# Structural characterization of bacterial proteins involved in antibiotic resistance and peptidoglycan biosynthesis

Tassoni, R.

# 8. General conclusions

Tuberculosis (TB) is an infectious disease that has accompanied the existence of humans for millennia [48,105], and that still remains a leading cause of death in the world [274]. The main causative agent of TB, the pathogen *Mycobacterium tuberculosis* (Mtb), shows intrinsic resistance to a wide range of clinically available antibiotics, including β-lactams, making TB a particularly difficult disease to eradicate [185]. Resistance to β-lactams is mainly caused by the chromosomally encoded, broad-spectrum β-lactamase enzyme, BlaC, which is able of hydrolyzing the β-lactam ring [32,42,82,262]. Due to BlaC, TB treatment by β-lactams has not been considered feasible until β-lactam/β-lactamase inhibitor combinations became available. In 1995, the first study proved that β-lactams could effectively kill Mtb in combination with β-lactamase inhibitors [32]. Later, much research focused on the structural characterization of BlaC [266], and on its inhibition by the four clinically approved β-lactamase inhibitors clavulanic acid, sulbactam, tazobactam, and avibactam [111,112,256,283]. However, still much remains to be uncovered on the mechanistic details of BlaC inhibition.

Recently, important findings in our group pointed out that the kinetics of BlaC recovery after inhibition by clavulanic acid is greatly influenced by phosphate, with enzyme activity recovering at a much faster rate in the presence of phosphate [62]. In this thesis work, the structure of BlaC was solved in its free state and in complex with phosphate (chapter 2). Phosphate, that was shown to bind to conserved residues close to the active site, might help recovery of BlaC by acting as an alternative nucleophile to water in the hydrolysis of the covalent clavulanate intermediate. Phosphate is a common ion in the human body and its effect on BlaC recovery from clavulanate inhibition should be taken into account when designing TB treatments. Furthermore, the effect of phosphate on BlaC recovery from inhibition by sulbactam, tazobactam, and avibactam is still not known and should be assessed for better designing therapies against TB.

Although clavulanate was shown to effectively inhibit Mtb growth in combination with β-lactam antibiotics, it might not be the inhibitor of choice for TB treatment, as it was also reported to induce expression of chromosomal β-lactamases [182], and in some cases, even induce production of drug-efflux pumps, or overexpression of BlaC itself. One promising alternative to clavulanate is the synthetic inhibitor avibactam, which was shown to stably inhibit BlaC for more than 48 h [283]. Nonetheless, avibactam shows poor affinity for BlaC compared to the other inhibitors [283], and the possibility of β-lactamases to evolve resistance against avibactam cannot be excluded as shown by recent studies on an engineered carbapenemase-2 (KPC-2) enzyme from *Klebsiella pneumoniae* that conferred resistance to ceftazidime/avibactam combination therapy [8].

Optimization of β-lactam/β-lactamase inhibitor combinations is for sure one readily available solution to treat TB. However, the fast mutability of Mtb and its high tendency to develop drug resistance require that additional antibiotic targets must be identified and new drugs developed. Several factors are already known to contribute to Mtb drug resistance and could represent possible drug targets, including the Mtb mycolic acid-containing cell envelope [123,185,194,220], and the products of genes such as *ermMT* [18], *tap* [2], *whiB7* [185], and *whiB4* [178]. Furthermore, Mtb-specific protein export systems were found to be essential for pathogenicity and are being considered as possible targets for drug development [71].

The second part of the thesis focused on the structural and biochemical characterization of the Alr and YlmE proteins of *S. coelicolor* A3(2) (chapters 5-7). Alr and YlmE share 48.1% amino acid sequence similarity (25% identity), but their structural similarity is basically limited to the eight stranded α/β-barrel fold, which characterizes the *N*-terminal domain of Alr and the entire YlmE. Both Alr and YlmE covalently bind the PLP cofactor *via* an internal aldimine bond with a Lys residue, Lys46 in Alr and Lys40 in YlmE, that is located in the core of the barrel domain. However, little conservation is found in the residues surrounding the PLP cofactors, where the only conserved residues are an Arg and a Ser (Arg237 and Ser222 in Alr, and Arg226 and Ser211 in YlmE) that stabilize the PLP in the active site by hydrogen bonding.

Besides the α/β-barrel fold at the *N*-terminal domain, the structure of Alr contains a β-strand-rich domain located at the *C*-terminal end of the protein, which mediates the dimerization of Alr by interacting in a head-to-tail manner with the *N*-terminal domain of another monomer. Each Alr dimer contains two active sites, which form at the interface between the α/β-barrel of one monomer and the β-strand domain of the other. The entryway to the active site is a narrow cavity at the dimerization interface, and both monomers contribute essential catalytic residues to the active site. Conversely, YlmE was shown to crystallize as a monomer, which only consists of the α/β-barrel domain. In other words, YlmE completely lacks a dimerization domain, such as the *C*-terminal, β-strand domain of Alr. The YlmE oligomerization state in solution was further studied by SEC and native-PAGE obtaining contrasting results. While the monomeric state of YlmE was confirmed by SEC, native-PAGE experiments revealed the presence of four bands, suggesting the existence of at least four YlmE oligomerization states.

While the alanine racemization activity of Alr was confirmed *in vitro*, and $K_m$ values of 6.3±0.1 mM and 8.9±0.3 mM could be determined for L- and D-Ala, respectively, YlmE did not show any binding to L- or D-Ala, or to any of the other amino acids and small molecules tested for binding by crystallographic soaking and ITC. Instead, a substantial amount of nucleic acids was enriched by pull-down experiments in which the raw lysates of the *S. coelicolor* parent strain and of its

*ylmE* deletion mutant were incubated with YlmE as the bait protein. The MS analysis of the enriched molecules revealed that they mostly consisted of $tRNA^{Thr}$ and $tRNA^{Gly}$, and that they also contained RNA molecules with a similar molecular weight as tRNAs but that could not be identified as any of the known tRNAs. YlmE binding to tRNA was confirmed by EMSAs, ITC, and HSQC-NMR using commercial tRNAs as a substrate. Interestingly, following the titration of commercial tRNAs into $^{15}N$-labelled YlmE (200 µM) by HSQC-NMR showed a progressive disappearing of YlmE peaks, already at concentrations of ligand as low as 2.5 and 5 µM. Since no protein aggregation could be observed, the disappearance of YlmE peaks in the HSQC spectrum might be possibly explained by the formation of YlmE oligomers with a large molecular weight and a long tumbling time. However, no structural information is available on the mode of self-protein-protein interaction of YlmE. The prediction of possible dimerization interfaces of YlmE by the PISA and Eppic webservers only found one possible interface, which cannot explain the results obtained by native-PAGE and HSQC-NMR titration of tRNAs.

The identification of YlmE as a tRNA-binding protein is totally unexpected and novel. Since *ylmE* is co-transcribed with the essential cell division gene *ftsZ*, it is likely that *ylmE* itself plays a role in (the control of) cell division. If I am to speculate on a possible function, a possibility is that YlmE mediates the incorporation of a rare amino acid into the pentapeptide of the peptidoglycan (PG) subunits, e.g. as an easily recognizable marker for future septum sites. Such a minor moiety is unlikely to be seen in total PG analysis. Rare D-amino acids have recently been reported by Lam *et al.* [145], but a function has not yet been assigned to them. An additional mystery is the function of YlmD. The *ylmD* gene is situated between (and co-transcribed with) *ftsZ* and *ylmE*. Surprisingly, deletion of both *ylmD* and *ylmE* does not have an effect on morphogenesis, but deletion of *ylmE* alone has a detrimental effect on sporulation. It was proposed that *ylmDE* may form a toxin-antitoxin system involved in tRNA maturation or modification, but this has yet to be verified.

In the future, the YlmE-tRNA interaction should be characterized in detail. The formation of YlmE filaments in the presence of tRNAs could be tested by sedimentation experiments by ultracentrifugation in a similar way as for FtsZ [139], and their low-resolution structure could be studied by negative stain electron microscopy. The effect of tRNAs on YlmE oligomerization could also be studied by native-PAGE, and the affinity of YlmE for specific tRNAs should be assayed *in vitro*. One unresolved question also remains on the identity of the unknown RNA molecules enriched by pull-down, and experiments should be design to further elucidate their nature. Furthermore, the biochemical function of YlmE should be put in the context of *Streptomyces* cell division, and YlmE interaction with other cell division proteins should be taken into account, particularly with YlmD.