Stress to progress

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Scope of this thesis

The aim of our research was to gain insight into the adaptive strategies and stress response systems that mycobacteria deploy when challenged with antibiotics that have proven to be successful in the treatment of TB. We used this knowledge to develop genetic tools that will allow a more rapid identification and assessment of new compounds, both *in vitro* as well as *in vivo* using the zebrafish embryo infection model.

In **Chapter 2** we set out to unravel the genetic network involved in the biosynthesis of the capsular α-glucan, by performing transposon mutagenesis. In doing so, we identified both capsule-deficient as well as overproduction mutants and discovered novel members that play a role in the production of this capsular layer during stress conditions. In this work, we link the overproduction of capsular α-glucan to the stringent response, which is normally induced by nutrient starvation. In addition, we constructed stress reporters that respond to low-phosphate states, to address the formation of a capsular α-glucan as a result of phosphate limitation in *M. marinum.* We used these tools *in vivo* to address the relevance of phosphate limitation that mycobacteria encounter during infection.

In **Chapter 3** we present work on the major antibiotic stress operon in mycobacteria: the *iniBAC* operon. This operon is induced by ethambutol and isoniazid in *M. marinum.* We investigated the origin of the stress signal that leads to induction of the operon by constructing a stress reporter in which the production of a fluorescent reporter is coupled to the *iniBAC* promoter. We used this reporter to identify transposon mutants with constitutive IniBAC production. Interestingly, we found a clear and strong link with IniBAC induction and vitamin B12 production and the methylmalonyl CoA pathway. We also attempted to identify the metabolic cue that is responsible for the induction of the operon.

In **Chapter 4** we describe the identification of the regulator of the *iniBAC* operon, IniR. We show that IniR belongs to a novel class of transcriptional regulators in both *M. marinum* and *M. tuberculosis.* IniR can activate *iniBAC* transcription and induction is abolished in knockout mutants. Finally, we show that *iniBAC* can be directly induced by free trehalose. This induction is amplified when cell wall permeability is increased.

In **Chapter 5** we set out to characterize the role of the IniBAC proteins and address the question whether the proteins form a functional complex. We performed localization studies and showed that both IniA and IniC can be found in the cytosol and that IniA partially associates with the cell membrane. IniB appeared to be present in large amounts in bacterial culture upon treatment with EMB or INH. Analysis of *ΔiniA* and *ΔiniC* mutants showed that the production and secretion of IniB is dependent on the presence of IniA and IniC. We used ESX-1-, ESX-5-, and SecA2-deficient mutants to show that secretion of IniB is independent of these alternative secretion systems.

In **Chapter 6** we present the first comprehensive overview of the mycobacterial stress responses that are induced in *M. tuberculosis* and *M. marinum* after treatment with first-line antibiotics ethambutol, isoniazid, streptomycin and rifampicin and the second-line antibiotic ciprofloxacin. In

RNA sequencing experiments we showed that *M. marinum* displays very distinct 'stress fingerprints', which we could utilize to distinguish antibiotics by mode of action. We then generated stress reporters to study whether mode of action prediction was possible with these stress reporters. We screened a compound library of 190 compounds and identified three compounds that likely cause cell wall damage.

In **Chapter 7** we describe the development of a reporter strain that can be used to measure cytochrome bd expression in mycobacteria. Since cytochrome bd is an important survival factor *in vivo*, studying its expression could lead to new insights in the dynamics of complexes that are crucial for energy metabolism. In this chapter, we show that the *cydA* reporter can be activated in culture by several antibiotics that target the respiratory chain: bedaquiline (BDQ), clofazimine (CFZ) and Q203. This activation is concentration dependent. We also address the induction levels of the CydA marker in infection studies and in a zebrafish embryo infection model to determine the importance of cytBD during infection.

In **Chapter 8**, we reflect on the work presented in thesis and in **Chapter 9,** an overall summary of the work is given.