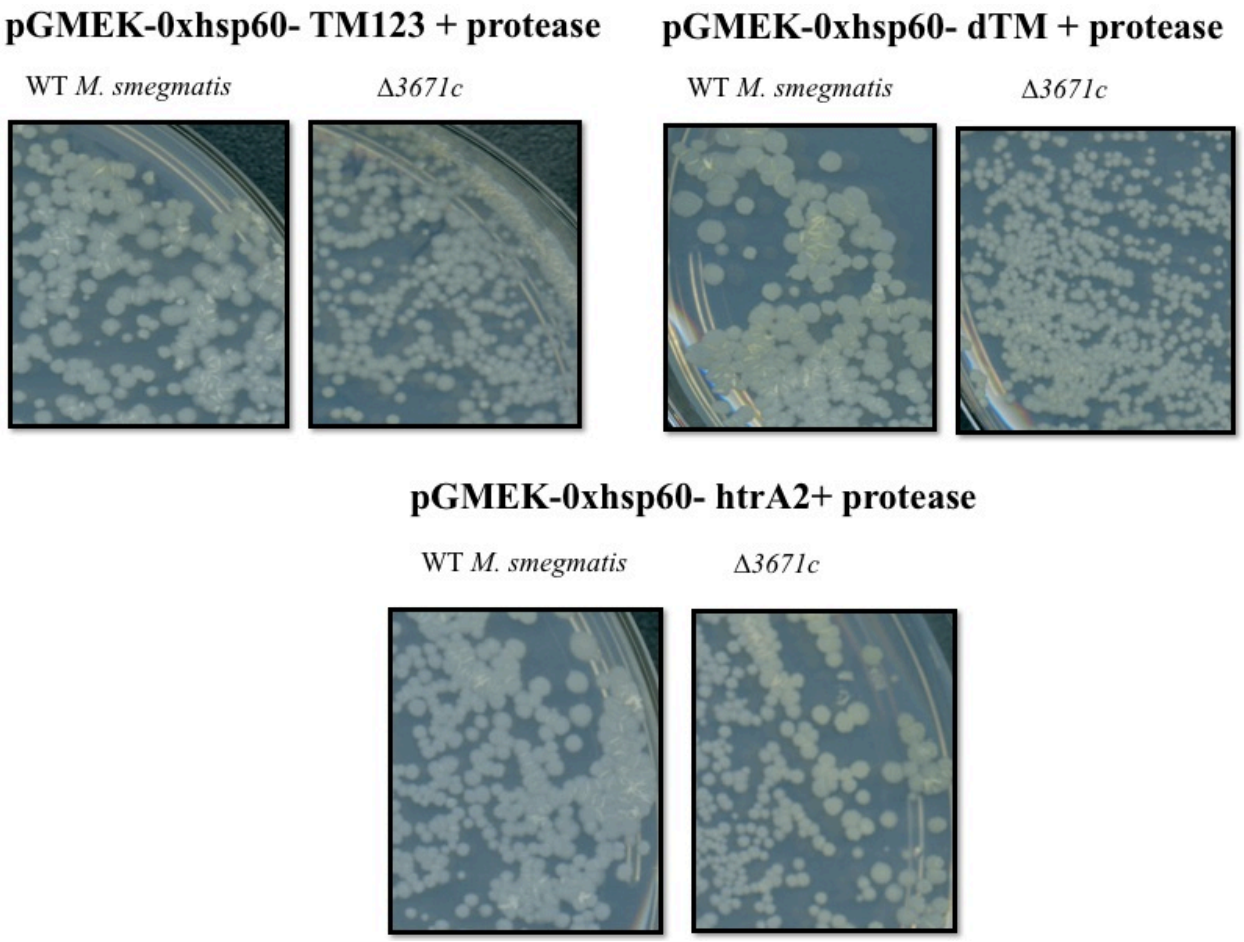

Fig 11: Growth phenotypes of WT *M. smegmatis* and Δ*3671c M. smegmatis* on 7H10 Kan20, both transformed with pGMEK TM123, pGMEK dTM and pGMEK HtrA2, respectively.

4.6. Transmembrane constructs in *M. tuberculosis*

The Western Blot with α-FLAG (Sigma) of the *M. tuberculosis* lysates showed expression of pGMEK TM1&2 (Fig. 12), but no expression of pGMEK 4TM (data not shown). Hence, pGMEK 4TM was not used anymore for further assays.

Also in *M. tuberculosis* a construct-specific smaller fragment (green arrow in Fig.) could be detected in the soluble fraction with both antibodies, α-FLAG (Sigma) and α-3671c (data not shown). This might also be due to a specific cleavage product of the construct.

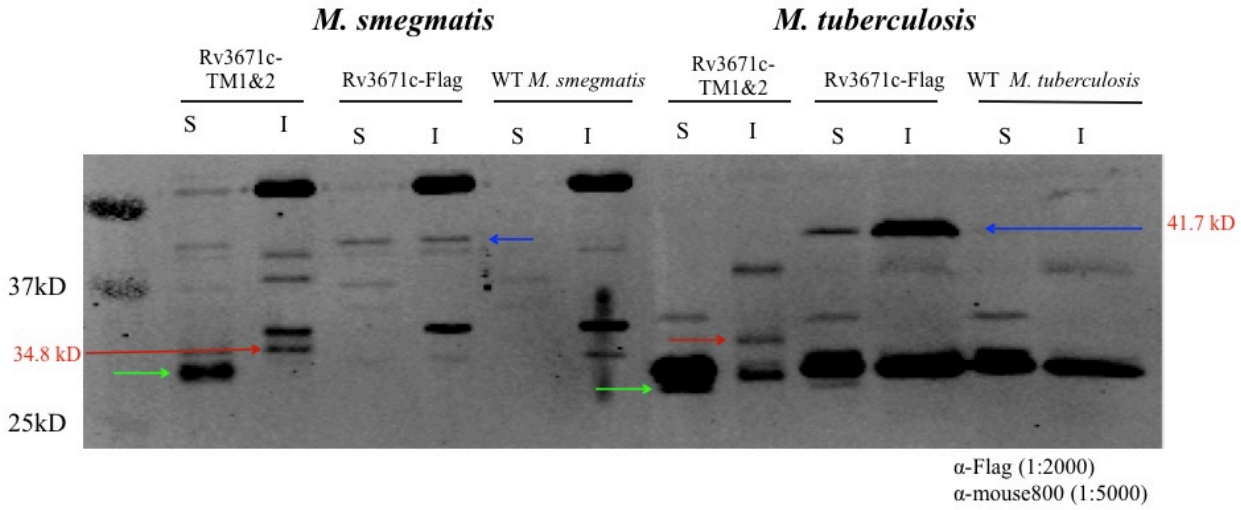

Fig 12: Detection of Rv3671c-TM1&2 (34.8 kD, red arrows) in the lysates of transformed Δ*3671 M. smegmatis* and Δ*3671 M. tuberculosis* by Western Blot. The protein is expected to be in the insoluble fraction. WT *M. smegmatis* and WT *M. tuberculosis (*H37Rv), respectively were used as negative controls, Δ*3671c M. smegmatis* + Rv3671c-Flag and Δ*3671c M. tuberculosis* + Rv3671c-Flag, respectively as positive controls (41.7 kD, blue arrows). The green arrows show construct-specific undefined fragments in the soluble fraction. The protein was detected using α-FLAG (1:2000) and α-mouse800 (1:5000) antibodies.

The Western Blot of dTM, TM123 and HtrA2 also showed expression of all three constructs in Mtb (see Fig. 13). A proteolytic product might be seen in the HtrA2-construct, however, unspecific detection occurs at the same size in every fraction, which makes it difficult to tell.

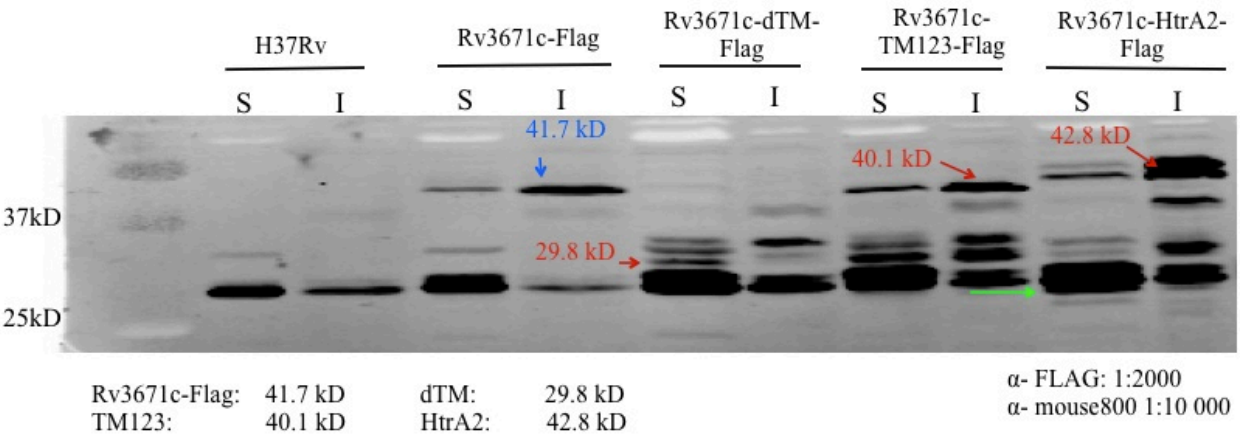

Fig 13: Detection of Rv3671c-dTM (29.8 kD), Rv3671c-TM123 (40.1 kD) and Rv3671c-HtrA2 (42.8 kD) (red arrows) in the lysates of transformed Δ*3671 M. tuberculosis* by Western Blot. dTM is expected to be in the soluble, TM123 and HtrA2 in the insoluble fraction. WT *M. tuberculosis (*H37Rv) was used as negative control, Δ*3671c M. tuberculosis* + Rv3671c-Flag as positive control (41.7 kD, blue arrow). The green arrow might be a proteolytic product of the HtrA2-construct. The protein was detected using α-FLAG (1:2000) and α-mouse800 (1:10000) antibodies.

The Spotting Assay on 7H10 and 7H11 Kan20 plates with the four constructs pGMEK TM1&2, pGMEK TM123, pGMEK dTM and pGMEK HtrA2 showed that neither pGMEK TM123, nor pGMEK dTM could complement the mutants phenotype. This result confirms the assumption that the protease domain needs to be located in the periplasm in order to be active. [18]
The result for pGMEK TM1&2 in *M. tuberculosis* is consistent with the result of the Spotting Assay in *M. smegmatis*. The construct with the two transmembrane domains is able to complement the knockout mutant on the plates up to a dilution of 10^{-3} on 7H10 and 10^{-2} on 7H11 (dilutions made from 10^{-1} to 10^{-4}). However, cells do not completely reach the same growth level as WT or Δ + Rv3671c-Flag.

pGMEK HtrA2 can partially complement the mutant, showing a slightly better growth at the 10^{-1} dilution than the knockout mutant.
The results of the Spotting Assay suggest that it is necessary for Rv3671c to have the protease domain in the periplasm and that the four transmembrane domains are needed for proper activity of Rv3671c. Thus, it seems that the four transmembrane regions cannot be replaced by the one transmembrane domain of HtrA2, implicating that the four TM domains have some kind of function, probably in the integration process of the enzyme into the membrane. They might also be needed for proper autocleavage in order to set the protease free for activity, an assumption that was already brought up by Vandal et al. [17] However, the observed site of autocleavage [18] was still contained in the constructs (near the N-terminus of the protease), so improper cleavage of the constructs might be due to a conformational change or steric hindrance resulting from the lack of one or more transmembrane domains which keeps the protease from reaching the proper cleavage site. However, the very low amounts of proteolytic product in the Western Blot of the positive control makes this hypothesis rather unlikely to be true.

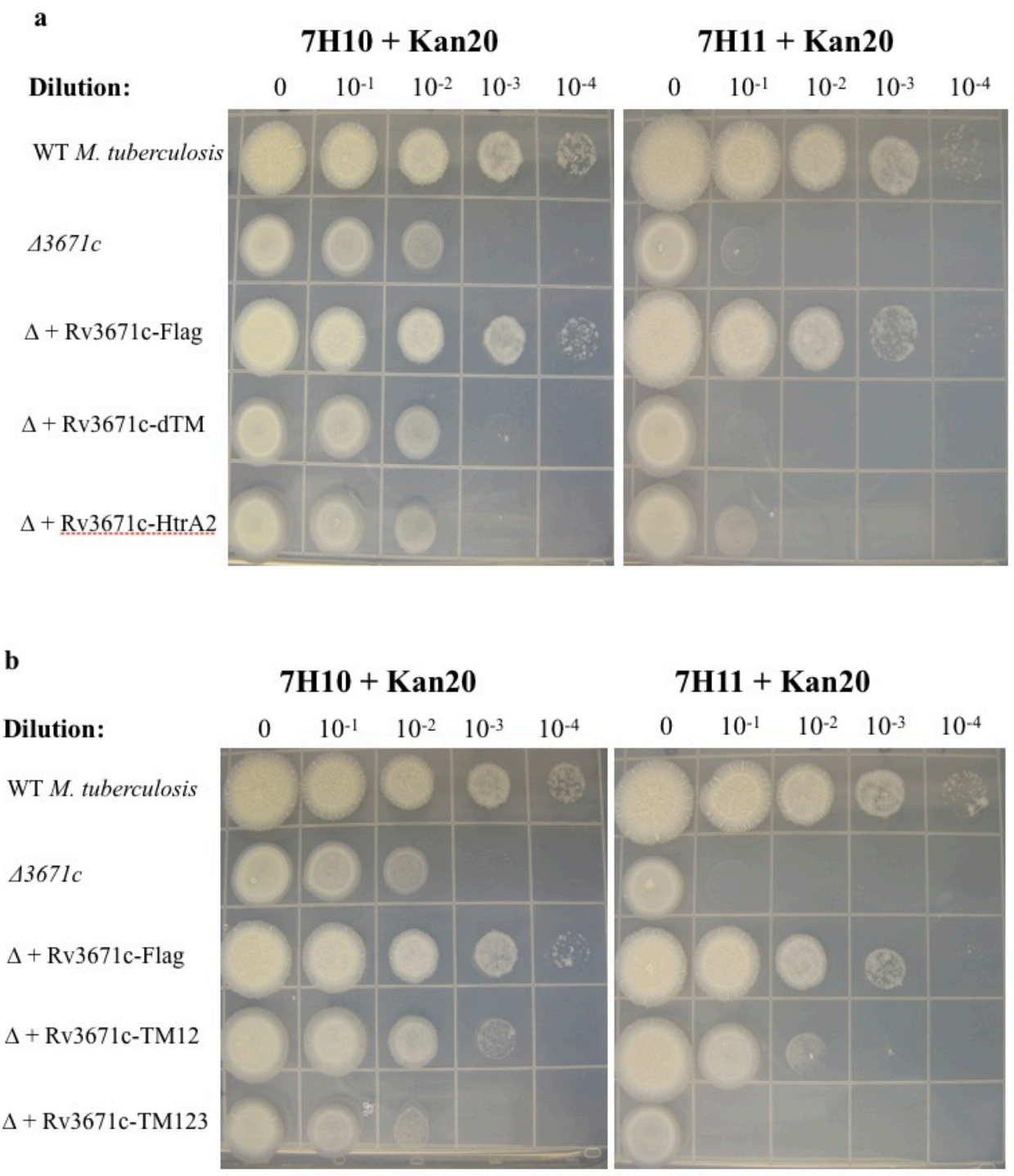

Fig 14: Spotting Assay used to determine if (**a**) Rv3671c-dTM, Rv3671c-HtrA2, (**b**) Rv3671c-TM1&2 and Rv3671c-TM123 are able to complement the *M. tuberculosis* Rv3671c-knockout mutant's growth deficiency on 7H10 and especially 7H11 (containing more malachite green) plates. WT *M. tuberculosis* and Δ*3671* + Rv3671c-Flag were used as positive controls, Δ*3671* as negative control. Dilutions (10^{-1} to 10^{-4}) of each cultures were made in 7H9-Media.

5. OUTLOOK

More assays need to be done in *M. tuberculosis* in order to see if the constructs can complement the mutant's deficiencies to cope with acid and H_2O_2. Also, an additional construct where the protease without TM domains is fused to a secretion signal that transports it into the periplasm could be made. This would address the question if the protease in its native environment needs to be anchored to the membrane. If not, this would further support the assumption that Rv3671c cleaves itself off the membrane in order to be active. [17]

As a final step, mice could be infected with the mutants containing the constructs and establishment of disease could be monitored. Insights into the function and significance of the four transmembrane domains could also give rise to a better understanding of the function of Rv3671c.

As an additional step, the experiment could be repeated by making the same constructs again, now containing a triple-mutation in the enzymatic core of the protease. This would answer the question if the construct-specific products in the soluble fraction are due to autocleavage.

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7. ABBREVIATIONS

att site	attachment site	kD	kilo Dalton
bp	base pairs	LB	Luria-Bertani (media)
BSL3	Biosafety Level 3	Mtb	*Mycobacterium tuberculosis*
Carb100	Carbenicillin 100 µg/ml	OD_{580}	optical density (580 nm)
dH_20	distilled water	ON	over night
DMSO	dimethyl sulfoxide	PCR	Polymerase Chain Reaction
dNTPs	deoxynucleotide triphosphates	rev	reverse
fw	forward	rpm	rounds per minute
HIV	Human Immunodeficiency Virus	RT	room temperature
HtrA	high-temperature requirement (protein)	TB	tuberculosis
IFN-γ	Interferon γ	TM	transmembrane
Kan50/Kan20	Kanamycin 50 µg/ml / 20 µg/ml	WT	wild type

4 TM	pGMEK-0x-hsp60-Rv3671c-4 transmembrane domains-Flag
dTM	pGMEK-0x-hsp60- Rv3671c-deleted transmembrane domains, only protease-Flag
HtrA2-TM	pGMEK-0x-hsp60-transmembrane domains of HtrA2 + Rv3671c protease-Flag
TM 1&2	pGMEK-0x-hsp60-Rv3671c-transmembrane domains 1 and 2 + protease-Flag
TM 123	pGMEK-0x-hsp60-Rv3671c-transmembrane domains 1, 2 and 3 + protease-Flag