Abstract

Mycobacterium tuberculosis, a causative agent of tuberculosis, is a worldwide spread pathogen that causes almost 2 million deaths each year (WHO report, 2009). Even though the combat against tuberculosis started many decades ago, due to complex, expensive, inconvenient and long term therapy the cure rates of the disease are not fully satisfactory. Moreover, in the recent years, there is an emergence of new multidrug resistant and extensively drug resistant strains, which worsens an already bad situation. The success of *Mycobacterium tuberculosis* as a human pathogen lies in the ability of this bacterium to precisely regulate its multiplication and sustain in the host in the non-replicative, latent state for very long periods of time. In addition, or maybe more importantly, mycobacteria possesses one of the most sophisticated cell envelopes, that have ever evolved in the prokaryotic world. Not only this structure protects the bacterium from extreme environmental conditions, like acidic pH of the phagosome, but also allows the pathogen to effectively modulate the response of the human immune system. Also, the mycobacterial cell wall is almost completely impermeable to most available antibacterial drugs. Due to their physiological importance, the processes of cell division and septal wall synthesis are potentially best targets for development of new drugs/ strategies to combat *Mycobacterium tuberculosis*. Nonetheless, poor understanding of unique for *Mycobacterium* features of FtsZ mediated cell division and peptidoglycan synthesis only restricted to midcell and cell poles remain large obstacles for finding new solutions to fight tuberculosis disease.

The cell division event follows replication of the genetic material of the bacterium and starts with assembly of a dynamic GTPase, FtsZ into circumferential ring at the cell's middle point. FtsZ ring serves as a cytoskeletal backbone needed for cell division and is later joined by several other proteins to form a division platform (divisome) between the two, newly forming cellular compartments. The main goal of the divisome components is to synthesize the latheral wall between the two evolving daughter cells, which are than splitted by cleaving the excess cell wall material with specific hydrolytic enzymes. All the proteins involved in the described above processes need to be recruited to the divisome in particular order since there

are two opposite forces – cell wall synthesis and hydrolysis, that need to be precisely orchestrated for successful cell multiplication.

This study concentrated on characterization of Rv0011c, a homolog of cell division implicated protein CrgA, only recently described in *Streptomycetes coelicolor*. This small, membrane bound protein was reported to be a negative regulator of FtsZ assembly in *S. coelicolor*, however was found to be essential for cell division progress of *S. arvemitilis*. Even though two opposite results were reported in very closely related species, both suggested the involvement of CrgA in FtsZ mediated cell division process. On the other hand *rv0011c* gene is placed in the immediate proximity of the cell morphology/ division cluster encoding proteins involved in cell wall synthesis RodA (*rv0017c*), PbpA (*rv0016c*) and serine/threonine kinases PknA (*rv0015c*) and PknB (*rv0014c*). Since it is commonly believed that genes placed in the proximity on the chromosome are likely to be involved in the same processes CrgA was predicted to be involved in cell wall synthesis as well.

Using a battery of molecular biology, genetic and biochemistry techniques the role of CrgA in the cell division and septal wall synthesis was investigated. Firstly, to check the importance of investigated protein for proper growth and cell division the strains with altered levels of this protein were created. The CrgA was found to be fairly abundant in the mycobacterial cells as its levels riches ~20,000 molecules per cell, which may support the relevance of this protein for growth of the bacteria. Growth rate, cell morphology and length were compaired between the wild type H37Rv, merodiploid strain overexpressing CrgA and strain partially depleted with this protein by antisensing. Increase in cell length by 36% and drastic drop in the growth rate was observed for the strain expressing antisense mRNA against *rv0011c* transcript, even though the immunoblotting reveled drop of cellular levels of CrgA by ~33% versus wild type. On the contrary, the strain overexpressing CrgA by almost 4,5 fold, revealed regular cell morphology and only slight change in growth rate, if compaired to wild type. The septum formation was also investigated by staining of cell's membranes with FM4-64 lipophylic dye. The strain depleted in CrgA revealed higher than wild type occurance of cells with midcell septum (~18 versus ~13% in the latter) however, many of long cells did not possess lateral membrane, suggesting that there are possible cell division defects in those cells.

The staining of nascent peptidoglycan with Vancomycin FL indicated that CrgA overproducing strain shows less septal signal (~9.4%) than the control strain (~14.5%). On the contrary antisense strain revealed vibrant mid-cell staining in ~19.5%. Additionally, the nascent peptidoglycan synthesis sites in *M. tuberculosis* were confirmed for the first time by

an alternative staining with fluorescent derivative of ramoplanin, known to bind murG protein and lipid I and II units of peptidoglycan.

CrgA subcellular localization was next tested by creating fusion protein with enhanced cyan fluorescent protein. The attempts to visualize ECFP-CrgA in the original host were unsuccessful due to demands of BSL-3 levels safety procedures and so it was decided to use only conditionally pathogenic surrogate host – *Mycobacterium smegmatis*. Fluorescent CrgA derived from *M. tuberculosis* was found to localize as a nice band at the mid-cell in ~13% of cells and as a foci at the cell poles of ~18% of *M. smegmatis* cells. Since there is 81% of overall homology between the two proteins derived from the two species they are predicted to be fully interexchangable. Localization of CrgA was found to be dependent on the proper FtsZ assembly into middle ring, and there was almost no mid-cell localization detected in the strain depleted in FtsZ. ECFP-CrgA colocalized nicely with FtsZ-EYFP protein at the cells middle point. Also, the intracellular N-terminal region of CrgA was predicted to be involved in subcellular trafficking of this protein since its removal resulted in significant reduction in localization.

CrgA protein was found to interact with FtsZ by biochemical pull down assay, and with other divisome components by bacterial two hybrid system. The positive interactions with FtsQ and two high molecular weight penicillin binding proteins – FtsI and PbpA, implicated CrgA in last steps of peptidoglycan synthesis. CrgA was also found to be able to self-interact which was further confirmed by an alternative method, size exclusion chromatography, showing CrgA to form dimmers.

Interactions of the CrgA with peptidoglycan synthesis components must have been relevant for the cells of *M. tuberculosis*, hence there were significant differences in the antibiotic resistant patterns discovered between the strains with altered CrgA levels versus the wild type H37Rv. The most striking was increased sensitivity of the strain overproducing the investigated protein to cephalexin, a beta lactam antibiotic with high affinity to FtsI. The same strain was also found to be more resistant to vancomycin, targeting the substrate for PBPs, including FtsI. Moreover, overall localization of PBPs was influenzed by overproduction of CrgA in *M. smegmatis* as shown by an innovative for mycobacteria staining with fluorescent analog of penicillin V - Bocillin FL. Similar observations were noticed when GFP-FtsI localization was investigated in CrgA overexpression background as well.

In this study CrgA protein from *M. tuberculosis* was characterized as a novel component of divisome, that is directly or indirectly implicated in the last steps of septal peptidoglycan synthesis. Importantly, its interactions with several septosomal proteins

including FtsZ, FtsQ, FtsI, PbpA and possibly other proteins influence dynamic of growth and antibiotic resistance patterns of pathogenic mycobacteria. The data gathered in the presented thesis expand still limited knowledge about the mycobacterial cell division and septum synthesis process. Moreover, from a practical angle, CrgA protein could potentially become a target for development of new drugs/ strategies to combat tuberculosis disease.