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Biochemical and Structural Characterization of the Interaction
between a Hybrid Antibiotic and an Antibiotic Resistance
Protein with their Target Enzymes

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Abstract

Most antibiotics are natural products evolved by microorganisms to target essential enzymes in other organisms. Extensive clinical use of antibiotics has driven resistance development, restricting many antibiotics' effectiveness. Therefore, exploring alternative antibiotic development strategies and deepening our understanding of resistance mechanisms is crucial.

The first topic of this thesis examines the putative self-resistance protein CszB, which is part of the biosynthetic gene cluster synthesizing the DNA gyrase inhibitor chloroszentirazine (Csz) in *Xenorhabdus szentirmai*. As part of the pentapeptide repeat proteins (PRP), CszB is hypothesised to associate with *X. szentirmai*'s gyrase, conferring self-resistance against Csz. Therefore, efforts were made to co-purify and co-crystallize CszB with a truncated fusion construct of the DNA-binding/cleavage domain of *X. szentirmai* gyrase and investigate their interaction with biochemical methods. The results showed no evidence for complex formation, suggesting that CszB doesn't associate with the DNA-binding/cleavage domain.

The second project examines how the hybrid antibiotic thiomarinol A interacts with its target isoleucyl-tRNA synthetase (IleRS). Thiomarinol A combines an analogue of the IleRS-inhibiting antibiotic mupirocin with a metal-chelating antibiotic. Despite their similarities, thiomarinol A remains effective against high-level mupirocin-resistant *Staphylococcus aureus*. Through thermal shift assays, we demonstrated a strong stabilisation of mupirocin-susceptible IleRS1 variants, while the mupirocin-resistant IleRS2 variant was stabilised significantly less. Cryo-electron microscopy analysis produced a preliminary structure at 3.5 Å resolution of *Priestia megaterium*'s PmIleRS1, bound to thiomarinol A. These results provide the first thiomarinol A-bound IleRS structure and the first quantitative comparison of thiomarinol A's interaction with IleRS1 and IleRS2 variants.

Zusammenfassung

Die meisten Antibiotika sind Naturstoffe, produziert von Mikroorganismen, die Schlüsselenzyme in fremden Organismen angreifen. Der übermäßige Einsatz von Antibiotika hat die Entwicklung von Resistenzmechanismen vorangetrieben und die Wirksamkeit vieler Antibiotika eingeschränkt. Daher müssen alternative Strategien zur Antibiotikaentwicklung erforscht und unser Verständnis von Resistenzmechanismen vertieft werden.

Das erste Projekt untersuchte das Antibiotikaresistenzprotein CszB, welches Teil des Biosynthese-Genclusters in *Xenorhabdus szentirmaii* ist, das den DNA-Gyrase-Inhibitor Chloroszentirazine (Csz) herstellt. Da CszB ein Pentapeptide-Repeat-Protein (PRP) ist, wurde vermutet, dass CszB an die Gyrase von *X. szentirmaii* bindet, um diese zu schützen. Es wurde versucht, CszB im Komplex mit einem trunkierten Fusionskonstrukt der DNA-Bindungs-/Spaltungsdomäne der Gyrase von *X. szentirmaii* zu reinigen und zu kristallisieren. Da es keine Hinweise auf Komplexbildung gab, ist die Schlussfolgerung, dass CszB nicht an die DNA-Bindungs-/Spaltungsdomäne der Gyrase bindet.

Das zweite Projekt erforschte, wie das Hybridantibiotikum Thiomarinol A die Isoleucyl-tRNA-Synthetase (IleRS) hemmt. Thiomarinol A vereint das IleRS-hemmende Antibiotikum Mupirocin mit einem Metall-chelatierenden Antibiotikum. Trotz der Ähnlichkeit zu Mupirocin bleibt Thiomarinol A wirksam gegen Mupirocin resistente *Staphylococcus aureus* Stämme. Durch biochemische Studien konnten wir eine starke Stabilisierung der Mupirocin empfindlichen IleRS1-Varianten durch Thiomarinol A demonstrieren, während die Mupirocin resistente IleRS2-Variante signifikant geringer stabilisiert wurde. Mit Cryo-Elektronenmikroskopie konnte eine vorläufige 3,5 Å auflösende Struktur von PmIleRS1 von *Priestia megaterium* gebunden an Thiomarinol A, erzielt werden. Diese Ergebnisse liefern die erste Struktur von IleRS gebunden an Thiomarinol A, und den ersten quantitativen Vergleich des Einflusses von Thiomarinol A auf IleRS1- und IleRS2-Varianten.

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1 Introduction

1.1 Natural Products and their Role as Antibiotics

In recent decades, antimicrobial resistance (AMR) has reached alarming levels, posing a persistent threat to global health (World Health Organisation, 2014) (World Health Organisation, 2025). To confront this concerning development, the continuous development of new antibiotics is crucial (Frieri et al., 2017) (Bernal et al., 2022). Most antibiotics used today are derived from secondary metabolites of bacteria or fungi, (Durand et al., 2019) that have been optimized through evolution as the organism's defence mechanism (Bernal et al., 2022).

The Golden age of antibiotic discovery started in the 1940s when Selman Waksman invented a systematic screening approach to identify antagonistic interactions between different bacteria (Waksman, 1947). Thereupon, the pharmaceutical industry developed high-throughput phenotypical screening methods (Williston et al., 1947), leading to the discovery of over 20 classes of antibiotic natural products (NPs) between the 1940s and the 1970s. With rising resistance and demand, however, this method has become too inefficient and expensive (Katz & Baltz, 2016). Not all antimicrobials exhibit their antimicrobial properties under laboratory conditions (Shi et al., 2022) and if they do, identifying the active compound among the background noise of known antimicrobials becomes increasingly difficult and makes the discovery of new NPs less likely (Durand et al., 2019) (Katz & Baltz, 2016). From the 1980s onward, pharmaceutical research mainly focused on scaffold modification and target-based screening but failed to generate enough new compounds to keep pace with rising AMR (Swinney & Anthony, 2011).

1.1.1 Antibiotic Combination Treatment

An alternative to *de novo* discovery is to enhance existing drugs through combination therapy. One strategy co-administers adjuvants with the antibiotic to increase bioavailability or inhibit resistance mechanisms, while another strategy uses antimicrobial combinations that act synergistically or target different pathways (Lee et al., 2026). In tuberculosis (TB) treatment, such combinations have improved efficacy and reduced resistance development compared to monotherapy (Davies et al., 2014). Apart from TB treatment, however, the clinical evidence for a prolonged clinical utility of current antibiotics through combination treatment is limited. Furthermore, the effects of two drugs administered together are heavily influenced by their dosage, pharmacodynamics, and pharmacokinetics: Despite simultaneous administration of the drugs, the components travel through the body individually and not necessarily to the same area or to the same extent. There are indications that differences in localisation and bioavailability even vary between patients, making the fine-tuning of the right dosage very

challenging (Lee et al., 2026) (Domalaon et al., 2018) (Klahn & Brönstrup, 2017). One strategy to circumvent this problem is the creation of antibiotic hybrids, which are discussed in **1.3** in more detail.

1.1.2 Antibiotic Discovery through Genomic Screening

Since high-throughput genome sequencing became more accessible and affordable, an alternative route to NP discovery has gained popularity: instead of relying on the observation of phenotypic effects, bioinformatic tools are used to identify biosynthetic gene clusters (BGCs) in bacterial genomes that encode the enzymes involved in NP biosynthesis (Blin et al., 2019). These enzymes within a BGC are often organized in recurring patterns that can be screened for through genome mining, leading to the discovery of an increasing number of secondary metabolites (Craney et al., 2013). This approach has shown that bacteria have the potential to produce a much larger number of secondary metabolites than previously predicted (Bentley et al., 2002), because only a fraction of them exhibit their antimicrobial properties under laboratory conditions (Shi et al., 2022).

1.2 The Pentapeptide Repeat Protein (PRP) CszB and its Interaction with Gyrase

The first topic of this thesis is a cooperation with *Prof. Dr. Helge Bode* (Zuo, 2024) and revolves around the pentapeptide repeat protein (PRP) CszB, which is part of the BGC involved in the synthesis of the NP chloroszentirazine (Csz) in *Xenorhabdus szentirmai* (Zuo, 2024). Csz exhibits antimicrobial activity against *Mycobacterium tuberculosis* by targeting the pathogen's gyrase (Liang et al., 2025). It is hypothesised that CszB protects the host's topoisomerase, conferring self-resistance (Zuo, 2024).

1.2.1 Biosynthetic Gene Cluster of Chloroszentirazine

Csz is a secondary metabolite produced by strains of the enterobacteria *Photorhabdus* and *Xenorhabdus* (Zuo, 2024) (Liang et al., 2025), which are obligate mutualistic symbionts of certain nematodes (Sajnaga & Kazimierczak, 2020). These enterobacteria produce a variety of secondary metabolites involved in the nematodes' food supply, including those that kill prey through insecticidal toxins or convert the cadaver into bioavailable nutrients (Bowen & Ensign, 1998) (Goodrich-Blair & Clarke, 2007). Antimicrobials like Csz, however, defend the prey against decomposition by bacterial competitors without harming the nematodes or themselves (Shi & Bode, 2018).

1 Introduction

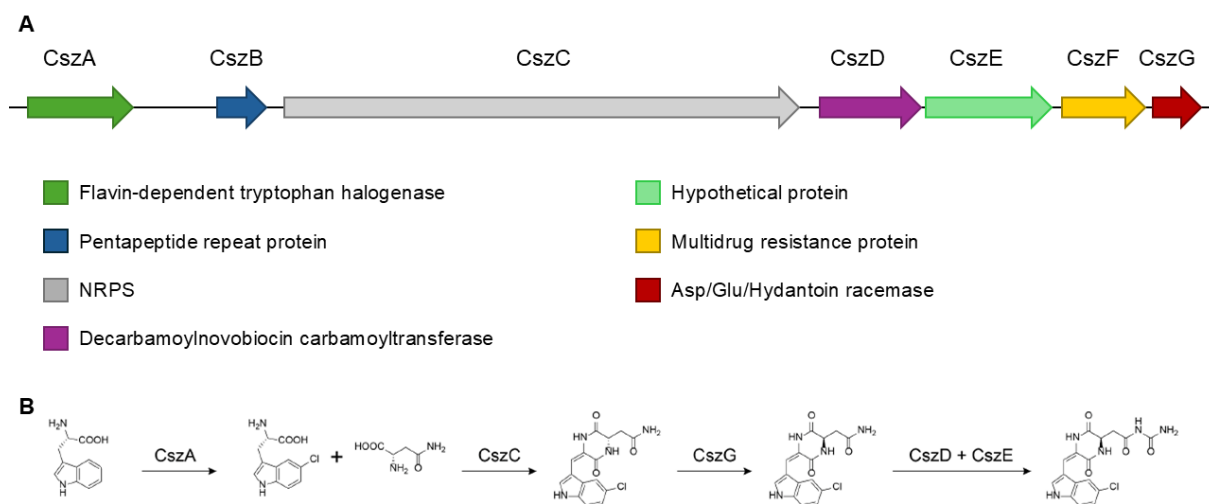


Figure 1: Biosynthesis of Csz.

A: Biosynthetic gene cluster encoding the seven proteins involved in Csz biosynthesis, transport and self-resistance. **B:** Proposed pathway of Csz biosynthesis. Kindly provided by Prof. Dr. Helge Bode (Max Planck Institute for Terrestrial Microbiology. Prof. Dr. Helge B. Bode., 2026).

The BGC of Csz encodes seven genes involved in antimicrobial biosynthesis, transport, and self-resistance (**Figure 1A**) and was identified by Prof. Dr. Bode and his group through self-resistance guided genome mining (Zuo, 2024) (Bode et al., 2019). This approach utilizes the presence of PRPs like CszB as indicators of BGCs that produce topoisomerase inhibitors, as some PRPs are known to confer self-resistance against these compounds (Zuo, 2024) (Bode et al., 2019) (Hug et al., 2019). CszF is a transporter, hypothesised to export the antimicrobial out of the host cell. Interestingly, omitting the gene for CszB only slightly suppresses growth in Csz-producing bacteria, and deleting the gene for CszF resulted in no growth suppression. However, deleting both genes significantly limited growth, indicating that both proteins are essential for the self-resistance mechanism (Zuo, 2024).

The remaining enzymes are involved in the biosynthesis of Csz (**Figure 1B**): CszC is a non-ribosomal peptide synthetase (NRPS), a large multi-modular enzyme complex (Singh et al., 2017) that assembles the 2,5-diketopiperazine scaffold by head-to-tail condensation of an L-tryptophan and an L-asparagine (Zuo, 2024). The cyclic form of the dipeptide enhances stability and bioavailability, making it a widely used framework for NPs with various purposes (Huber, 2022) (Bojarska et al., 2021). Four different tailoring enzymes are proposed to modify the precursor: The halogenase CszA adds a chlorine atom to L-tryptophan, the racemase CszG transforms the asparagine from L to D-configuration, and the carbamoylation is performed by CszD and CszE (Zuo, 2024).

1.2.2 Gyrase

Genomic screening of Csz-resistant *M. tuberculosis* colonies confirmed that the NP targets the DNA gyrase, as no other mutations were shared between the resistant strains (Liang et al., 2025). Gyrase is part of the type IIA topoisomerase family, including Topo IV in prokaryotes and Topo II in eukaryotes (Forterre et al., 2007). Type IIA topoisomerases use ATP hydrolysis to introduce transient DNA double-strand breaks to transfer a DNA segment through the gap (Mizuuchi et al., 1980). While Topo IV is usually responsible for decatenation during replication (Zechiedrich et al., 1997), gyrase removes torsion accumulating in front of DNA and RNA polymerases by actively introducing negative supercoiling into the circular DNA (Gellert et al., 1976). However, in some bacteria, including *M. tuberculosis*, gyrase is the only type IIA topoisomerase and must compensate for the missing Topo IV (Hirsch & Klostermeier, 2021) (Stracy et al., 2019).

1.2.2.1 Gyrase Structure and Catalytic Mechanism

Gyrase consists of the two subunits GyrA and GyrB, assembling into an A₂B₂ hetero-tetramer with three molecular interfaces called gates (**Figure 2**) (Mizuuchi et al., 1978) (Roca et al., 1996). The GyrB subunit has an N-terminal ATPase domain, a transducer domain, and a Mg²⁺-dependent topoisomerase-primase (TOPRIM) domain on the C-terminus (Fu et al., 2009). GyrA's N-terminus consists of a Winged-helix domain (WHD), accommodating the catalytic tyrosines, the tower domain, and a coiled-coil domain. Together, these domains form a heart-shaped dimer called the breakage-reunion domain (BRD) that is involved in DNA binding and cleavage (Morais Cabral et al., 1997). After a connector, the C-terminal domain (CTD) follows in a β-pinwheel fold, which is involved in wrapping the DNA in position (Reece & Maxwell, 1991) (Corbett et al., 2004) (Broeck et al., 2019).

The DNA-gate is formed by the two TOPRIM domains of GyrB and the WHDs of GyrA (Morais Cabral et al., 1997). The two N-terminal ATPase domains of GyrB dimerise to form the N-gate (Brino et al., 2000). The C-gate is formed by the coiled-coil domains of GyrA (Rudolph & Klostermeier, 2013).

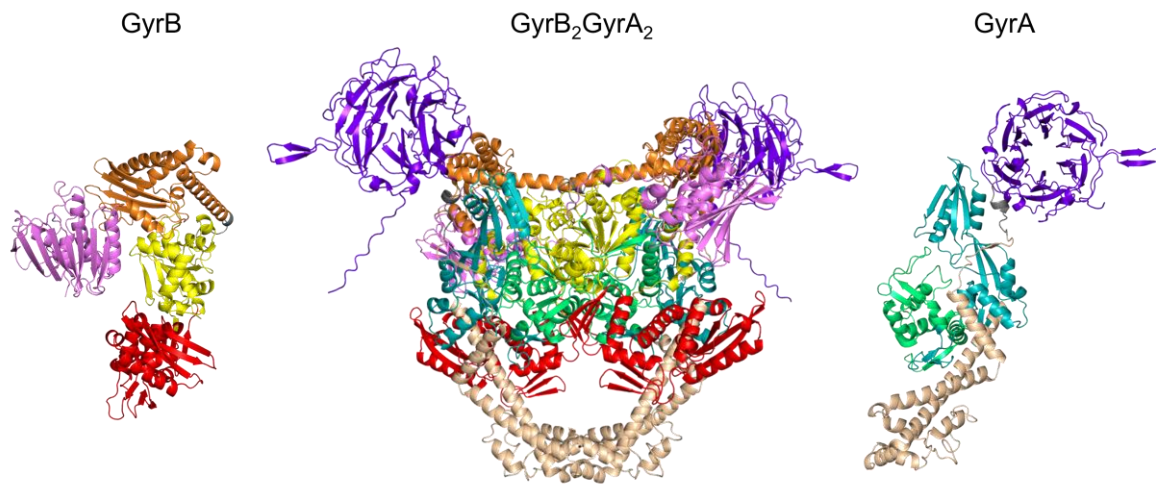
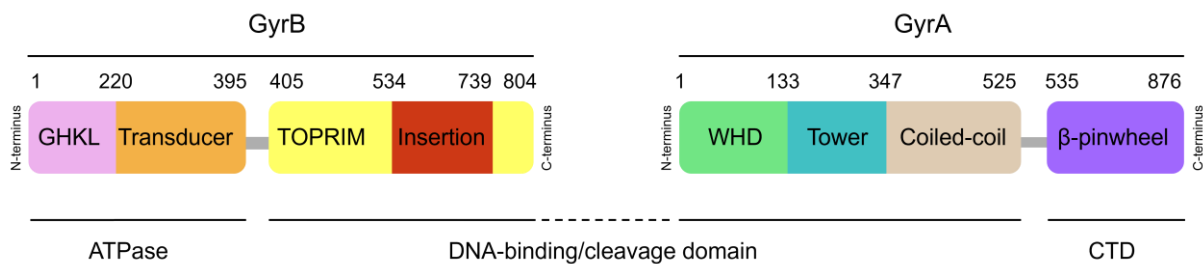
A**B**

Figure 2: Gyrase of *X. szentirmaii* with color-coded domains.

A: AlphaFold predictions (Abramson et al., 2024) of *X. szentirmaii*'s gyrase subunits GyrB (left) and GyrA (right) and the assembled hetero-tetramer GyrB₂GyrA₂ (middle). **B:** Domain architecture of *X. szentirmaii*'s gyrase subunits GyrB (left) and GyrA (right), color-coded according to the cryo-EM structure of the full-length *E. coli* gyrase (Broeck et al., 2019).

To start the catalytic process, the gyrase captures two segments of the circular DNA: The gate-segment (G-segment) is captured at the DNA-gate (Morais Cabral et al., 1997), the adjacent DNA is wrapped around the GyrA-CTD, and the transported-segment (T-segment) is placed above the G-segment, forming a positive node within the same circular piece of DNA (Reece & Maxwell, 1991). Upon ATP binding, the two N-terminal ATPase domains of GyrB dimerise and close the N-gate (Brino et al., 2000). The catalytic tyrosines of WHD covalently bind to the 5' ends of the DNA via a phosphodiester bond, and the G-segment is cleaved (Horowitz & Wang, 1987). The DNA-gate parts, and upon hydrolysis of one ATP, the T-segment is translocated through the gap to the bottom cavity, introducing negative supercoiling (Gubaev et al., 2009). The DNA-gate closes, the G-segment is re-ligated, and upon hydrolysis of the second ATP, the C-gate opens to release the DNA (Rudolph & Klostermeier, 2013).

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1.2.2.2 Structure Determination of Gyrase

Due to the high degree of flexibility of the outer regions, crystallisation of the native gyrase hetero-complex is very difficult. A common strategy is to crystallize single domains if a certain area of the protein is of interest (Lamour et al., 2002). To investigate as much of the complex as possible, a popular approach is to delete the GyrB N-terminus (Dales et al., 1999) and the GyrA C-terminus and fuse the adjacent GyrB C-terminus with the GyrA N-terminus (Bax et al., 2010) (Blower et al., 2016). The resulting fusion protein of the DNA-binding/cleavage domain reduces flexibility through truncation and promotes correct complex formation through the fusion of the subunits (Kobe et al., 2015). Recently, a 6.60 Å cryo-EM structure of the complete *Escherichia coli* (*E. coli*) DNA gyrase heterocomplex with DNA has further demonstrated an alternative route for structure determination (Broeck et al., 2019).

1.2.3 Small Molecules as Gyrase Inhibitors

Gyrase's crucial role in DNA remodelling, paired with their phylogenetic difference to eukaryotic topoisomerase II homologues (Forterre et al., 2007) makes them an excellent target for antibiotics. Although there are plenty of toxins targeting gyrases (Ruan et al., 2024), only two classes of gyrase inhibitors are currently in clinical use (Khan et al., 2018):

Aminocoumarins, including the most common NPs novobiocin, clorobiocin and coumermycin A1, are catalytic inhibitors that prevent ATP hydrolysis by competitively targeting the ATPase domain (Khan et al., 2018) (Ali et al., 1993). Despite their potency, these inhibitors are limited by their spectrum of activity, toxicity, and solubility (Khan et al., 2018).

One of the most successful antibacterial drugs, on the other hand, are the quinolones, including the especially popular fluoroquinolones (FQ), which act as gyrase poisons (Suda et al., 2013). The small molecules stack between the DNA base pairs and non-covalently bind to the GyrA domain at the cleavage site (Blower et al., 2016) (Wohlkonig et al., 2010). This interaction traps the gyrase after DNA cleavage with a closed DNA-gate and physically blocks re-ligation of the G-segment. By converting gyrases and Topo IVs into stabilized cleavage complexes, quinolones increase the number of double-strand breaks and thereby poison the cell (Drlica et al., 2007) (Aldred et al., 2014).

Structural similarities and RNA-sequencing analysis comparing Csz to FQs and other antibiotics groups Csz close to FQs (Liang et al., 2025) (Zuo, 2024). Csz is highly potent and selective against *Micrococcus luteus* and *M. tuberculosis* while exhibiting low toxicity for mammalian cells and commensals (Liang et al., 2025). Genomic screening of Csz-resistant strains revealed that all mutations occur within the DNA-binding cleft of GyrA, comparable to mutations associated with resistance to the fluoroquinolone moxifloxacin. A cross-resistance study, however, revealed that mutations conferring resistance to one compound do not

necessarily lead to resistance against the other, suggesting an independent mode of action between FQs and Csz (Liang et al., 2025).

1.2.4 PRPs as Gyrase Protectors

Apart from these small molecules acting as catalytic inhibitors or poisons, there are also proteins targeting gyrase in a regulatory or protective way: A small subset within the PRP superfamily associates with their host gyrase to protect it from quinolones and other gyrase inhibitors (Ruan et al., 2024) (Zhang & Kennedy, 2021). PRPs are characterized by a repetitive, five amino acids long sequence, folding into the characteristic right-handed quadrilateral β -helix (Hegde et al., 2005) (**Figure 3**). Typically, two PRP monomers associate via their C-termini to form a rod-shaped dimer with the approximate dimensions of a short double-stranded DNA. Although many PRP gyrase inhibitors have been described structurally, their mode of interaction is not completely understood, and different models have been proposed so far (Ruan et al., 2024) (Zhang & Kennedy, 2021).

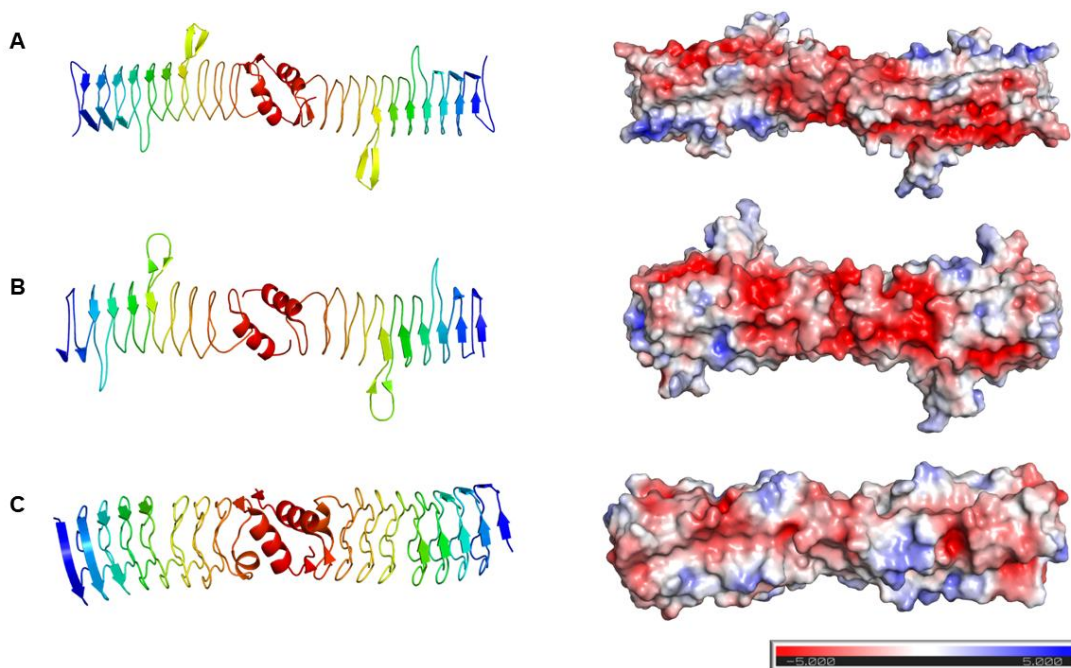


Figure 3: Structures (left) and charge distribution (right) of the PRPs CszB, QnrB1 and MfpA.

A: AlphaFold prediction (Abramson et al., 2024) of CszB from *X. szentirmaii*. **B:** Crystal structure of QnrB1 (PDB: 2XTW) from *Klebsiella pneumoniae* (Vetting et al., 2011). **C:** Crystal structure of MfpA (PDB: 6ZT4) from *Mycobacterium smegmatis* (Feng et al., 2021).

When the first PRP structure, MfpA from *M. tuberculosis*, was resolved, its structural and electrostatic similarities to DNA were discovered. This observation formed the initial hypothesis of the G-segment mimic model: The model proposes that PRPs competitively bind to the

gyrase's DNA-binding/cleavage domain instead of the G-segment, thereby reducing the formation of toxic stabilized cleavage complexes (Hegde et al., 2005). However, this model is inconsistent with most PRPs since they don't inhibit the supercoiling activity of DNA gyrase (Vetting et al., 2011).

The more recent T-segment mimic model, on the other hand, proposes that the PRP competes with the T-segment for binding at the N-gate. This model is strongly supported by the crystal structure of MfpA from *Mycobacterium smegmatis* bound to the ATPase domain from GyrB (Feng et al., 2021). In a quinolone-stalled gyrase, it is proposed that MfpA interacts with the N-terminal ATPase of GyrB, causing conformational changes that open the DNA-gate and dissociate the quinolones, reconstituting the gyrase (Feng et al., 2021). The T-segment model is widely accepted for MfpA from *M. smegmatis*, however, there are still open questions when it comes to other PRPs:

While MfpA from *M. smegmatis* can rescue the gyrase, the direct homologue in *M. tuberculosis* needs the small GTPase (MfpB) to confer quinolone resistance (Tao et al., 2013). Studies have shown that the PRP QnrB1 binds GyrB ATPase and depends on ATP hydrolysis to release FQs from the stalled gyrase (Mazurek et al., 2021). However, biochemical analysis demonstrated that the PRP binds both subunits GyrB and GyrA independently (Tran et al., 2005) (Kim et al., 2015). Further, QnrB1 has protruding loops (Xiong et al., 2011) that are absent in MfpA, interacting with the tower domain in GyrA (C. Chen et al., 2021). A current model for QnrB1 therefore proposes a stabilizing interaction with the ATPase domain and an additional interaction with the tower domain, which is associated with G-segment stabilisation, stimulating the DNA-gate to allow T-segment passage and resealing of the double-strand break (Mazurek et al., 2021) (C. Chen et al., 2021).

Comparing the AlphaFold structure of CszB to the crystal structure of QnrB1 (**Figure 3**) reveals that they share remarkable structural similarities. They share the same locations and dimensions of the small loop (8 amino acids) and the large loop (11 amino acids). Apart from their sequence, CszB differs from QnrB1 by length, as it is 58 amino acids longer, increasing its dimensions by approximately one quadrilateral β -helix turn (Mazurek et al., 2021). Since phylogenetic studies locate CszB closest to QnrB1, a similar mode of gyrase interaction is suggested (Zuo, 2024).

1.3 The Hybrid Antibiotic Thiomarinol A and its Interaction with Isoleucyl-tRNA synthetase (IleRS)

The second topic of this thesis is a cooperation with *Prof. Dr. Li Bo* (R. A. Johnson et al., 2021) and focuses on the interaction of the hybrid antibiotic thiomarinol A with its target, isoleucyl-tRNA synthetase (IleRS). Hybrid antibiotics consist of an antimicrobial that is covalently linked either to another antimicrobial or an adjuvant (Klahn & Brönstrup, 2017). This innovative approach has the potential to achieve a combinatorial effect while avoiding the previously mentioned problems of combination treatment (1.1.1) (Domalaon et al., 2018).

1.3.1 Thiomarinol A and its Constituents

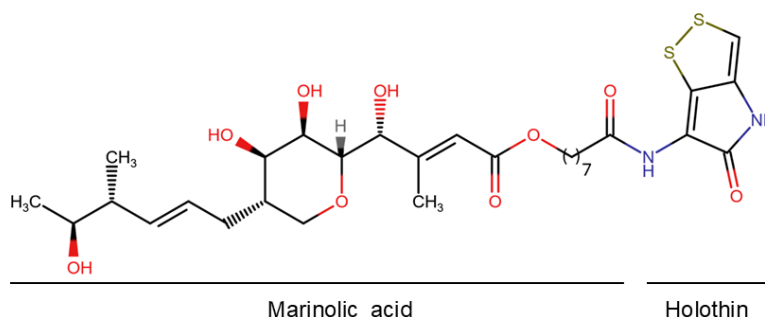


Figure 4: Chemical structure of the hybrid antibiotic thiomarinol A.

Thiomarinol A is an NP that was discovered in *Alteromonas rava* via phenotypical screening and exhibits a strong inhibitory effect against both Gram-positive and Gram-negative bacteria (Shiozawa et al., 1993). The plasmid pTML1 encoding thiomarinol A's BGC is the first documented example containing the biosynthetic pathways of two individual antibiotics that are conjugated in the final step of biosynthesis (Fukuda et al., 2011). Thiomarinol A (**Figure 4**) is composed of marinolic acid, a close analogue to the IleRS inhibitor mupirocin (**Figure 6**), and the dithiopyrrolone (DTP) holothin (**Figure 5**) (Shiozawa et al., 1993).

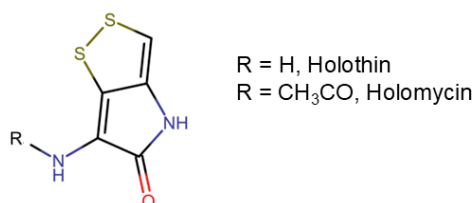


Figure 5: Chemical structure of the dithiopyrrolones (DTPs) holothin and holomycin.

In the reducing environment of a cell, DTPs become high-affinity metal chelators with a preference for coordinating zinc ions. They stagger bacterial growth by disrupting metal

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homeostasis and removing available co-factors for survival-dependent metalloenzymes. This mechanism makes DTPs toxic to human cell lines (Chan et al., 2017) (Kwon et al., 2019).

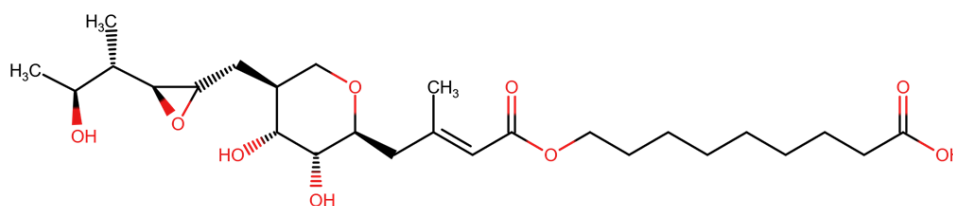


Figure 6: Chemical structure of mupirocin.

Mupirocin is a powerful bisubstrate inhibitor of IleRS (Nakama et al., 2001). Its main constituent, pseudomonic acid A, was first isolated in the 1970s from *Pseudomonas fluorescens* (Fuller et al., 1971). Its high potency combined with minimal toxicity to eukaryotic cells (Tucaliuc et al., 2019) makes it a widely used antibiotic against topical infections with Gram-positive bacteria like *Streptococci* and *Staphylococci*, including the high-priority pathogen (Sati et al., 2025) methicillin-resistant *Staphylococcus aureus* (MRSA) (Sutherland et al., 1985) (Tucaliuc et al., 2019). However, resistance to mupirocin emerged shortly after its clinical introduction, creating a demand for improved strategies to combat mupirocin-resistant MRSA strains (Tucaliuc et al., 2019) (Brdová et al., 2024).

1.3.2 IleRS

1.3.2.1 IleRS Structure and Catalytic Mechanism

Aminoacyl-tRNA synthetases (aaRS) catalyse a key step in protein biosynthesis by specifically selecting and covalently coupling the transfer RNA (tRNA) to its corresponding amino acid (Schimmel, 1979) (Kwon et al., 2019). Chemical genomic screening confirmed the class I aaRS (Eriani et al., 1990) bacterial IleRS to be thiomarinol A's main target (R. A. Johnson et al., 2021).

The structure of IleRS can be divided into seven domains: an editing domain, which is connected to the catalytic domain by two connective peptide domains, and three subdomains (1, 2, and 3) that form the anticodon-binding region (**Figure 7**) (Nureki et al., 1998) (Brkic et al., 2023).

The editing domain sits over the active site and removes incorrectly aminoacylated amino acids (Nureki et al., 1998) (Silvian et al., 1999). The catalytic domain has the characteristic class I Rossmann fold and accommodates the active site with the conserved HXGH motif (X representing a hydrophobic amino acid) and the KMSKS/R loop (Eriani et al., 1990) (Nureki

1 Introduction

et al., 1998). The catalytic mechanism joining isoleucine to tRNA^{Ile} is divided into two steps: After ATP and isoleucine have entered the active site of IleRS, the carboxyl group of the amino acid is activated by ATP, forming the active intermediate isoleucyl-adenylate (Ile-AMP) (DeMoss et al., 1956) (Kingdon et al., 1958). Next, tRNA^{Ile} associates with IleRS and Ile-AMP is esterified to the 2'-hydroxyl group of the tRNA's 3'-terminal adenosine (Hecht et al., 1959).

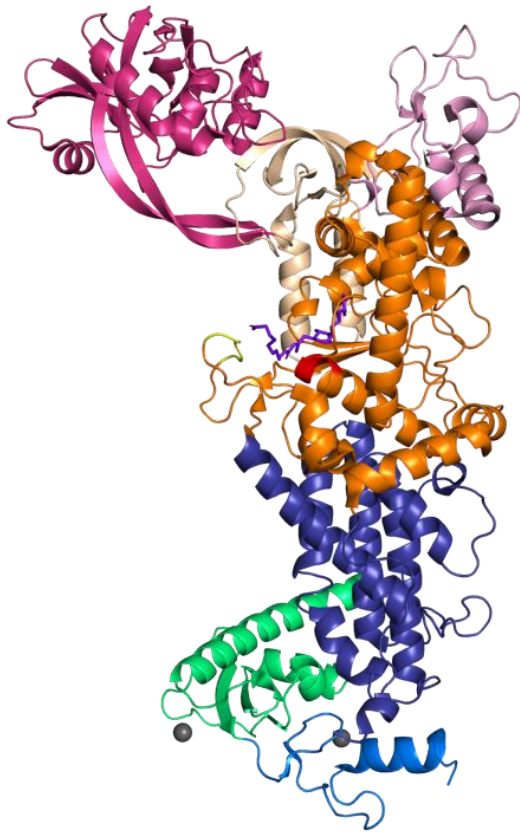
1.3.2.2 IleRS Variants

Although the aaRS enzyme family predates the last universal common ancestor (LUCA), bacterial and eukaryotic IleRS have developed sequential and structural differences (Nagel & Doolittle, 1995) (Wolf et al., 1999). This caused a selective susceptibility of most bacterial IleRS to the antibiotic mupirocin, while eukaryotic homologues remain largely unimpaired (Chung et al., 2020).

Mupirocin is a bisubstrate inhibitor competing with both isoleucine and ATP for the isoleucyl-binding site (Silvian et al., 1999) (Nakama et al., 2001). Contrary to simple competitive inhibitors like Isoleucyl-AMP, mupirocin and IleRS first form an initial enzyme-inhibitor complex that transitions to a much tighter complex via isomerisation (Pope et al., 1999). This leads to a blocked IleRS and disrupts protein biosynthesis (Hughes & Mellows, 1980).

Low-level mupirocin resistance (^{LL}MR) typically arises from mutations in the mupirocin binding site of IleRS, whereas high-level mupirocin resistance (^{HL}MR) results from the plasmid-mediated acquisition of a eukaryotic-like IleRS variant that was likely established through multiple horizontal gene transfers (Wolf et al., 1999) (Thomas et al., 1999). The reason for the difference in susceptibility most likely lies in a small structural difference in the active site: The crystal structures of eukaryotic IleRS (Chung et al., 2020) and eukaryotic-like IleRS2 (Brkic et al., 2023) reveal a narrower active site compared to IleRS1 (**Figure 7**). This conformation obstructs H-bond stabilisation of mupirocin's carboxylate with the KMKS, which enhances stable complex formation in IleRS1 (Brkic et al., 2023). This structural difference most likely impairs mupirocin's activity in eukaryotic IleRS and eukaryotic-like IleRS2 (Chung et al., 2020) (Brkic et al., 2023). Many IleRS enzymes contain zinc-binding domains in subdomain 3 (Silvian et al., 1999) or the connective peptide domains (Chung et al., 2020), often in the form of a cysteine-rich zinc-finger domain. These zinc-binding motifs also vary between different IleRS variants: While IleRS1 typically has a zinc-finger motif in subdomain 3, IleRS2 typically contains one or more zinc-fingers in the connective peptide domains (Chung et al., 2020) (**Figure 7**).

A



B

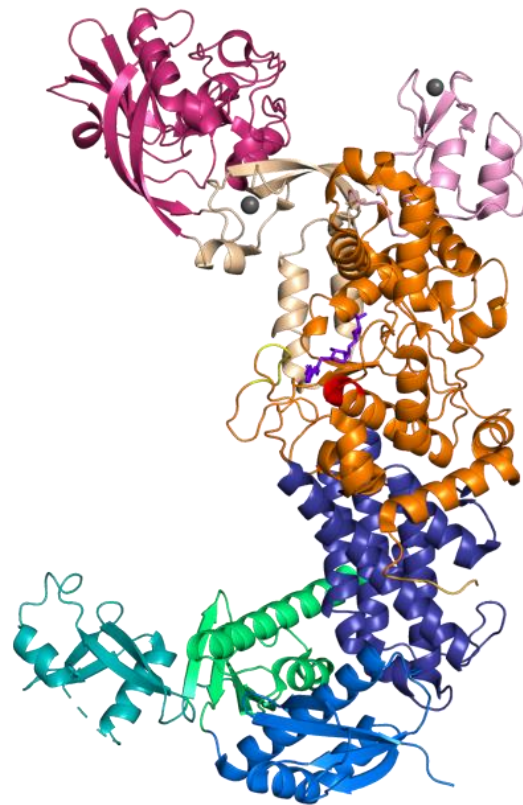


Figure 7: Crystal structures of the *P. megaterium* IleRS variants PmlleRS1 and PmlleRS2.

The editing domain (magenta) is connected by the connective peptide 1 domain (beige) and the connective peptide 2 domain (light pink) to the catalytic domain (orange), harbouring the KMSKS loop (yellow) and the HXGH motif (red). The anticodon-binding region is composed of three subdomains: subdomain 1 (dark blue), subdomain 2 (green) with an insertion in PmlleRS2 (teal), and subdomain 3 (light blue). Both proteins are bound to mupirocin (purple) and have complexed Zn^{2+} ions (grey).

A: Crystal structure of PmlleRS1 bound to mupirocin (PDB: 8C9G) (Brkic et al., 2023). Zinc-finger motif in subdomain 3: 890, 910. **B:** Crystal structure of PmlleRS2 from *P. megaterium* bound to mupirocin (purple) (PDB: 8C8U) (Brkic et al., 2023). Zinc-finger motifs: connective peptide 1 domain: 184, 390. Connective peptide 2 domain: 462, 503.

Usually, bacteria rely on only one of the two IleRS paralogues: IleRS1 has a faster aminoacylation turnover and is therefore preferred by fast-growing bacteria. Slower-growing organisms, however, often use the less efficient IleRS2, which confers mupirocin resistance (Cvetesic et al., 2016) (Zanki et al., 2022). Apart from the organism producing mupirocin (Yanagisawa & Kawakami, 2003), a small group of bacteria have kept both genes, including *Priestia megaterium*, using IleRS1 for efficiency and upregulating IleRS2 when exposed to

mupirocin (Zanki et al., 2022) (Yanagisawa & Kawakami, 2003). Increasing usage of mupirocin, however, has led to increased plasmid-mediated acquisition of IleRS2 variants as a high-level resistance mechanism (Pérez-Roth et al., 2013). For ^{HL}MR MRSA, two IleRS2 variants were identified so far: first, MupA (Chalker et al., 1994) (Hodgson et al., 1994), and more recently, MupB (Seah et al., 2012).

1.3.3 Potency of Thiomarinol A

Previous studies investigating thiomarinol A's mode of action compared the hybrid antibiotic to analogues of its constituents, holothin and mupirocin. Although both thiomarinol A and mupirocin primarily target IleRS, their interaction pattern doesn't overlap completely, indicating at least slightly different modes of action (R. A. Johnson et al., 2021):

Thiomarinol A exhibits substantially higher potency than mupirocin. *In vitro*, its affinity for MRSA COL IleRS1 (from here on called SalleRS1) is approximately 1600-fold greater than mupirocin's. *In vivo*, thiomarinol A is 125-fold more potent against MRSA COL containing SalleRS1 and 1250–2500-fold more potent against ^{LL}MR MRSA COL (R. A. Johnson et al., 2021). While mupirocin has no potency in ^{HL}MR BAA-1556 containing the IleRS2 variant MupA, thiomarinol A retains reduced antibacterial activity (Fukuda et al., 2011) (R. A. Johnson et al., 2021). However, it should be noted that in this case the minimum inhibitory concentration (MIC) is only six-fold lower than the cytotoxic concentration observed in human cell lines (R. A. Johnson et al., 2021).

The chemical-genomic screen showed that none of the tested DTP's target IleRS or overlap with thiomarinol A's other interaction patterns. The potency of holomycin, a stable analogue of holothin, remained largely indifferent against any tested mupirocin resistance. Interestingly, like the other DTPs, thiomarinol A can chelate zinc ions under reducing conditions and affect zinc availability in *S. aureus* (R. A. Johnson et al., 2021).

Combination treatment *in vitro* showed an additive effect of mupirocin and holomycin. However, a 100-fold dosage was required compared to the natural or synthetic hybrids (R. A. Johnson et al., 2021) (Fukuda et al., 2011). Exposure to increasing levels of thiomarinol A resulted in a modest development of resistance to a much lesser degree compared to cells exposed to mupirocin. Further, low-level thiomarinol-resistant cells remained partially susceptible to mupirocin, underlining the differences in their mechanism of action (R. A. Johnson et al., 2021).

1.3.4 Antimicrobial Spectrum of Thiomarinol A

Additionally, thiomarinol A has a broader antimicrobial spectrum compared to mupirocin, as it exhibits a strong inhibitory effect against Gram-negative bacteria (Shiozawa et al., 1993). Gram-negative bacteria have an intrinsic resistance against a broad range of antibiotics, which

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largely comes from their almost impermeable outer membrane (Nikaido, 2003) and efflux pumps that actively expel toxic compounds from the cell (Li et al., 2015) (Krishnamoorthy et al., 2017).

Mupirocin is unable to enter Gram-negative bacteria, since it most likely relies on passive diffusion (R. M. Johnson et al., 2025). The DTP holomycin, on the other hand, passes the outer membrane of Gram-negative bacteria and accumulates in high amounts in the cells, most likely due to its planar, hydrophobic structure (R. M. Johnson et al., 2025). This indicates that the high accumulation of thiomarinol A in contrast to mupirocin results from the DTP moiety either increasing the uptake or retention of the small molecule. Fusing other components to the mupirocin analogue known to enhance cellular uptake also resulted in increased accumulation in the cell, but the antimicrobial activity was low or diminished. This indicates that the DTP's mode of action is not limited to increased accumulation but is necessary for the antimicrobial's broad antimicrobial spectrum and high potency of thiomarinol A (R. M. Johnson et al., 2025).

1.4 Objectives

This research explored the hybrid antibiotic thiomarinol A and the putative antibiotic resistance protein CszB, aiming to characterize their interaction with their respective target enzyme using structural and biochemical methods.

The first project investigated a potential complex formation between the putative self-resistance protein CszB and the DNA gyrase from *X. szentirmaii*. For the primary goal of a co-crystal structure of the protein complex, a truncated fusion construct of the gyrase's DNA-binding/cleavage domain was designed. We planned to obtain an apo-crystal structure of the gyrase construct and then exploit a potential complex formation to co-purify and co-crystallize CszB with the gyrase construct. Further, we aimed to assess the involved protein-protein and protein-DNA interactions via thermal shift assays (TSA), native PAGE, and Electrophoretic Mobility Shift Assay (EMSA).

The second project aimed to explore the macromolecular interaction of the hybrid antibiotic thiomarinol A with different variants of bacterial IleRS. We aimed to characterize the thermal stabilisation of the different IleRS variants upon binding of thiomarinol A via TSA. The primary objective of this project, however, was to obtain structures of the IleRS variants bound to thiomarinol A, to get structural insights into their macromolecular interaction, including potential conformational changes in the active site and the influence of the metal-chelating moiety on the complexed zinc ions.

2 Materials and Methods

2.1 Materials

2.1.1 Primers and Plasmids

All primers and oligonucleotides were obtained from *Eurofins Genomics*.

Table 1: List of all oligonucleotides and primers used. For: forward, rev: reverse.

Gibson assembly	Sequence 5' → 3'
GyrB_PB_for	AACCTGTACTTCCAAGGAATGACCCGTCGCAAGGG
1b_PB_rev	TCCTTGGAAGTACAGGTTTTTCAGAACC
GyrA_GyrB_for	GCAGCGAATATAGATATTAGCGACATTGCCAGAGAAATCAC
GyrB_GyrA_rev	GGCAATGTCGCTAATATCTATATTCGCTGCTTTCAGGGCA
GyrA_Ga1_rev	TTTCTGTTTCGACTTAAGCATTATTCGGTCAGCTCAGTCCG
ga1_DuetM1-for	TGCTTAAGTCGAACAGAAAGTAATCGTATTG
ga6_CszB_for	AGTATAAGAAGGAGATATACATATGTTCTGAATACATCCTGAACGTTT CTGAAGAAAC
ga6_DuetM2-rev	ATGTATATCTCCTTCTTATACTTAACTAATATACTAAGATGGGGAA
ga5_DuetM2-for	CCCTCGAGTCTGGTAAAGAAACCG
ga5_CszB_rev	TTTACCAGACTCGAGGGTTAGTTGTTCTGGGTGGATGATGAAAC
ga6_XzGyrB_for	TTAAGTATAAGAAGGAGATATACATATGACCCGTCGCAAGGG
ga5_XzGyrA_rev	TTTACCAGACTCGAGGGTTATTCGGTCAGCTCAGTCCGACGC
Q5 Mutagenesis	Sequence 5' → 3'
1b_ct_for	TAACTGCAGGTCGAC
PmlleRS2ct_rev	TAACGAATGCGTATTTTCG
Sequencing	Sequence 5' → 3'
s5_pET	ATGCGTCCGGCGTAGA
s6_DuetUP2	TTGTACACGGCCGCATAATC
s7_DuetDOWN1	GATTATGCGGCCGTGTACAA
s8_T7 Terminator	GCTAGTTATTGCTCAGCGG
s9_DuetSUMO-Seq	GACCTGGACATGGAAGACAAC
XZ_seq01	GCAGATGTCCGCTTTCTGG
XZ_seq02	ATCAAGCCTTCAATACTGATG
PM1_seq01	AGTTCTTTGTCCAGTTGATAG
PM2_seq01	GGACAGTACACGTCTGAAGTG

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Oligonucleotides for Protein Characterisation	Sequence 5' → 3'
MT_oligo_for	GGTCATGAATGACTATGCACGTAA
MT_oligo_rev	TTACGTGCATAGTCATTCATGACC

Table 2: List of all plasmids used.

Plasmid	Expressed in	Size [bp]	Source
pETDuet-His ₆ -SUMO		5736	Derived from pETDuet-1 <i>Novagen</i>
His ₆ -Throm-MBP-TEV		6521	Derived from pETDuet-1, <i>Novagen</i>
His ₆ -Throm-MBP- TEV_XzGyrBA	<i>E. coli</i> Rosetta2 (DE3) pLysS	9332	Gibson assembly, this thesis
His ₆ -Throm-MBP- TEV_XzGyrBA_CszB	<i>E. coli</i> Rosetta2 (DE3) pLysS	10036	Gibson assembly, this thesis
pETDuet-His ₆ - SUMO_CszB		6470	<i>Eurofins genomics</i>
pETDuet-His ₆ - SUMO_CszB_XzGyrBA	<i>E. coli</i> Rosetta2 (DE3) pLysS	9229	Gibson assembly, this thesis
pLIC-His ₆ -TEV- SalleRS1	<i>E. coli</i> BL21 gold (DE3)	8043	<i>Prof. Dr. Bo Li</i> (R. A. Johnson et al., 2021)
pETDuet-His ₆ - SUMO_PmlleRS1	<i>E. coli</i> BL21 gold (DE3)	8480	<i>Azenta life sciences</i>
pETDuet-His ₆ - SUMO_PmlleRS2	<i>E. coli</i> BL21 gold (DE3)	8813	<i>Azenta life sciences</i>
pETDuet-His ₆ - SUMO_PmlleRS2ct	<i>E. coli</i> BL21 gold (DE3)	8150	Q5 Mutagenesis, this thesis

2.1.2 Bacterial Strains, Media and Antibiotics

Table 3: List of all bacterial strains used.

Bacterial Strain	Competence	Method	Source
<i>E. coli</i> DH10 β	Electrocompetent	Cloning	<i>New England Biolabs</i>
<i>E. coli</i> XL1-blue	Electrocompetent	Cloning	<i>Agilent</i>
<i>E. coli</i> Rosetta2 (DE3) pLysS	Electrocompetent	Protein expression	<i>Novagen</i>
<i>E. coli</i> BL21 gold (DE3)	Electrocompetent	Protein expression	<i>Agilent</i>

Table 4: List of all media used.

Medium	Composition
LB medium	1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl
LB agar	LB Medium, 2 % (w/v) agar, 100 μ g/mL ampicillin
Autoinduction medium	1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05 % (w/v) glucose, 0.5 % (v/v) glycerol, 0.2 % (w/v) lactose monohydrate, 2 % (w/v) MgSO ₄ heptahydrate, 2.5 % (w/v) Na ₂ HPO ₄ dihydrate, 2.5 % (w/v) KH ₂ PO ₄ , 5 % (w/v) NH ₄ Cl, pH 6.8
SOC medium	2 % (w/v) peptone, 0.5 % (w/v) yeast extract, 20 mM glucose, 10 mM MgSO ₄ , 10 mM MgCl ₂ , 10 mM NaCl, 2.5 mM KCl

Table 5: List of all antibiotics used.

Antibiotic	Concentration [μ g/ml]
Ampicillin	100

2.1.3 Buffers

Table 6: List of all buffers used for protein purification.

Buffer	Composition
XzGyr IMAC A	100 mM Tris/HCl pH 7.5, 500 mM NaCl, 20 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr IMAC B	100 mM Tris/HCl pH 7.5, 500 mM NaCl, 500 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr Dialysis	100 mM Tris/HCl pH 7.5, 150 mM NaCl, 2 mM β -mercaptoethanol, 2.5 % (v/v) glycerol
XzGyr SEC	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 0.3 mM dithiothreitol
XzGyr_CszB IMAC A1	100 mM Tris/HCl pH 7.5, 100 mM NaCl, 20 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB IMAC B1	100 mM Tris/HCl pH 7.5, 100 mM NaCl, 500 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB Dialyse1	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB SEC1	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 10 % (v/v) glycerol
XzGyr_CszB IMAC A2	100 mM Tris/HCl pH 7.5, 300 mM NaCl, 20 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB IMAC B2	100 mM Tris/HCl pH 7.5, 300 mM NaCl, 500 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB Dialyse2	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol, 10 % (v/v) glycerol
XzGyr_CszB SEC2	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 10 % (v/v) glycerol
XzGyr_CszB IMAC A3	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol, 10 mM MgCl_2 , 10 % (v/v) glycerol
XzGyr_CszB IMAC B3	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 200 mM imidazole pH 7.5, 2 mM β -mercaptoethanol, 10 mM MgCl_2 , 10 % (v/v) glycerol
XzGyr_CszB Dialyse3	20 mM Tris/HCl pH 7.5, 100 mM NaCl, 2 mM β -mercaptoethanol, 10 mM MgCl_2 , 10 % (v/v) glycerol
IleRS IMAC A	25 mM HEPES pH 7.0, 500 mM NaCl, 10 mM imidazole pH 7.5, 10 mM β -mercaptoethanol
IleRS IMAC B	25 mM HEPES pH 7.0, 500 mM NaCl, 250 mM imidazole pH 7.5, 10 mM β -mercaptoethanol
IleRS Dialysis	25 mM HEPES pH 7.0, 150 mM NaCl, 10 mM β -mercaptoethanol
IleRS SEC	25 mM HEPES pH 7.0, 50 mM NaCl, 2 mM dithiothreitol
Saline	0.9 % (w/v) NaCl

2 Materials and Methods

Table 7: List of all buffers used for agarose gel electrophoresis.

Agarose Gel Electrophoresis	Composition
TAE buffer	40 mM Tris/HCl pH 8.5, 1 mM EDTA, 20 mM acetic acid

Table 8: List of all buffers used for protein characterisation.

SDS-PAGE	Composition
5x Laemmli buffer	60 mM Tris/HCl pH 6.8, 30 % (v/v) glycerol, 10 % (w/v) saccharose, 5 % (w/v) SDS, 0.02 % (w/v) bromophenol blue, 3 % (v/v) β -mercaptoethanol
SDS separating gel buffer	1.5 M Tris/HCl pH 8.8, 0.4 % (w/v) SDS
SDS stacking gel buffer	0.5 M Tris/HCl pH 6.8, 0.4 % (w/v) SDS
10x SDS running buffer	1.92M glycine, 1 % (w/v) SDS, 0.25M Tris
Comassie staining solution	0.05 % (w/v) Coomassie Brilliant Blue R250, 10 % (v/v) acetic acid, 25 % (v/v) isopropanol
Destaining solution	10 % (v/v) acetic acid

Native PAGE	Composition
10 x native PAGE running buffer	1.92 M glycine, 0.25 M Tris
5 x native loading dye	62.5 mM Tris/HCl pH 6.8, 40 % (v/v) glycerol, 1 % (w/v) bromphenol blue

EMSA	Composition
Oligo annealing buffer	20 mM Tris/HCl pH 7.5, 50 mM NaCl
5 x EMSA binding buffer	20 % glycerol, 5 mM MgCl ₂ , 2.5 mM EDTA, 2.5 mM DTT 250 mM NaCl, 50 mM Tris/HCl pH 7.5
10 x EMSA loading buffer	20 % (v/v) glycerol, 250 mM Tris/HCl pH 7.5, spatula tip bromphenol blue, 3 μ L xylencyanol
5 x TBE buffer	450 mM Tris base, 444 mM boric acid, 10 mM EDTA

2 Materials and Methods

Table 9: List of all commercial crystallisation screens used, purchased at Qiagen.

Screen Nr.	Description
1	pHClear Suite
2	pHClear II Suite
3	Classics Suite
4	Classics II Suite
5	JCSG+ Suite
6	ComPAS Suite
7	Protein Complex Suite
8	PEGs Suite
9	PEGs II Suite
10	PACT Suite

For co-crystallisation with thiomarinol A, a 100 mM stock solution was prepared from powdered thiomarinol A, kindly provided by *Prof Dr. Bo Li (R. A. Johnson et al., 2021)*, and Dimethylsulfoxide-d6 (DMSO) (Cas-Number: 2206-27-1).

2.1.4 Instruments and Software

Table 10: List of all instruments used, listed alphabetically.

Instrument	Source
Agarose gel electrophoresis system	<i>Appligene</i>
ÄKTA pure™ chromatography system + Frac-920	<i>Cytiva</i>
ÄKTA Start™ chromatography system + Frac30	<i>Cytiva</i>
Analytical Balance, LA 124i Classic	<i>VWR</i>
Autoclave Prestige Medical 2100 classic	<i>Soleni</i>
Autoclave Varioklav Classic 500	<i>HP Medizintechnik</i>
Centrifuge 3-30K, Rotor 12150-H	<i>Sigma</i>
Centrifuge 4K15, Rotor 11150	<i>Sigma</i>
Centrifuge 6-16KS, Rotor 12500-H	<i>Sigma</i>
Centrifuge 8K, Rotor 11805	<i>Sigma</i>
CryoLoop	<i>Hampton</i>
CrystalCap HT for CryoLoop	<i>Hampton</i>
CrystalWand Magnetic	<i>Hampton</i>
Digital Sonifier® 450	<i>Branson</i>
Electrophoresis Power Supply EPS 600	<i>Pharmacia Biotech</i>
Electroporation Cuvette (2mm gap)	<i>Peqlab</i>

2 Materials and Methods

Electroporator Eporator ®	<i>Eppendorf</i>
Falcon4i	<i>Thermo Fisher Scientific</i>
Glacios Cryo-TEM	<i>Thermo Fisher Scientific</i>
GloQube Plus	<i>Thermo Fisher Scientific</i>
Incubator ED53	<i>Binder</i>
Incubator Multitron Standard	<i>INFORS HT</i>
Intelli-Plates® 96-3-Well	<i>Art Robbins Instruments</i>
Magnetic Stirrer MR Hei-Standard	<i>Heidolph</i>
Microscope SZX10/ KL 1600 LED	<i>Olympus Life Science</i>
Microscope Camera Olympus SC50	<i>Olympus Life Science</i>
Microcentrifuge 1-14 (Rotor 91673)	<i>Sigma</i>
Microcentrifuge 1-14K (Rotor 12084)	<i>Sigma</i>
Mini-PROTEAN® Tetra System	<i>Bio-Rad</i>
M-power Toploading Balance AZ4101	<i>Satorius</i>
NanoDrop 2000c	<i>Thermo Fisher Scientific</i>
Oribtal Shaker VXR basic Vibrax®	<i>IKA</i>
Power Supply PowerPac	<i>Bio-Rad</i>
Precision Balance TE4101	<i>Satorius</i>
Quantum CX5 Edge	<i>Vilber</i>
Quick Combi Sealer Plus	<i>HJ-Bioanalytik</i>
Robot Crystal Gryphon	<i>Art Robbins Instruments</i>
Robot Hamilton Microlab STAR	<i>Hamilton</i>
Robot Oryx4	<i>Douglas Instruments</i>
Storage Dewar HC20	<i>Worthington Industries</i>
Superdex 200 pg 16/600	<i>Cytiva</i>
Superdex 200 10/300 increase	<i>Cytiva</i>
Synapt XS	<i>Waters</i>
Thermal Cycler CFX96, Real-Time	<i>Bio-Rad</i>
Thermal Cycler T100	<i>Bio-Rad</i>
Thermomixer comfort (1.5 mL)	<i>Eppendorf</i>
Ultrospec 10 Cell Density Meter	<i>Amersham Biosciences</i>
UVEX Microscope	<i>JAN Scientific inc.</i>
Vacuum pump	<i>VACUUBRAND</i>
Vitrobot Mark IV	<i>Thermo Fisher Scientific</i>

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Table 11: List of all software used, listed alphabetically.

Software	Source
AlphaFold Server	Deepmind (Abramson et al., 2024) https://alphafoldserver.com/welcome
CellSens Entry	Olympus Life Science
CCP4i	https://www.ccp4.ac.uk/ (Dectris, 2026a)
CFX Maestro (ThermoFisher)	Bio-Rad
Coot	https://www2.mrc.lmb.cam.ac.uk/personal/pemsley/coot/ (Emsley et al., 2010)
cryoSPARC version 3.4.0	Structura Biotechnology Inc.
CrystalDetect	JAN Scientific inc.
ExPASy ProtParam tool	https://web.expasy.org/protparam/
ExPASy translate tool	https://web.expasy.org/translate/
Google Colaboratory	https://colab.research.google.com/
Grammarly*	https://app.grammarly.com/
MassLynx V4.2 software	Waters Corporation
Mendeley reference manager	https://www.mendeley.com/
Microsoft Office	Microsoft
NanoDrop2000	Thermo Fisher Scientific
NEBaseChanger Tool	New England Biolabs
Perplexity AI**	https://www.perplexity.ai/
PyMol	DeLano Scientific LLC, Schrödinger Inc.
Phaser crystallographic software	https://www.phaser.cimr.cam.ac.uk/ (McCoy et al., 2007)
RCSB Chemical Sketch Tool	https://www.rcsb.org/search/chemical
Smart EPU Software	Thermo Fisher Scientific
SnapGene	GSL Biotech LLC
Unicorn	Cytiva

*Grammarly was exclusively used to detect spelling and punctuation mistakes.

**Perplexity AI was exclusively used to improve tone, clarity and readability in distinct sections of the text.

2.2 Methods

2.2.1 Molecular Cloning and Nucleic Acids

2.2.1.1 Q5 Polymerase Chain Reaction (PCR)

To amplify genes of interest or vectors via polymerase chain reaction (PCR), a high-fidelity Q5-DNA polymerase (*NEB*) was employed according to the manufacturer's instructions (New England Biolabs, 2026b). The reagents were combined in a nuclease-free PCR tube as shown in **Table 12**, and the standard protocol in **Table 13** was run on a T100 thermocycler (*Bio-Rad*). After the amplification was confirmed on a 1 % (w/v) agarose gel (**2.2.1.6**), the PCR product was purified with the Promega Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions (Promega, 2026a).

Table 12: Pipetting scheme for Q5 PCR.

Reagent	Volume [μl]
DNA template (1ng/μl)	1
Primer for (20 pmol/μl)	1.25
Primer rev (20 pmol/μl)	1.25
dNTPs (10 mM)	1
5x Q5 reaction buffer	10
Q5-polymerase	0.5
ddH ₂ O	35
total	50

Table 13: Q5 PCR protocol for thermocycler.

Time	Temperature [°C]
30 sec	98
35x	10 sec
	30 sec
	30-40
	sec/kb
2 min	72
∞	4

2.2.1.2 Gibson Assembly

To generate a new construct a Gibson assembly was performed. For each assembly site, a pair of primers was designed using the SnapGene built-in Gibson Assembly Primer Designer (Snapgene, 2026). The vector backbones and genes of interest were PCR amplified as described in **2.2.1.1** from genomic DNA (GyrB and GyrA) or previous constructs (all vector backbones and CszB). The annealing temperature was estimated using the NEB T_m calculator (New England Biolabs, 2026c). The resulting vector backbones and inserts are listed in **Table 14**. For each construct, 40 ng of the vector were mixed with the insert(s) in the described molar ratio in a 4 μL volume. The DNA was combined with 4 μL HiFi DNA Assembly MasterMix (*NEB*) and incubated for 1 h at 50°C.

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Table 14: List of all vector backbones and inserts created for Gibson assembly with their corresponding primers and the used molar ratio (vector : insert).

Construct	Backbone	Primers	Inserts	Primers	Ratio
His ₆ -Throm-MBP-TEV_XzGyrBA	His ₆ -Throm-MBP-TEV, 6297 bp	ga1_DuetM1-for 1b_PB_rev	GyrB ₃₉₁₋₈₀₄ , 1242 bp GyrA ₂₋₅₂₃ , 1566 bp	GyrB_PB_for GyrB_GyrA_rev GyrA_GyrB_for GyrA_ga1_rev	1:2:2
His ₆ -Throm-MBP-TEV_XzGyrBA_CszB	His ₆ -Throm-MBP-TEV_XzGyrBA, 9332 bp	ga5_DuetM2-for ga6_DuetM2-rev	CszB, 753 bp	ga6_CszB_for ga5_CszB_rev	1:2
pETDuet-His ₆ -SUMO_CszB_XzGyrBA	pETDuet-His ₆ -SUMO_CszB, 6470 bp	ga5_DuetM2-for ga6_DuetM2-rev	GyrBA, 2811 bp	ga6_XzGyrB_for ga5_XzGyrA_rev	1:5

2.2.1.3 Q5 Mutagenesis

Q5 Mutagenesis was used to create C-terminally truncated protein constructs. The primer pairs for Q5 mutagenesis (**Table 1**) were designed with the NEBaseChanger Tool (New England Biolabs, 2026a). The Q5 PCR was run as described in section 2.2.1.1 but with a reduced amount of template DNA (0.5 ng per reaction) and without the PCR cleanup step. Next, a Kinase-Ligase-DpnI (KLD) reaction was performed by combining the reagents as detailed in **Table 15** and incubating the reaction for 2 h at room temperature.

Table 15: Pipetting scheme for KLD reaction.

Reagent	Volume [μl]
PCR product (no cleanup)	3.5
10 x T4 Ligase Buffer	1
DpnI	0.5
T4 Ligase	0.5
T4 Polynucleotide Kinase	0.5
ddH ₂ O	4
Total Volume	10

2.2.1.4 Transformation via Electroporation

To transform a construct into electrocompetent *E. coli* cells, 40 μL of the corresponding strain were thawed on ice. For molecular cloning, 1 μL of the Gibson or KLD reaction was added to

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the cells, while for protein expression, 1 ng of the construct was added. The suspension was transferred into a cuvette and electroporated (set voltage: 2500 V, actual discharge time: 5.3-6.0 ms). The cells were supplemented with 1 mL of SOC medium, transferred into a 1.5 mL reaction tube, and incubated for 1 h at 37 °C. Subsequently, the cells were plated on 0.2 mM ampicillin-supplemented LB agar for selection. For cloning, all cells were plated, while for expression only 50 µL suspension were used. The plates were incubated for 18 h at 37 °C and stored at 4 °C until further use.

2.2.1.5 Plasmid Preparation and Validation

After transformation of the electrocompetent *E. coli* cells with the plasmid of interest (**2.2.1.4**), single colonies were picked from the agar plates and transferred into 3 mL LB medium containing 0.2 mM ampicillin. The cells were incubated overnight at 37 °C at 130 rpm and subsequently processed with the *Promega* Wizard® Plus SV Minipreps DNA Purification System according to the manufacturer's instructions (Promega, 2026b).

To assess the plasmids' identity, 5 µL of the isolated plasmids were digested with restriction enzymes (*NEB*) (**Table 16**) by incubating the mixture at 37 °C for 1 h. For pETDuet-His₆-SUMO constructs, BamHI-HF and XhoI were used, for His₆-Throm-MBP-TEV constructs and pLIC-His₆-TEV-SalleRS1 XbaI and XhoI. The result was evaluated on a 1 % (w/v) agarose gel (**2.2.1.6**).

Table 16: Pipetting scheme for control digest.

Reagent	Volume [µl]
Plasmid	5
10 x CutSmart Buffer	1
Restriction enzyme 1	0.2
Restriction enzyme 2	0.2
ddH ₂ O	3.6
Total Volume	10

Samples that showed a positive result in the restriction digest were sequenced by *Azenta* (*Azenta*, 2026) to avoid unwanted mutations. In a total volume of 10 µL, 200-500 ng of the isolated plasmid were supplemented with 25 ng sequencing primer (**Table 1**) and sent to Sanger sequencing.

2.2.1.6 Agarose Gel Electrophoresis

To evaluate the length of DNA, a 1 % (w/v) agarose gel was prepared. 1 g of agarose (*SERVA*) was dissolved in 100 mL TAE buffer upon heating. After cooling, 5 µL of DNA Stain Clear G (*SERVA*) were added, and the gel was cast. DNA samples were mixed with Gel Loading Dye,

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Purple (6X) (*NEB*) and pipetted into the pockets with the 1kb Plus DNA Ladder (*NEB*). The gel was run at a constant voltage of 120 V for 30-60 min in 1 x TAE buffer. After electrophoresis, the gel was assessed under UV light with the Quantum CX5 Edge imager (*Vilber*).

2.2.1.7 DNA Concentration Measurement via Nanophotometer

The NanoDrop2000c (*Thermo Fisher Scientific*) was used to measure DNA concentration (Desjardins & Conklin, 2010). First, the instrument was blanked with ddH₂O, then 2 µL of DNA were pipetted on the nanophotometer. The absorbance was measured at $\lambda = 260$ nm, 1 absorption unit corresponding to 50 ng/µL.

2.2.1.8 Oligonucleotide Annealing

To create dsDNA from ssDNA, of two complementary single-stranded oligonucleotides with a concentration of 100 pmol/µl were mixed in a PCR tube and heated in a T100 Thermal Cycler (*Bio-Rad*) for 10 minutes at 98 °C. Subsequently, the temperature was lowered in 0.9 °C increments. Finally, the annealed oligonucleotide was kept for 2 min at 8°C. The 50 pmol/µL dsDNA was stored short-term at 4 °C or long-term at – 20 °C.

2.2.2 Recombinant Protein Expression and Purification

2.2.2.1 Protein Expression

After successful transformation of the construct into the respective expression strain (**2.2.1.4**), single colonies were picked from the agar plates to inoculate precultures containing 150 mL LB Medium and 0.2 mM ampicillin. After incubation for 18 h at 37 °C at 130 rpm, the cells were transferred into a 2 L expression culture containing either LB or autoinduction media:

For LB cultures, the autoclaved medium was freshly supplemented with 0.2 mM ampicillin and inoculated with 40 mL preculture. The culture was incubated at 37 °C and 130 rpm until an optical density (OD) of 0.5-0.7 was reached ($\lambda = 600$ nm). The culture was cooled down at 4 °C for 30 min to reach the expression temperature of 20 °C before inducing gene expression by adding 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (*Biosynth*). The culture was incubated at 20 °C overnight at 130 rpm. For autoinduction cultures, the medium containing only tryptone and yeast extract had to be freshly supplemented with the remaining components (**Table 4**) before inoculation. After adding buffers, sugars, and 0.2 mM ampicillin, 40 mL preculture were added, and the culture was incubated at 25 °C at 130 rpm for approximately 48 hours.

All cells were expressed in autoinduction medium except for *E. coli* Rosetta 2 pLysS cells expressing the His₆-SUMO_CszB_XzGyrBA construct.

The cells were harvested using an 8K centrifuge (*Sigma*) at 5000 xg for 20 min. After discarding the supernatant, the pellets were resuspended in saline solution and transferred into Falcon

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tubes. After pelleting the cells at 4000 xg (4K15, *Sigma*), the clear supernatant was discarded, and the pellets were stored at -20 °C until purification.

2.2.2.2 Cell Lysis

The pellets were thawed on ice and resuspended in the corresponding buffer IMAC A (**Table 6**). The cells were transferred into a dicer in an ice bath, and a spatula tip DNase I (*Sigma Aldrich*) and Pefa bloc®SC (*ROTH*) were added to remove DNA and inhibit proteases. The cells were sonicated in a Digital Sonifier®450 (*Branson*) using an amplitude of 80% and an on-off cycle (on 1sec, off 1sec) for 4min. The lysate was cleared by high-speed centrifugation at 41415 xg (3-30K, *Sigma*) for 30 minutes at 4 °C.

2.2.2.3 Immobilized-Metal Affinity Chromatography (IMAC)

All proteins were expressed with a cleavable hexahistidine-tag (His₆-tag). To capture the protein of interest (POI) from the lysate, an immobilized-metal affinity chromatography (IMAC) was performed using a nickel nitrilotriacetic acid (Ni-NTA) agarose column connected to an ÄKTA pure™ or ÄKTA Start™ chromatography system (*Cytiva*). The flow rate for each step was set at 5 ml/min. The 5 mL HisTrap™ HP column (*Cytiva*) was equilibrated in the respective IMAC A buffer (**Table 6**) using at least 5 column volumes (CV) or until the UV signal reached 0. After the lysate was applied to the column, it was washed with at least 5 CVs of buffer IMAC A or until the UV signal fell below 40 mAU before eluting the bound protein with a gradient from 0 to 100 % of buffer IMAC B over 10 CV. The fractions that were collected after initiation of the gradient were analysed via SDS-PAGE (**2.2.5.2**) along with the cells, the lysate, and the flow-through, if collected.

2.2.2.4 Dialysis and Hexahistidine-Tag Removal

To remove the imidazole, the protein was dialyzed overnight. Further, the His₆-tag was cleaved during this step by addition of His₆-tagged SUMO or TEV protease (prepared in-house by *Katrin Gärtner*). 2 L of dialysis buffer were prepared (**Table 6**) and fractions containing the POI were pooled into a previously in dialysis buffer equilibrated dialysis tube with a size cut-off of 14 kDa (*Carl Roth*). The dialysis was set up overnight on a magnetic stirrer at 100-200 rpm at 4 °C.

2.2.2.5 Reverse IMAC

To remove the cleaved His₆-tag, the His₆-tagged protease, and nonspecific binding proteins, a reverse IMAC was performed. Analogous to IMAC, the flow rate for each step was set at 5 ml/min, and the Ni-NTA agarose column was equilibrated with at least 5 CV or until the UV signal reached 0 in buffer IMAC A (**Table 6**). The dialysate was transferred into a Falcon tube and centrifuged at 4°C at 10000 xg for 10 min to remove precipitate before applying it to the nickel column. Fractions were collected immediately at the beginning of sample application

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and throughout the wash step, lasting at least 5 CV or until the UV signal fell below 40 mAU. The fractions were evaluated on an SDS-PAGE (2.2.5.2).

2.2.2.6 Preparative Size Exclusion Chromatography (SEC)

To refine the protein's purity after the reverse IMAC, a preparative size exclusion chromatography (SEC) was performed. In case of a low yield, an analytical SEC was performed instead (2.2.5.5). The fractions containing the POI were pooled and concentrated at 4 °C to a volume of 2-3 mL at 4000 xg using an Amicon® ultra - 15 centrifugal concentrator (Merck) with a molecular weight cut-off of 20 kDa for purifications containing CszB, 50 kDa for PmlleRS2ct, and 100 kDa for XzGyrBA, PmlleRS1, and PmlleRS2.

The ÄKTA pure™ chromatography system (Cytiva) and the HiLoad™ 16/600 Superdex™ 200 pg column (Cytiva) were previously equilibrated with the corresponding degassed and filtered SEC buffer (Table 6). To remove precipitate, the concentrated protein solution was centrifuged at 16602 xg for 10 min at 4°C before manually applying it to the column through a 5 mL injection loop. The system was run at a flow rate of 1 mL/min for 1.5 CV, and fractions were collected after 0.3 CV. After evaluation via SDS-PAGE (2.2.5.2), the fractions containing the protein were pooled and concentrated to the desired concentration, as previously described, and used for further experiments.

2.2.3 Protein Crystallisation and X-ray Crystallography

2.2.3.1 Crystal Plate Setup

To obtain a protein structure of the POI crystallisation trials were set up. For all plates the sitting drop vapor diffusion method was used in Intelli-Plates® 96-3-Well (Art Robbins Instruments). For each condition, three different protein-buffer ratios were prepared: 0.2 µL protein with 0.2 µL buffer, 0.2 µL protein with 0.1 µL buffer, and 0.3 µL protein with 0.1 µL buffer. While screens 1-10 are standard high-throughput screens from QIAGEN (Table 9), fine screens (FS) were freshly pipetted with the Hamilton Microlab STAR robot and are shown in Table 18.

Table 17: List of all crystal plates set up.

Protein	Additive	c [mg/ml]	T [°C]	Screen Nr.
XzGyrBA	4 mM MgCl ₂	15	20	1-10
XzGyrBA	4 mM MgCl ₂	10	20	1-10
SalleRS1	0.01 mM ZnAc	20	4	1-10
SalleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	20	1-10
SalleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	4	1-10
SalleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	16	4	10
SalleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	4	FS1
PmlleRS1	0.01 mM ZnAc	20	20	1-10

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PmlleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	20	1-10
PmlleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	20	FS2, FS3
PmlleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	4	FS2, FS3
PmlleRS1 + 1mM thiomarinol A	0.01 mM ZnAc	20	20	FS4
PmlleRS2	0.01 mM ZnAc	16.5	4	1-10
PmlleRS2	0.01 mM ZnAc	14.5	4	1-10
PmlleRS2*	0.01 mM ZnAc	16.5	4	1-10
PmlleRS2 + 1 mM thiomarinol A	0.01 mM ZnAc	16.5	4	1-10
PmlleRS2ct	0.01 mM ZnAc	16.5	4	1-10

*New purification with fewer contaminants

Table 18: List of all fine screens created.

Fine screen	Conditions
FS1	0.2 mM NaCl, 0.1 mM HEPES pH 6.8 – 7.5, 2-24 % PEG 6000
FS2	0.2 M Magnesium chloride 0.1 M Tris pH 7.5 – 8.2 , 10-32 % (w/v) PEG 6000
FS3	FS2 + seeds
FS4	400 - 900 mM LiCl and 10 - 20% PEG3350, 25 mM Hepes-KOH pH=7.5 at 20 °C, 50 mM NaCl

Crystal plates were pipetted with the Crystal Gryphon robot (*Art Robbins Instruments*) except for seeding plates, which were prepared with the Oryx4 robot (*Douglas Instruments*). First, the reservoir was filled with 50 μ L of the buffer from the crystallisation screen. Next, the respective amount of buffer was pipetted onto the wells. Finally, the protein was added in the respective amount to the droplet. The plate was sealed using the Quick Combi Sealer Plus (*HJ-Bioanalytik*) and stored at 4 or 20 °C. The crystals were documented with the Olympus Microscope Camera SC50 (*Olympus Life Science*) and the software CellSensEntry (*Olympus Life Science*). Additionally, the crystals were tested under polarized light and a UV microscope (*JAN Scientific inc.*) to confirm the presence of protein.

2.2.3.2 Soaking of Apo-Crystals

Some apo-crystals of IleRS variants were soaked with thiomarinol A in an attempt to obtain a structure of the complex. The corresponding well was opened with a scalpel and 0.1 μ L of 10 mM thiomarinol A was added. The crystals were incubated from 5 min to 10 h before picking.

2.2.3.3 Diffraction Measurement

To send crystals to data collection, they had to be protected with a cryoprotectant, and flash frozen in liquid nitrogen. First, the cryoprotectant (**Table 19**) was mixed 1:1 with the reservoir

buffer to achieve the final concentration and added to the crystal. The crystals were picked in a CryoLoop (*Hampton*) and plunged into liquid nitrogen.

Table 19: List of all Cryoprotectants used.

Cryoprotectant	Final concentration [%]
Butanediol	20
Glycerol	20
	35
PEG400	20
Ethylene glycol	20

Crystals were measured at the Deutsches Elektronen-Synchrotron (DESY, Germany). Diffraction data were obtained from single crystals and processed with the X-ray Detector Software (XDS) Program Package (Dectris, 2026b). An AlphaFold prediction (Abramson et al., 2024) was used for Molecular Replacement in Phaser (McCoy et al., 2007) (CCP4 suite (Dectris, 2026a)). The model was refined with Refmac5 (Murshudov et al., 2011) and finally Coot (Emsley et al., 2010).

2.2.4 Cryogenic Electron Microscopy (Cryo-EM)

2.2.4.1 Cryo-EM Sample Preparation

Parallel to crystallization trials, cryo-electron microscopy (cryo-EM) was performed. The POI was supplemented with 0.01 mM ZnAc and 1 mM thiomarinol A using the same molar ratios as for crystallization (**Table 17**). Dilutions were prepared at 2, 4, 6, and 8 μ M protein concentration. For each concentration, one additional sample was prepared containing 10% β -OG. The C-Flat 2/1 copper holey carbon (mesh 200) grids (*Merck*) were glow-discharged using the GloQube Plus (*Quorum*) for 15 s at 50 mA. 5 μ L of the sample were applied to the grids using a Vitrobot Mark IV (*Thermo Fisher Scientific*).

2.2.4.2 Image Processing and Reconstruction

For both the screening process and the -40° tilted dataset, data acquisition was performed on a Glacios Cryo-TEM (*Thermo Fisher Scientific*) operated at 200 kV, using Smart EPU Software (*Thermo Fisher Scientific*) for automated image acquisition and a Falcon4i (*Thermo Fisher Scientific*) for movie collection. Motion correction, particle picking, 2D classification, and initial 3D reconstruction were performed in cryoSPARC version 3.4.0 (*Structura Biotechnology Inc.*).

2.2.5 Protein Characterisation

2.2.5.1 Protein Concentration Measurement via Nanophotometer

To measure a protein's concentration, the NanoDrop2000c (*Thermo Fisher Scientific*) was used. First, the instrument was blanked with the protein's current storage buffer, then 2 μL of the protein solution were pipetted on the nanophotometer. The absorbance at $\lambda = 280 \text{ nm}$ was measured and divided by the absorbance coefficient previously calculated using the ProtParam tool (*ExPASy*) to obtain the protein concentration in mg/mL .

2.2.5.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To estimate a protein's size, an SDS-PAGE was performed. To cast the gels, stacking and separation gel buffers were prepared and supplemented with ammonium persulfate (APS) and tetramethylethylenediamine (TEMED) to initiate polymerisation (**Table 8**). Protein samples were mixed with 5 x Laemmli buffer and denatured at 95°C for 5 min. Depending on the protein(s) size(s), either 10 % ($> 100 \text{ kDa}$) or 12 % ($< 100 \text{ kDa}$) SDS gels were selected and submerged in 1 x SDS running buffer. 10 μL of the protein samples were loaded onto the gel along with 5 μL of the Unstained Protein Standard, Broad Range (10-200 kDa) (*NEB*) or the Unstained Protein Standard IV (*SERVA*). The gel was run at a constant current of 35 mA for 40-60 minutes. The gel was submerged in Coomassie staining solution, heated up, and incubated under light shaking. After at least 15 minutes, the gel was transferred into destaining solution until the bands were sufficiently visible. Images were taken with the Quantum CX5 Edge imager (*Vilber*).

2.2.5.3 Native PAGE

To estimate protein complex formation, a native PAGE was performed. The protein samples were combined in different ratios and, after 10 min incubation, mixed with 5 x native PAGE sample buffer in a 20 μL volume. The Mini-PROTEAN TGX 4-15% gel (*Bio-Rad*) was submerged in 1 x native PAGE running buffer and 20 μL of the protein samples were applied to the gel pockets. The native PAGE was run at 4°C at 25 mA for 2.5 h. Subsequently, the gel was stained and photographed according to **2.2.5.2**.

2.2.5.4 Electrophoretic Mobility Shift Assay (EMSA)

An EMSA was performed to analyse the proteins' ability to bind DNA under different conditions. A total mass of 5 μg of protein was used (When both proteins were combined, 4 μg of XzGyrBA and 1 μg of CszB were used), and 600 ng MToligo (**Table 1**). The components were prepared accordingly, and after 10 min of incubation, they were supplemented with 5 x binding buffer and 10 x loading buffer in a 20 μL volume. A 5 % precast polyacrylamide TBE gel (*Bio-Rad*) was submerged in 0.5 x TBE buffer, and 20 μL of the protein samples were applied to each pocket. The gel was run at 4°C at 150 V for 45 min. First, the DNA was stained by submerging

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the gel in 50 μ L 0.5 x TBE buffer supplemented with 5 μ L SYBR gold (*Invitrogen*) for 30 min. The gel was imaged under UV light in the Quantum CX5 Edge imager (*Vilber*) before staining the gel with Coomassie staining solution according to **2.2.5.2**.

2.2.5.5 Analytical SEC

An analytical SEC was run to estimate the oligomerisation state of a protein by its retention time. The protocol is analogous to **2.2.2.6** with the following distinctions: the column that was used is the Superdex 200 10/300 GL (*Cytiva*), the flow rate was reduced to 0.75 mL/min, and fractions were collected right after a maximum of 500 μ L of protein were injected via a 1 mL loop.

2.2.5.6 Mass Spectrometry

To confirm the proteins' identity, the POI was diluted to 0.1-0.2 mg/mL and sent to the core facility to perform Electrospray Ionization Mass spectrometry (ESI-MS). For examination of a specific protein band, it was excised from the SDS gel and analysed via matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS) with the MassLynx V4.2 software (*Waters Corporation*).

2.2.5.7 Thermal Shift Assay (TSA)

TSAs were performed to examine the protein's thermal stability under different buffer conditions or in the presence and absence of ligands. 1 μ L of a 1:40 SYPRO ORANGE® (*Merck Millipore*) stock was added to 5 μ g of protein in a final volume of 20 μ L to a clear, unskirted Multiplate 96-Well PCR Plate (*Bio-Rad*) and sealed with adhesive optical Microseal 'B' PCR Plate Sealing Film (*Bio-Rad*). To observe protein-ligand interactions, three technical replicates were prepared manually for each condition. For buffer screens, 1 μ L of each buffer was added with the Gryphon robot (*Art Robbins Instruments*) from previously prepared deep well blocks to each condition. The plate was spun at 1000 xg for 10 min at 4 °C before it was placed into the qPCR Thermal Cycler CFX96 (*Bio-Rad*). Starting at 4 °C, the program increased the temperature in 0.5 °C increments to 98 °C while measuring the fluorescence (excitation λ =470 nm, emission λ =570 nm). The melting points were calculated in the *Bio-Rad* CFX Maestro program by the first derivative of the melting curve. For protein-ligand interactions, the final melting temperatures were calculated from the technical triplicates and reported as mean $\pm$ standard deviation.

3 Results

3.1 Investigation of the Interaction between CszB and the Gyrase Fusion Construct XzGyrBA

3.1.1 Construct Design of XzGyrBA

The first project aimed to co-crystallize the putative self-resistance protein CszB from *X. szentirmaii* with the bacterium's gyrase. To increase the chances of obtaining a co-crystal structure, the gyrase construct design focused on high solubility and good crystallisation properties. Therefore, an established strategy for gyrase crystallisation was employed (1.2.2.2), deleting the N-terminal ATPase domain of GyrB and the C-terminal DNA-wrapping domain of GyrA and seamlessly attaching GyrA₂₋₅₂₃ to GyrB₃₉₁₋₈₀₄. This resulted in the 105.8 kDa fusion protein XzGyrBA, containing the DNA-binding/cleavage domain (**Figure 8**).

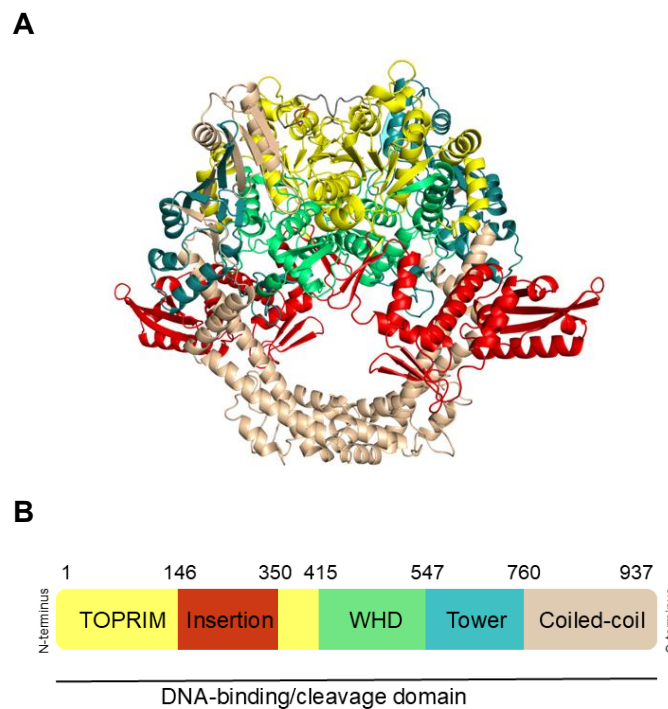


Figure 8: Fusion construct XzGyrBA. **A:** AlphaFold model (Abramson et al., 2024) of the dimerized fusion protein XzGyrBA₂. **B:** Domain architecture coloured according to the full-length *E. coli* gyrase (Broeck et al., 2019).

3.1.2 Apo-Structure Determination of XzGyrBA

XzGyrBA was first expressed without CszB, aiming to obtain an apo-crystal structure to aid in molecular replacement for a co-complex. The N-terminally His₆-MBP-tagged fusion protein was expressed in *E. coli* Rosetta2 (pLysS) cells and purified (**Figure 9**): After the initial IMAC, the relevant fractions were dialyzed overnight, and the His₆-MBP-tag was cleaved via His₆-TEV-protease. After the reverse IMAC, the proteins' purity was refined via SEC and their identity confirmed via ESI-MS (**Figure S9**).

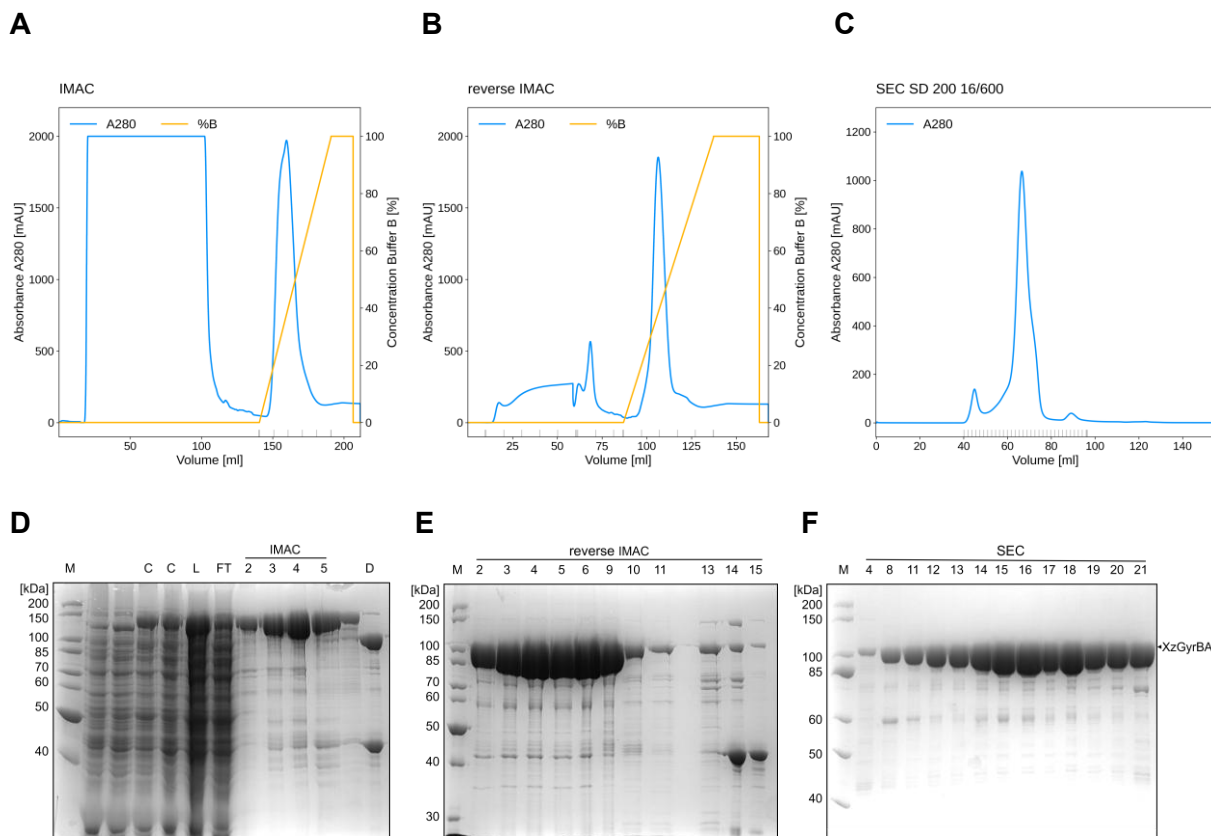


Figure 9: Purification of XzGyrBA

MW XzGyrBA = 105.8 kDa. M = marker, C = cells, L = cell lysate, FT = flow-through, D = dialysate.

A: Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-MBP-tag removal via His₆-TEV-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of the cells expressing XzGyrBA and of the fractions collected during IMAC. The two lanes after the marker and before the cells correspond to a different purification. **E:** SDS-PAGE of the fractions collected during reverse IMAC. **F:** SDS-PAGE of the fractions collected during SEC. The arrow indicates XzGyrBA.

Since XzGyrBA remained soluble in low salt and low glycerol buffers after tag removal, the glycerol was phased out and salt lowered to encourage crystallisation. The retention time on an analytical SEC (**Figure S8**) falls between the standards IgG and ferritin with molecular masses of 158 kDa and 440 kDa (Cytiva, 2020), indicating the fusion construct elutes as a dimer.

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4 mM MgCl₂ were added to the protein to complement the ionic interactions in the TOPRIM domain (Sissi & Palumbo, 2009), before setting up crystal screening plates. The initial protein concentration of 15 mg/ml yielded several crystals after 48 h (**Figure 10**). However, to improve morphology, the concentration was reduced to 10 mg/mL, yielding more promising-looking morphologies after three weeks. All crystals exhibited birefringence under polarized light and strongly absorbed UV-light at 280 nm, indicating the presence and tight packing of protein. Three datasets were collected (**Table 20**). All exhibited the same space group P1 and their resolutions ranged from 2.1 to 1.7 Å.

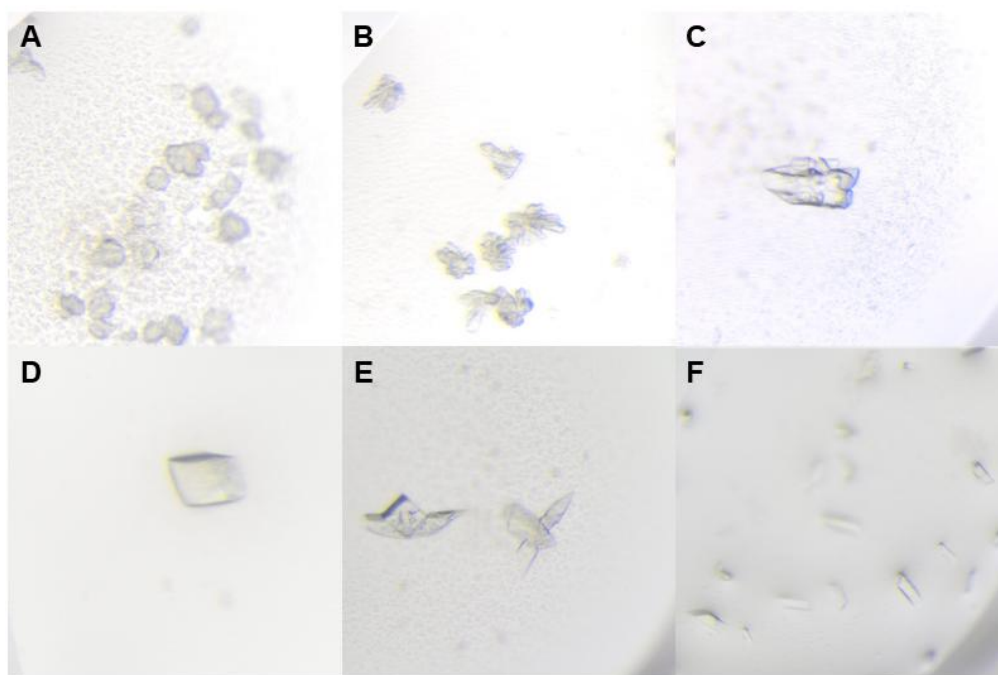


Figure 10: Representative crystals of XzGyrBA at 15 mg/ml (A, B, C) and 10 mg/ml (D, E, F). The corresponding crystallisation conditions are described in Table 20.

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Table 20: Crystallization conditions of XzGyrBA and resolution of the collected dataset. The protein was stored in the buffer XzGyr SEC (Table 6).

Figure	Protein	c [mg/ml]	T [°C]	Buffer composition	Drop ratio [protein:buffer]	Resolution [Å]
10A	Fresh	15	20	0.1 M HEPES, 2 M Ammonium sulfate	3:1	
10B	Fresh	15	20	0.1 M HEPES pH 7, 15 % (w/v) PEG 200007	2:1	
10C	Fresh	15	20	0.2 M Sodium chloride, 0.1 M Na/K phosphate pH 6.5, 25 % (w/v) PEG 1000	3:1	
10D	Thawed	10	20	0.1 M Tris pH 8, 1.5 M Ammonium sulfate	2:1	2.1
10E	Thawed	10	20	0.2 M Ammonium chloride, 20 % (w/v) PEG 3350	3:1	1.7
10F	Thawed	10	20	0.2 M Potassium/sodium tartrate, 0.1 M Bis Tris propane pH 6.5, 20 % (w/v) PEG 3350	3:1	1.8

During model building of the dataset obtained from the crystal depicted in **Figure 10E**, the sequence of GyrB₃₉₁₋₈₀₄ could not be fitted into the electron density. Further analysis revealed the reason: The fusion construct was not intact within the crystal. Instead of the expected XzGyrBA dimer, the electron density corresponds to four XzGyrBA₄₄₇₋₉₃₇ fragments, corresponding to the GyrA₃₃₋₅₂₃ domain, lacking approximately 30 amino acids at the N-terminus near the linker region. To assess whether the thawing process caused this issue, crystals set up right after purification were also analysed (**Figure 10C**). These crystals exhibited the same space group, suggesting a similar composition and conformation.

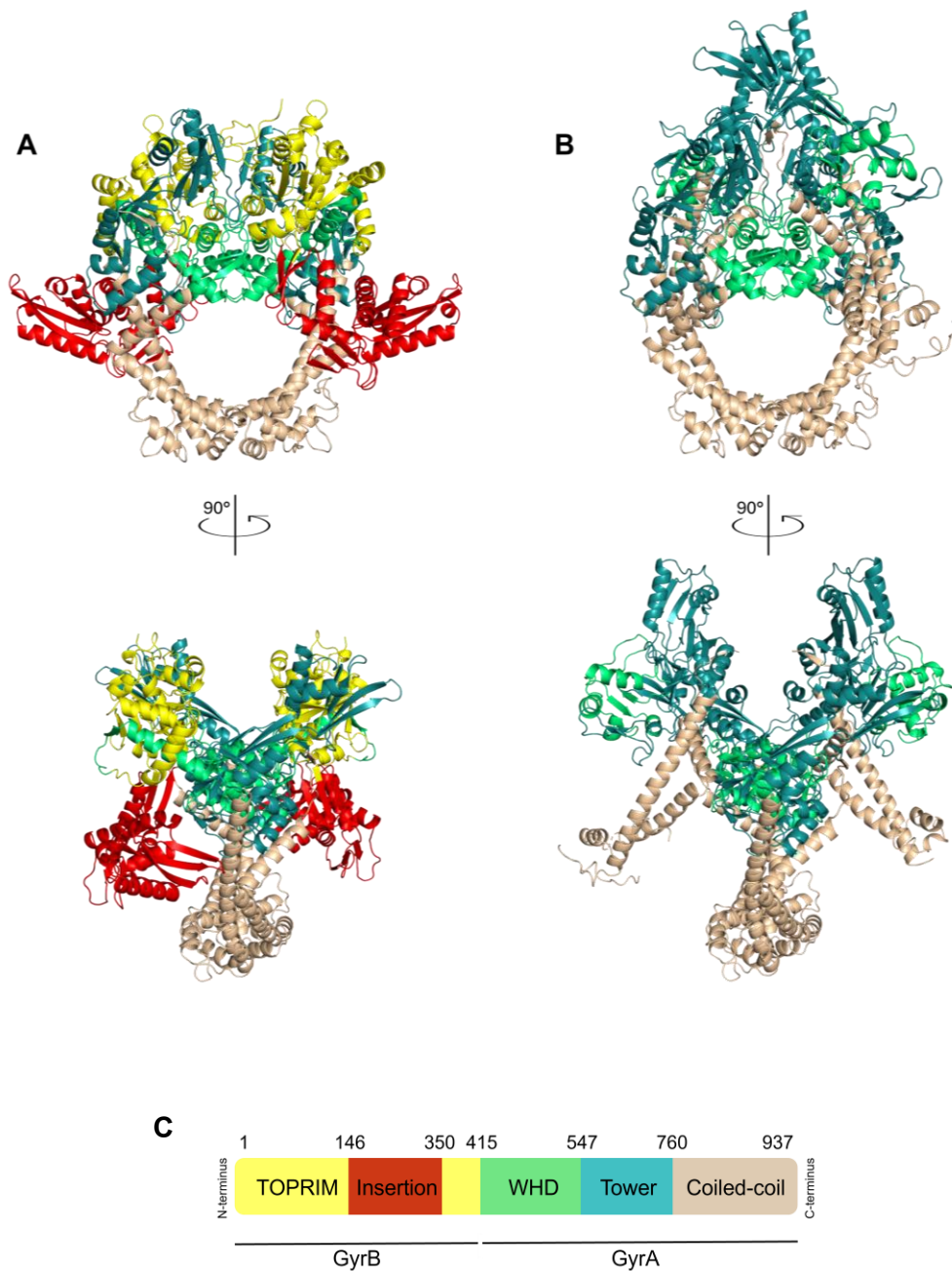


Figure 11: Crystal structure of XzGyrBA₄₄₇₋₉₃₇ compared to AlphaFold prediction of XzGyrBA.

A: AlphaFold model (Abramson et al., 2024) of XzGyrBA. **B:** Crystal structure of the homo-tetramer XzGyrBA₄₄₇₋₉₃₇. **C:** Domain architecture of XzGyrBA.

3.1.2.1 Investigation of Proteolysis in XzGyrBA

For further investigation, an aliquot of XzGyrBA was thawed and remeasured by ESI-MS over a wider range. The fragment that was assigned to the electron density has a calculated mass of 55.25 kDa. In the freshly thawed XzGyrBA, only the intact fusion protein was detected at 105.8 kDa with no evidence for degradation (**Figure S10**).

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Microcrystals from the crystals shown in **Figure 10E** were dissolved and analysed by ESI-MS to directly evaluate the protein's mass inside the crystal (**Figure S11**). In this sample, the fusion protein's mass of 105.8 kDa was absent, confirming that no intact construct crystallized under these conditions. Instead, two new peaks were detected, one of them at 55.86 kDa, which is identical to the calculated mass of the slightly longer fragment XzGyrBA₄₄₁₋₉₃₇. The corresponding hypothesis is that the crystallisation conditions facilitated breakage of the fusion construct between M440 and S441 as depicted in **Figure 12**, resulting in four GyrA fragments associating and crystallizing with the N-terminal six amino acids too flexible to be resolved in the electron density map. To investigate if prolonged storage in solution also leads to proteolysis, an aliquot of XzGyrBA was thawed and incubated at 4 °C for four weeks prior to ESI-MS measurement. In this sample, the mass spectrum showed the intact fusion protein alongside new species with masses around 60 kDa, suggesting partial degradation of the fusion product (**Figure S12**).

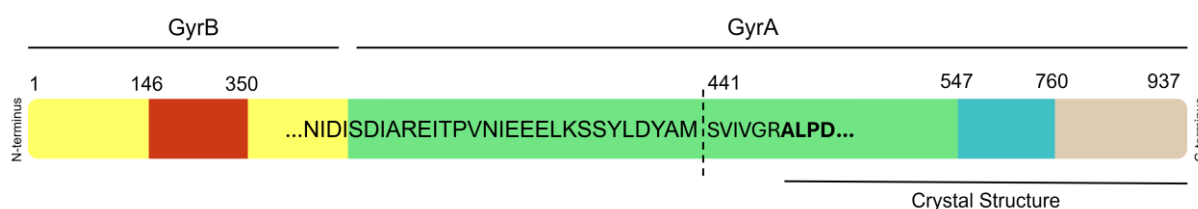


Figure 12: Sequence of XzGyrBA linker region with proposed breakage point between M440 and S441 (**dotted line**), six amino acids before the beginning of the crystal structure (**bold**).

Comparing the protein masses obtained from the dissolved crystal (**Figure S11**) with the masses in the aged sample (**Figure S12**) shows that, although not identical, the degradation products fall within a similar range. Taken together, these results indicate that the fusion protein is susceptible to proteolysis in the linker region, both under crystallization conditions and during prolonged storage in solution. This instability suggests that the current construct is not sufficiently robust and requires modification, such as mutation of the linker to prevent breakage at the fusion site.

3.1.3 Co-purification of XzGyrBA and CszB

In the next section, the co-purification of CszB with XzGyrBA was attempted by selectively tagging one of the putative interaction partners. Successful co-purification requires that the two proteins form a complex that remains intact throughout the chromatography steps. Two alternative constructs were designed, with either CszB or XzGyrBA carrying the tag, to account for possible steric interference caused by tagging.

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3.1.4 *His₆-MBP-tagged XzGyrBA with CszB*

In the first approach, the His₆-MBP-tagged construct previously used for XzGyrBA expression was expanded by inserting CszB into the multiple cloning site (MCS) 2. The proteins were expressed in *E. coli* Rosetta 2 pLysS cells. The initial co-purification used the same protocol and buffers as for XzGyrBA. Although a faint band was visible in the right range in the SDS-PAGEs, in the final SEC step the band shifted to the aggregation peak and XzGyrBA eluted alone. Since the buffers used until SEC contained 5-10 % glycerol and the two bands eluted together until SEC and separated during this step, it was assumed that the purification failed due to the absence of glycerol, causing aggregation of CszB.

In the next attempt, the glycerol was kept at 10% throughout the purification to increase solubility of CszB. Further, the initial salt concentration was lowered from 500 to 300 mM to avoid shielding of electrostatic interactions of the complex.

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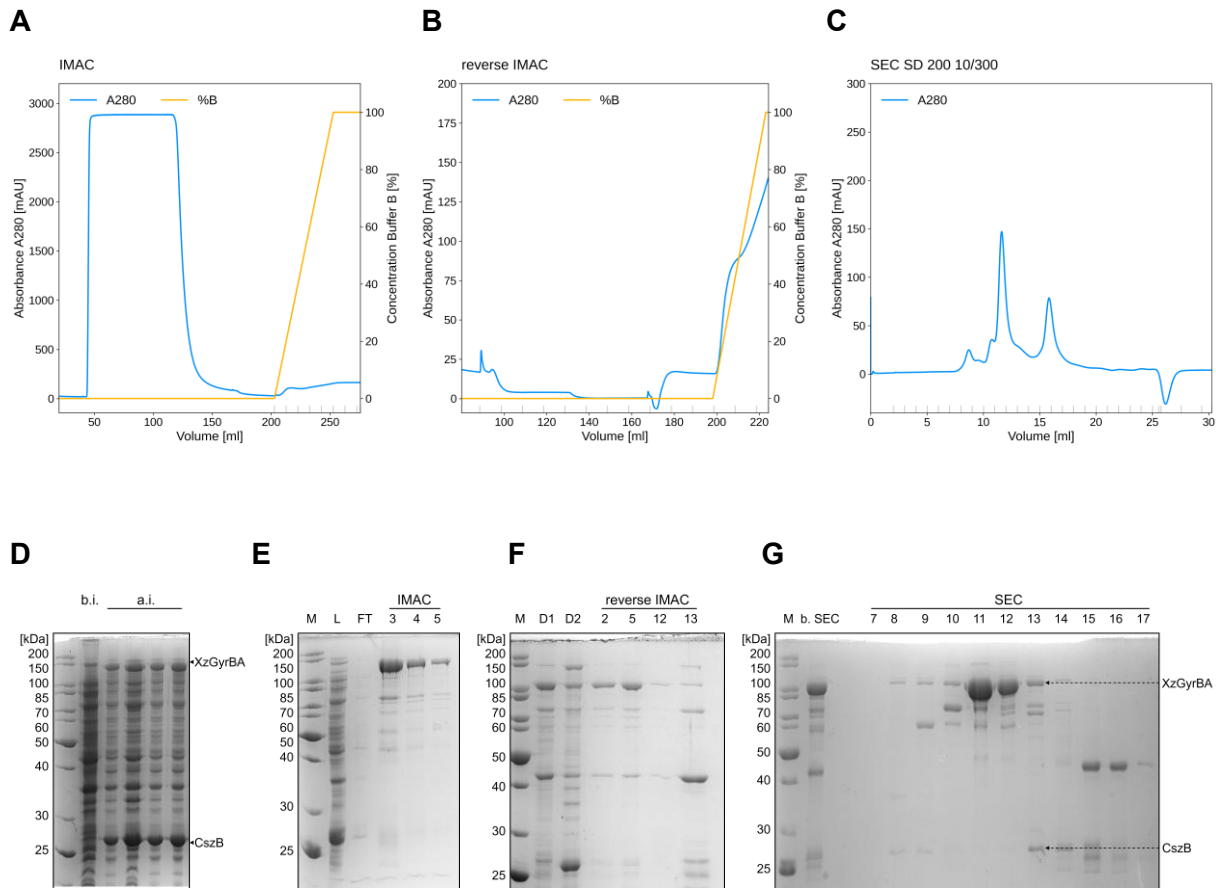


Figure 13: Purification of XzGyrBA:CszB with adapted buffer conditions

MW XzGyrBA = 105.8 kDa, MW CszB = 28.7 kDa. M = marker, b.i. = cells before induction, a.i. = cells after induction, L = cell lysate, FT = flow-through, D1 = dialysate 1, D2= dialysate 2, b. SEC = before SEC. **A:** Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-MBP-tag removal via His₆-TEV-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of the cells exhibiting strong expression of XzGyrBA and CszB, indicated by arrows. **E:** SDS-PAGE of the fractions collected during IMAC. **F:** SDS-PAGE of the dialysate and fractions collected during reverse IMAC. **G:** SDS-PAGE of the sample before SEC and of the fractions collected during SEC with arrows indicating both XzGyrBA and CszB in fraction 13.

The cells exhibited strong expression of CszB that far exceeded the amount of XzGyrBA (**Figure 13D**). However, most of the CszB passed through the Ni-NTA without associating with the column-bound XzGyrBA (**Figure 13E**). The SEC Chromatogram (**Figure 13C**) showed several peaks with different combinations of protein (**Figure 13G**), including XzGyrBA and faint bands in the region 25-30 kDa. Analysis via ESI-MS (**Figure S13**) confirmed that only fraction 13 contained both XzGyrBA and CszB. This fraction corresponded to the shoulder after the main peak, harbouring most of the XzGyrBA. Since the fraction containing both XzGyrBA and CszB elutes after the peak corresponding to the likely dimeric form of XzGyrBA, the current hypothesis is that CszB interacts non-specifically with monomeric XzGyrBA. This interaction may also involve additional co-eluting proteins in the 69–85 kDa range, resulting in

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heterogeneous complexes with apparent molecular weights below 158 kDa, based on the column calibration standards (Cytiva, 2020).

3.1.4 His₆-SUMO-tagged CszB with XzGyrBA

In an alternative approach, co-purification of His₆-SUMO-tagged CszB was attempted with the untagged XzGyrBA inserted in the MCS2. While CszB was successfully purified (**Figure 14**), confirmed by ESI-MS (**Figure S14**), no co-elution of XzGyrBA was observed. The purified CszB was used for further experiments, including a buffer screen (**Figure S18**), confirming that CszB strongly prefers glycerol, high salt (500-750 mM), and a pH between 6 and 8.

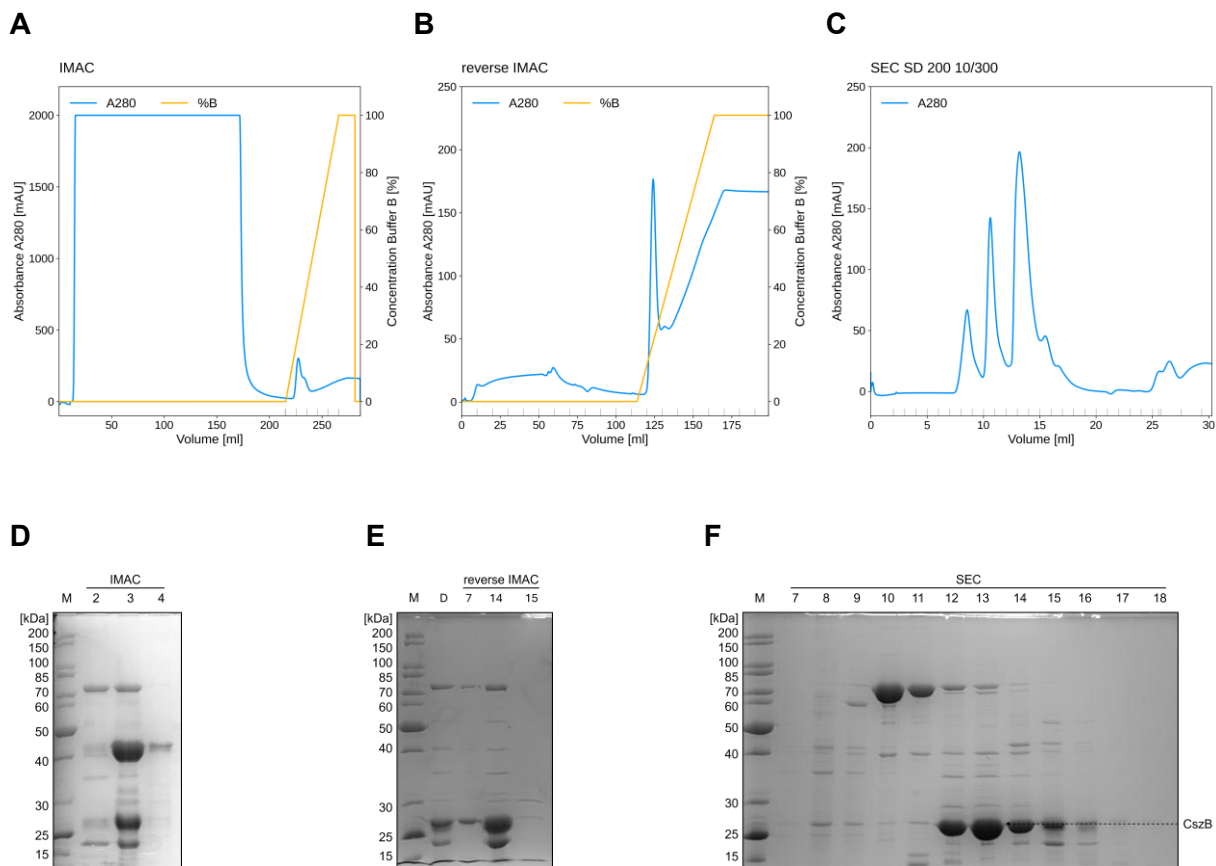


Figure 14: Purification of CszB:XzGyrBA.

MW XzGyrBA = 105.8 kDa, MW CszB = 28.7 kDa. M = marker, D = dialysate. **A:** Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-SUMO-tag removal via His₆-SUMO-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of the fractions collected during IMAC. **E:** SDS-PAGE of the dialysate and the fractions collected during reverse IMAC. **F:** SDS-PAGE of the fractions collected during SEC with an arrow indicating CszB in fraction 13.

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3.1.5 Biochemical Investigation of CszB-XzGyrBA Complex Formation

The results above suggest that XzGyrBA and CszB associate to a certain extent but that this interaction is either transient or nonspecific and does not result in a stable complex. To further assess their interaction, a native PAGE (**Figure 15A**) and a TSA (**Figure 15B**) were performed using different ratios of XzGyrBA and CszB.

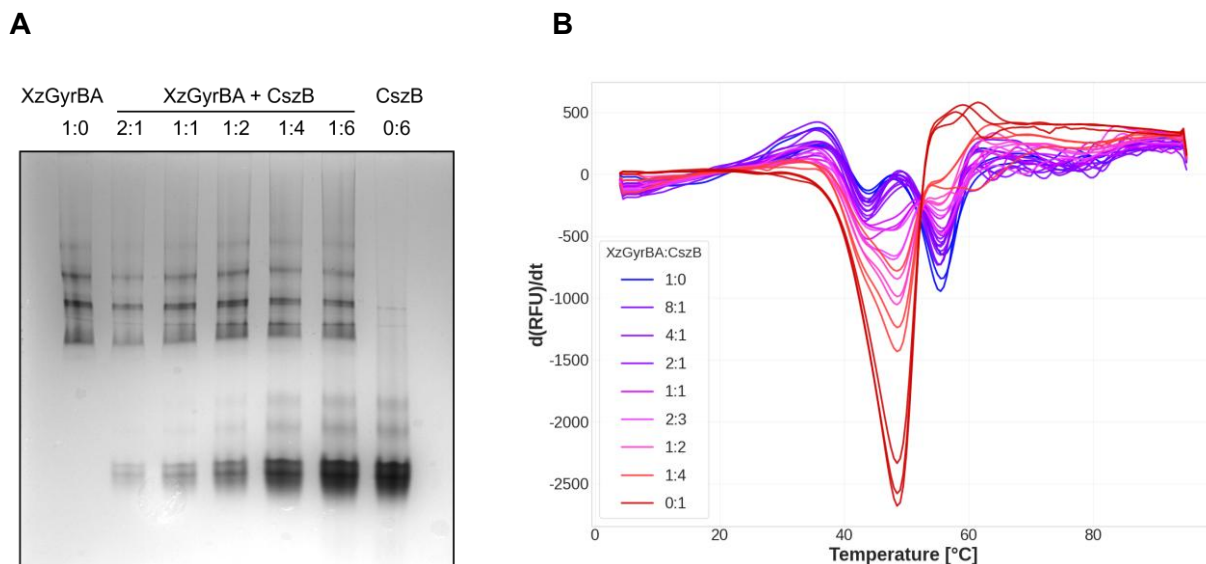


Figure 15: Biochemical investigation of complex formation between XzGyrBA and CszB.

A: Native PAGE of XzGyrBA with CszB in different ratios. **B:** Thermal shift assay of XzGyrBA with CszB in different ratios.

To compare the migration pattern of the combined proteins in the native PAGE to the apo-proteins, each protein was applied alone on either side of the gel (**Figure 15A**). The apo-proteins display distinct band patterns with little overlap. The upper bands are attributed to XzGyrBA, potentially in different multimeric states, while CszB most likely corresponds to the lower bands. It should be noted that the CszB sample was derived from the purification shown in **Figure 14**, and therefore some of the observed bands may originate from co-purified contaminants. Since all the combinations exhibit an additive effect on the migration pattern, there is no indication for complex formation.

Different ratios of XzGyrBA and CszB were also investigated via TSA (**Figure 15B**) to visualize potential interactions that influence the thermal stability of the constituents. In accordance with the native PAGE, only additive effects were observed in the TSA, indicating no measurable interaction between the two proteins under the tested conditions.

Lastly, the fusion protein's ability to bind and protect DNA with and without CszB was investigated with an EMSA (**Figure 16**). The DNA used in this experiment, MToligo (**Table 1**),

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is the same oligonucleotide that was used during crystallisation of the *M. tuberculosis* gyrase fusion construct (Blower et al., 2016).

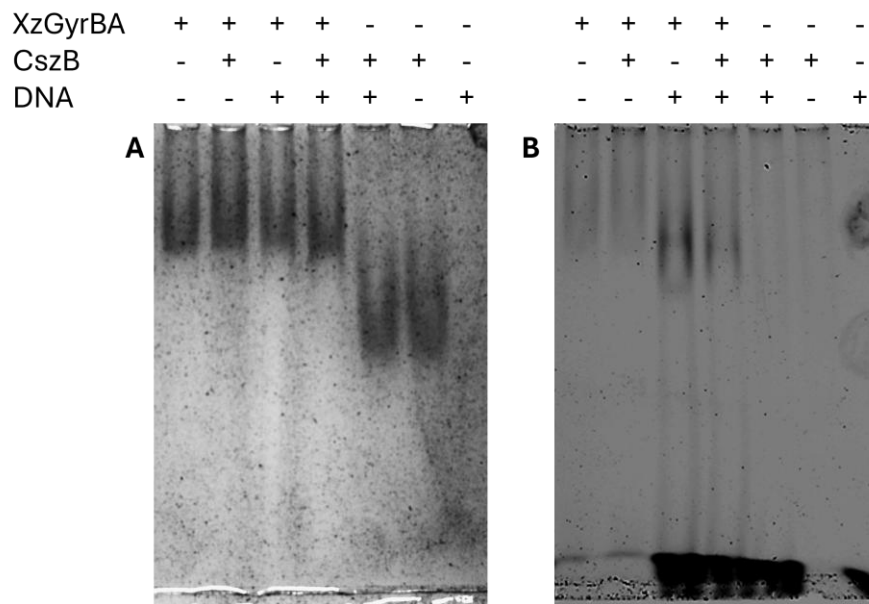


Figure 16: EMSA investigating the fusion construct XzGyrBA's ability to bind DNA in the presence and absence of CszB. **A:** Coomassie staining of protein bands. **B:** SYBR gold staining of DNA bands.

The controls on the right end of the gel confirm that CszB and DNA have no detectable interaction. XzGyrBA shows a slight UV signal at the same height as the protein, indicating that a small amount of DNA was co-purified with the protein. However, adding MToligo results in an increased UV signal, suggesting that XzGyrBA was able to bind more DNA. The addition of CszB results in minimally reduced DNA binding of XzGyrBA, suggesting that CszB might slightly decrease the gyrase's ability to bind DNA.

3.2 Investigation of Thiomarinol A's Interaction with different IleRS Variants

3.2.1 Purification of different IleRS Variants

The second project investigates the macromolecular interaction between the hybrid antibiotic thiomarinol A and different IleRS variants with biochemical and structural methods. While previous studies with thiomarinol A focused on SalleRS1 from *S. aureus* (R. A. Johnson *et al.*, 2021) this thesis also investigates its direct homologue PmIleRS1 and the mupirocin-resistant variant PmIleRS2 from *Priestia megaterium*. Furthermore, a C-terminally truncated variant of PmIleRS2, PmIleRS2ct, was generated for crystallisation trials based on a previously reported crystal structure of the IleRS2 variant in *Thermus thermophilus* (Nureki *et al.*, 1998).

All proteins were expressed in *E. coli* BL21 gold (DE3) and purified via IMAC, dialysis, reverse IMAC, and SEC. The proteins' identities were confirmed via ESI-MS (**Figures S15-S17**), excluding PmIleRS2ct due to problems at the in-house ESI-MS facility. While SalleRS1 (**Figure 17**), PmIleRS1 (**Figure 18**), and PmIleRS2 (**Figure 19**) were expressed in high quantities and good purity, the yield of PmIleRS2ct (**Figure 20**) was much lower and showed more impurities.

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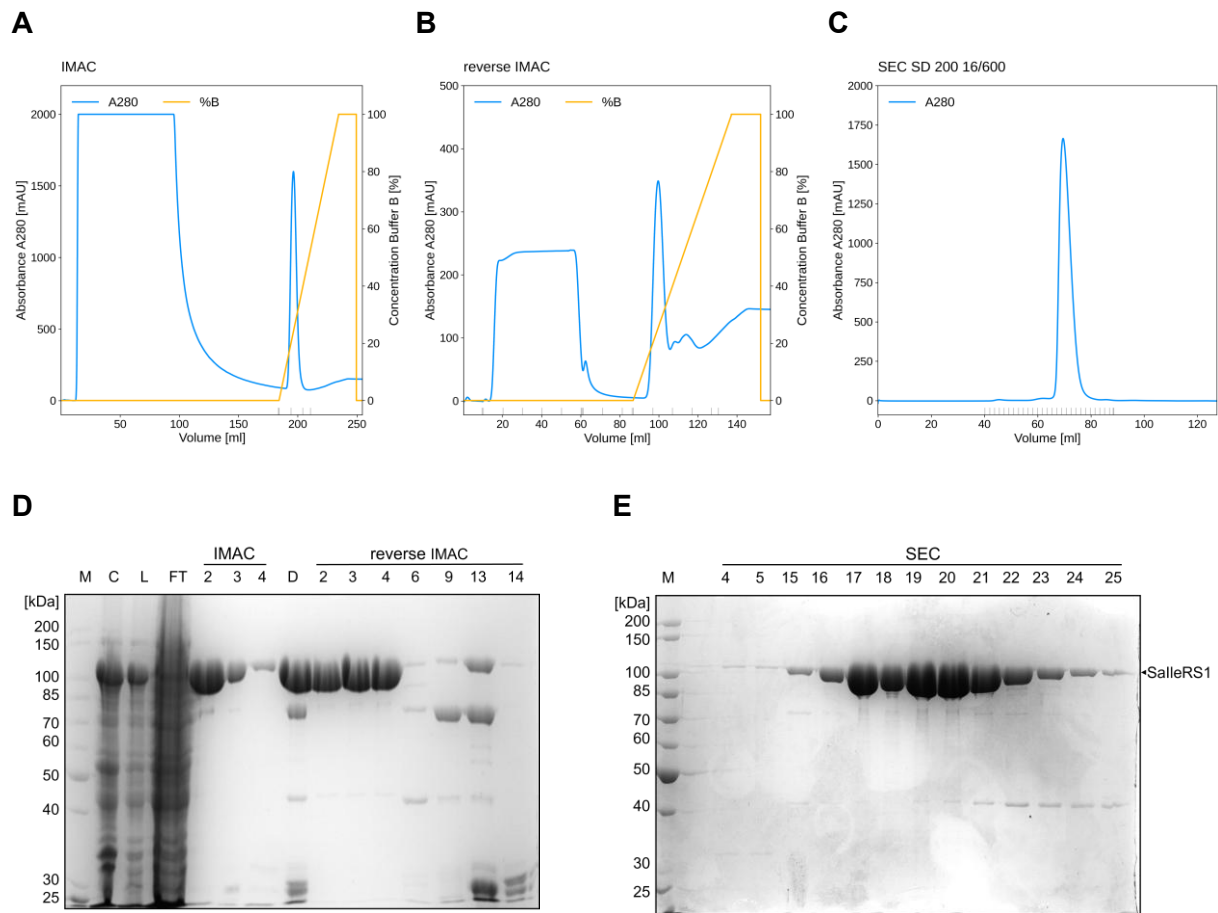


Figure 17: Purification of SalleRS1.

MW SalleRS1 = 105.2 kDa. M = marker, C = cells, L = cell lysate, FT = flow-through, D = dialysate. **A:** Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-tag removal via His₆-TEV-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of the cells exhibiting expression of SalleRS1, fractions collected during IMAC, the dialysate, and fractions collected during reverse IMAC. **E:** SDS-PAGE of fractions collected during SEC with an arrow indicating SalleRS1.

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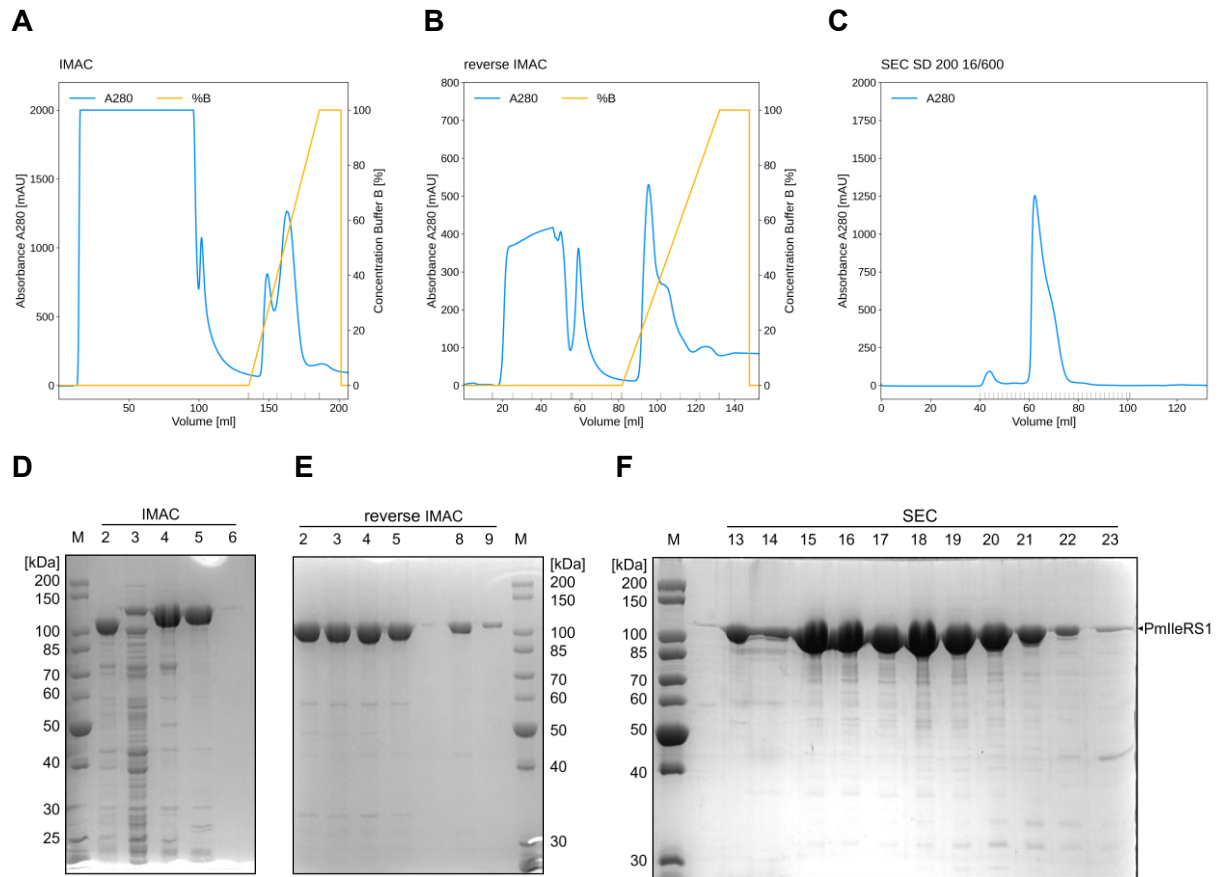


Figure 18: Purification of PmlleRS1.

MW PmlleRS1 = 104.9 kDa. M = marker. **A**: Chromatogram of IMAC. **B**: Chromatogram of reverse IMAC after His₆-SUMO-tag removal via His₆-SUMO-protease. **C**: Chromatogram of SEC. **D**: SDS-PAGE of fractions collected during IMAC. **E**: SDS-PAGE of fractions collected during reverse IMAC. **F**: SDS-PAGE of fractions collected during SEC with an arrow indicating PmlleRS1.

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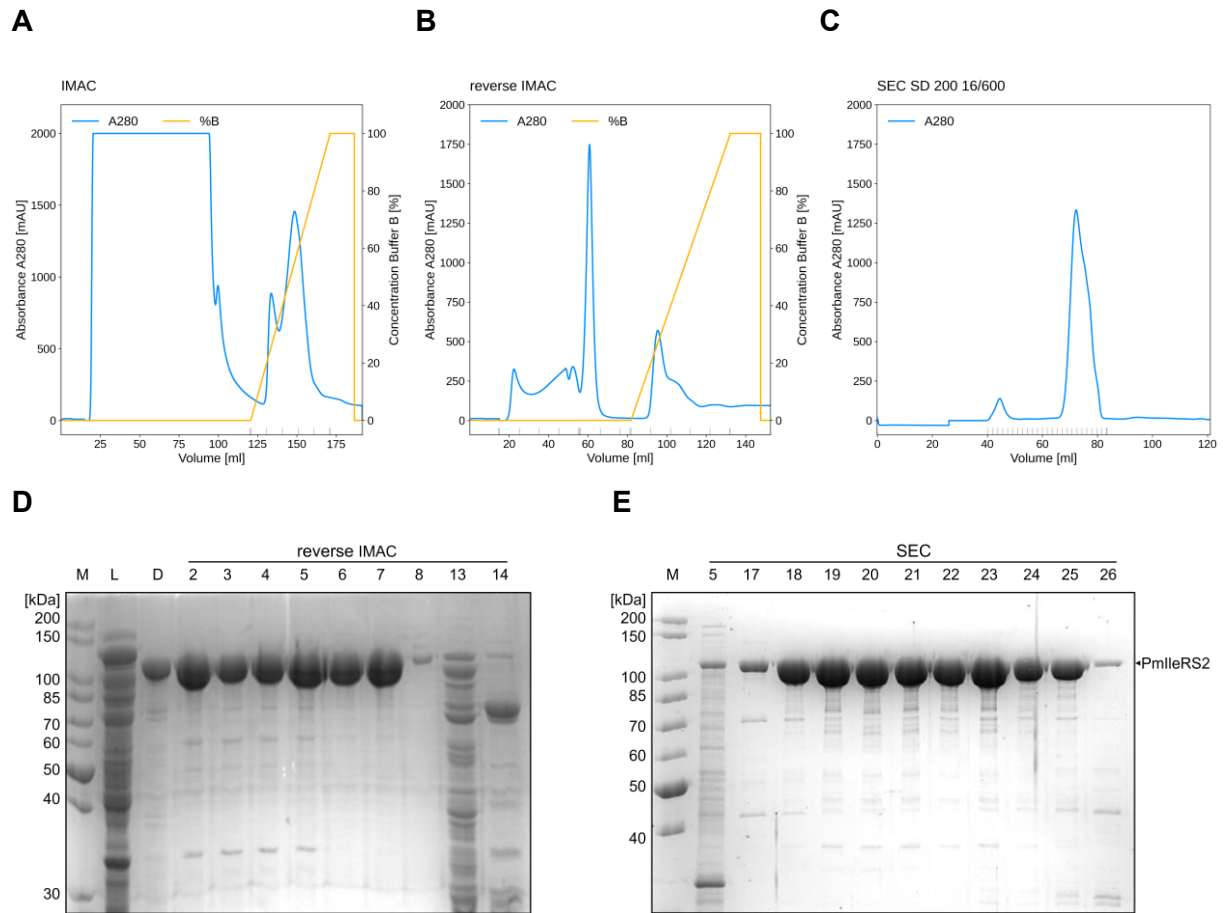


Figure 19: Purification of PmlleRS2.

MW PmlleRS2 = 117.5 kDa. M = marker, L = cell lysate, D = dialysate. **A:** Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-SUMO-tag removal via His₆-SUMO-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of the lysate, the dialysate, and fractions collected during reverse IMAC. **E:** SDS-PAGE of fractions collected during SEC with an arrow indicating PmlleRS2.

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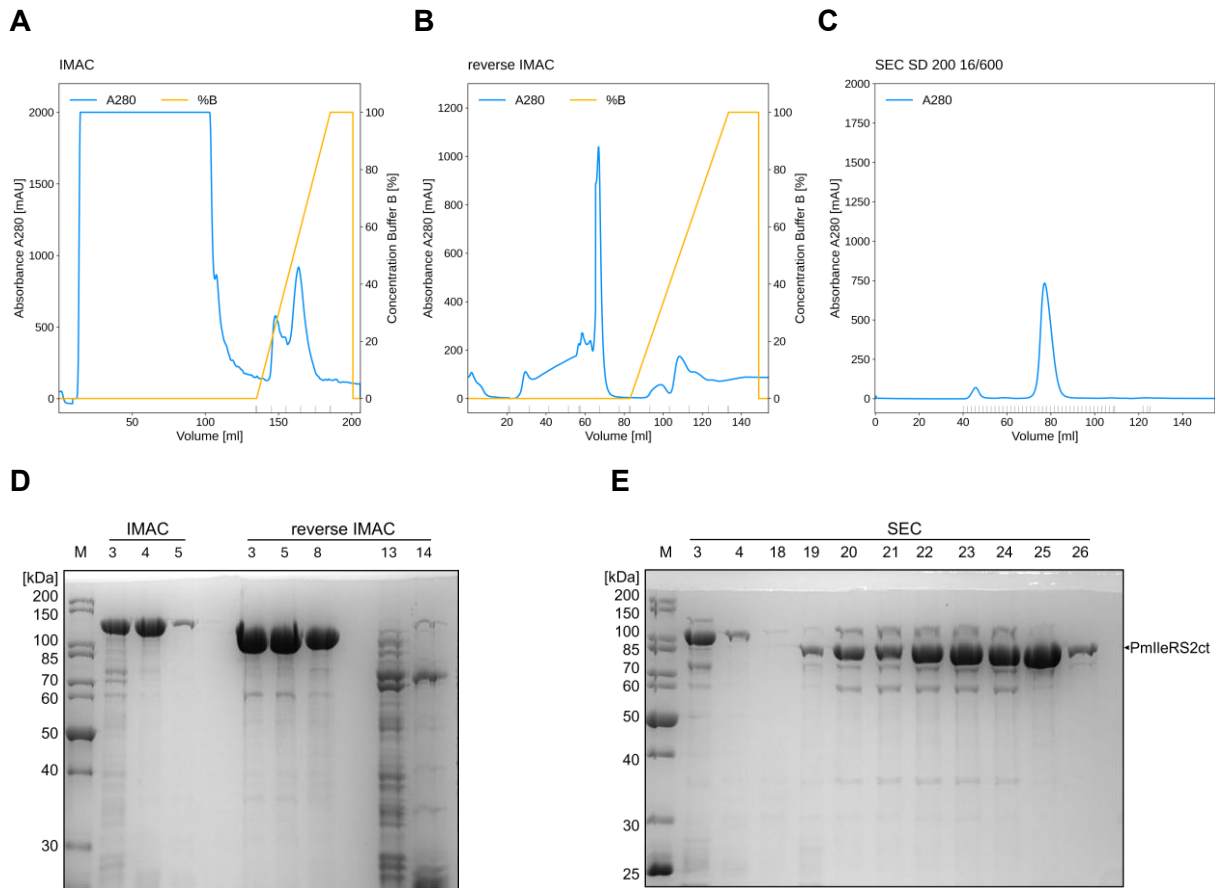


Figure 20: Purification of PmlleRS2ct.

MW PmlleRS2ct = 92.7 kDa. M = marker. **A:** Chromatogram of IMAC. **B:** Chromatogram of reverse IMAC after His₆-SUMO-tag removal via His₆-SUMO-protease. **C:** Chromatogram of SEC. **D:** SDS-PAGE of fractions collected during IMAC and reverse IMAC. **E:** SDS-PAGE of fractions collected during SEC with an arrow indicating PmlleRS2ct.

3.2.2 Biochemical Investigation of the Interaction of different IleRS Variants with Thiomarinol A

Previous *in vitro* studies with thiomarinol A focused on the mupirocin-susceptible variant SallerS1 (R. A. Johnson et al., 2021), although thiomarinol A was shown to remain potent *in vivo* against the high-level mupirocin-resistant IleRS2 variant MupA (Fukuda et al., 2011) (R. A. Johnson et al., 2021). To compare thiomarinol A's influence on IleRS1 and IleRS2 variants, the thermal stability of SallerS1, PmlleRS1, and PmlleRS2 was investigated via TSA in the presence and absence of the hybrid antibiotic (**Figure 21**).

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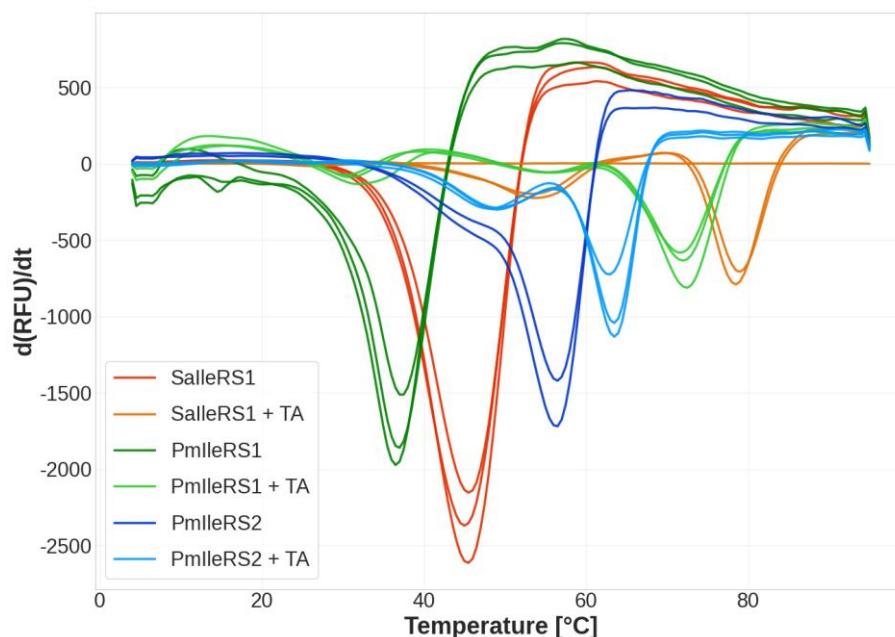


Figure 21: TSA of the IleRS variants SalleRS1, PmIleRS1 and PmIleRS2 with and without thiomarinol A (TA). Plot of the 1st derivative of RFU against temperature.

Table 21: Melting temperatures T_m ' of the IleRS variants SalleRS1, PmIleRS1 and PmIleRS2 with and without thiomarinol A (TA) obtained from the TSA in Figure 21. All values are reported as mean $\pm$ standard deviation.

Protein	T_m [°C]	$T_m + TA$ [°C]	ΔT_m [°C]
SalleRS1	45.3 $\pm$ 0.3	78.5 $\pm$ 0.5	+ 33.17 $\pm$ 0.29
PmIleRS1	37.0 $\pm$ 0.5	72.0 $\pm$ 0.5	+ 35.00 $\pm$ 0.50
PmIleRS2	56.5 $\pm$ 0.0	63.3 $\pm$ 0.3	+ 6.83 $\pm$ 0.29

Upon addition of thiomarinol A, the melting temperature for SalleRS1 rose by 33.17 $\pm$ 0.29 °C (all values are reported as mean $\pm$ standard deviation). The direct homologue PmIleRS1 has a comparable temperature increase of 35.00 $\pm$ 0.50 °C. In contrast to these observations, PmIleRS2 has a comparably modest temperature increase of only 6.83 $\pm$ 0.29 °C (**Table 21**). These results suggest that all tested IleRS variants associate with thiomarinol A, but IleRS1 variants bind significantly tighter to the hybrid antibiotic, with PmIleRS1 exhibiting the highest shift in melting temperature.

3.2.2 Crystallisation of Different IleRS Variants with and without Thiomarinol A

For structural investigation of the macromolecular interaction between the hybrid antibiotic and the IleRS variants, crystallisation trials were set up for SalleRS1, PmIleRS1, PmIleRS2, and PmIleRS2ct. All proteins were supplemented with 0.01 mM ZnAc to support the integrity of their zinc-finger motifs (Chung et al., 2020). For co-crystallisation trials, the proteins were preincubated with 1 mM thiomarinol A for 10 min on ice. Despite the low solubility of thiomarinol A in water (Shiozawa et al., 1993), there was only minor precipitation after spinning. For all full-length proteins, both co-crystallisation and apo-protein crystallisation plates were set up, while only apo-protein screens were set up for PmIleRS2ct, due to the low yield.

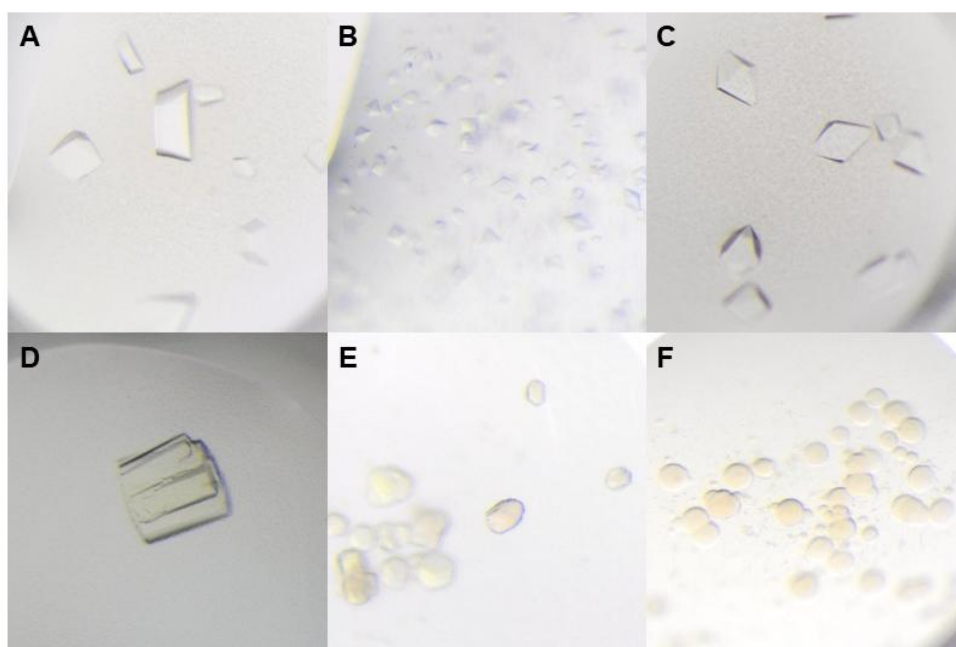


Figure 22: Representative crystals of SalleRS1 (G), PmIleRS1 (A, E) and PmIleRS2 (B, C, F). The corresponding crystallisation conditions are described in Table 22.

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Table 22: Crystallization conditions of SalleRS1, PmlleRS1 and PmlleRS2. The protein was stored in buffer IleRS SEC (Table 6).

Figure	Protein	c [mg/ml]	T [°C]	Buffer composition	Drop ratio [protein:buffer]
22A	PmlleRS1	20	20	0.2 M Sodium acetate, 20%, (w/v) PEG 3350	3:1
22B	PmlleRS2	16.5	4	0.1 M MES, 2.0 M Sodium chloride, pH 6.0	3:1
22C	PmlleRS2*	16.5	4	0.1 M Sodium acetate, 0.2 65 % (v/v) MPD, pH 5.0	3:1
22D	SalleRS1 + TA	20	20	0.2 M Ammonium chloride, 0.1 M MES pH 6 20%, (w/v) PEG 6000	3:1
22E	PmlleRS1 + TA	20	20	0.2 M Magnesium chloride, 0.1 M Tris pH 8, 20% (w/v) PEG 6000	3:1
22F	PmlleRs2 + TA	16.5	4	0.1 M Sodium acetate, 0.2 65 % (v/v) MPD, pH 5.0	2:1

* Cleaner purification

A few representative crystals are shown in **Figure 22** with the corresponding conditions in **Table 22**. PmlleRS2ct produced no crystals under the tested conditions. The full-length protein PmlleRS2 produced very small apo-crystals in one condition (**Figure 22B**). After improving the protein purity, several big crystals grew (**Figure 22C**). Unfortunately, they were not stable and completely disintegrated during picking or the soaking process. When PmlleRS2 was set up with thiomarinol A, only spherulites were produced in one condition (**Figure 22F**).

PmlleRS1 produced plenty of very promising-looking apo-protein crystals (**Figure 22A**) and several co-crystals (**Figure 22E**). Unfortunately, the apo-protein crystals were also not very stable, however, a few crystals were successfully soaked with thiomarinol A. SalleRS1 produced a variety of apo-protein crystals and co-crystals (**Figure 22D**) of various morphologies. A selection of soaked crystals, apo-protein crystals, and co-crystals from SalleRS1 and PmlleRS1 was sent for data collection. All crystals exhibited birefringence under polarized light and showed strong UV absorption at $\lambda = 280$ nm, indicating the presence of protein in the crystal.

Although diffraction patterns confirmed that the crystals contained protein, none of the crystals diffracted sufficiently for structure determination. The best result was obtained from the

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co-crystal of SalleRS1 shown in **Figure 22D**, which diffracted to approximately 5 Å resolution, followed by the co-crystal of PmlleRS1 shown in **Figure 22E** with a resolution of 8 Å.

Several attempts were made to improve crystal quality. For SalleRS1, a fine screen was designed around the condition resulting in the 5 Å diffraction (**Figure 22D**). For PmlleRS1, seeds were produced from the remaining co-crystals (**Figure 22E**) and used on a fine screen around the same condition at 4 and 20 °C. Another fine screen was set up for PmlleRS1 testing the crystallisation conditions from a publication where PmlleRS1 was co-crystallized with mupirocin (Brkic et al., 2023). However, none of the crystals resulted in a diffraction pattern usable for structure determination.

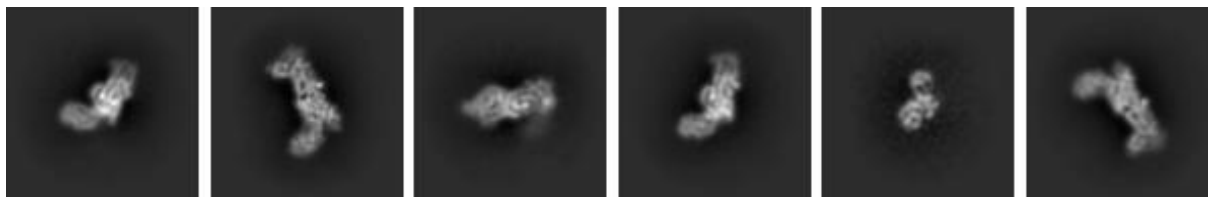
3.2.3 Cryo-EM Structure Determination of PmlleRS1 with Thiomarinol A

To improve the chances for a structure of lleRS bound to thiomarinol A, cryo-electron microscopy studies were conducted in parallel to the crystallisation trials. Since PmlleRS1 exhibited the largest thermal stabilisation upon thiomarinol A binding during TSA (**Figure 21**), it was selected for this experiment. PmlleRS1 was supplemented with ZnAc and thiomarinol A according to the molar ratios used for crystallisation. The protein was diluted to 2-8 µM. For each concentration, one grid was prepared with and one without β-OG. First pictures of the atlas quickly revealed that samples treated with β-OG showed much thinner and more homogeneous ice, and the concentration 4 µM was selected as the best compromise for sufficient population without overlapping of the particles.

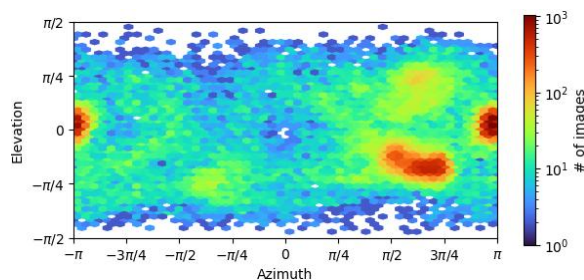
After collection of a small dataset, the 2D classification showed a strong preference for two orientations (**Figure 23A**) on the same side of the L-shaped protein, while the other sides were less populated, as indicated by the lack of orientations in the middle of the orientation distribution plot (**Figure 23B**). The initial 3D reconstruction showed a very good resolution in the preferred view, a very low resolution in the back view, and medium resolution in the top and bottom views, because the projections could not be assigned accurately. In the next step, a -40° tilted acquisition dataset was collected to compensate for the lacking orientations. The protein concentration was set to 1 µM to reduce the risk of particle overlap at the steeper angle. Merging the datasets filled in many missing orientations (**Figure 23C**), and a preliminary structure of 3.5 Å resolution was achieved (**Figure 23D**). Although the resolution at this moment is not good enough to see thiomarinol A, these results are a promising first step towards a cryo-EM structure of the complex.

3 Results

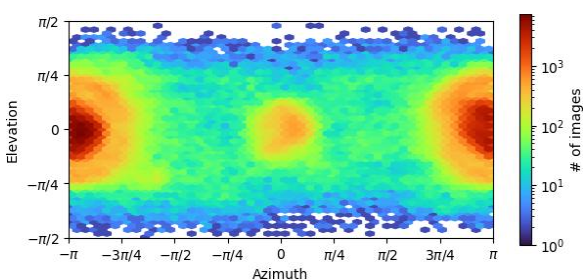
A



B



C



D

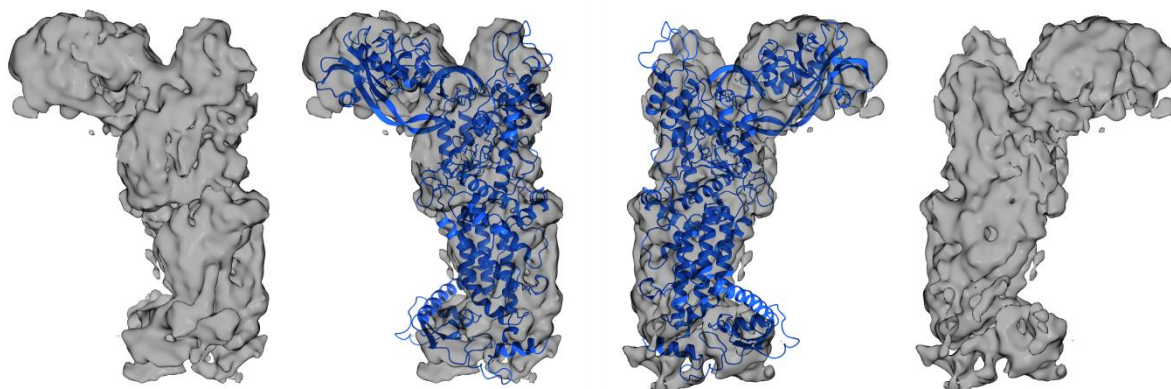


Figure 23: Results of the cryo-EM studies of *PmlleRS1* with thiomarinol A.

A: 2D classes of *PmlleRS1*. **B:** Orientation distribution plot visualizing the distribution of orientations after the first dataset was collected. **C:** Orientation distribution plot visualizing the distribution of orientations after the - 40° tilted acquisition was merged to the initial dataset. **D:** Preliminary 3.5 Å model of *PmlleRS1* bound to thiomarinol A (grey) compared to crystal structure 8C9G of *PmlleRS1* bound to mupirocin (blue).

4 Discussion

4.1 Interaction of CszB with XzGyrBA

4.1.1 XzGyrBA is prone to Proteolysis in the Linker Region

For this project, a truncated fusion construct of the DNA-binding/cleavage domain of *X. szentirmaii*'s gyrase was designed after a strategy commonly used for gyrase crystallisation (Blower et al., 2016). To encourage correct complex formation, the GyrB C-terminus was seamlessly fused to the GyrA N-terminus, and to reduce flexibility, the N-terminal ATPase domain of GyrB and the C-terminal wrapping domain of GyrA were deleted. Although the fusion protein's mass was confirmed after purification by ESI-MS, the apo-crystal structure of XzGyrBA contained four C-terminal fragments of the fusion protein, corresponding to most of GyrA. All measured crystals exhibited the same space group, suggesting a similar composition of their asymmetric unit. Analysis via mass spectrometry indicated that extended storage of XzGyrBA in solution resulted in proteolytic cleavage in the linker region of the fusion protein around the linker region. The resulting accumulation of C-terminal fragments led to their preferred incorporation in the protein crystal, resulting in the structure of the GyrA homo-tetramer. Therefore, the construct is unsuitable for further crystallisation trials and needs to be revised, for example by mutation of the linker region to increase flexibility.

4.1.2 XzGyrBA and CszB don't form a stable Complex

The main goal of this project was to co-purify and co-crystallize CszB with XzGyrBA. Previous attempts to purify and crystallize N-terminally His₆-tagged CszB on its own indicated low solubility of the PRP (Angerer, 2025). To increase solubility and get insights into the protein-protein interaction, several attempts were made to co-purify CszB and XzGyrBA as a complex by tagging one of the constituents: When CszB was tagged, no untagged XzGyrBA was co-purified, indicating no interaction. When XzGyrBA was tagged, only a heterogeneous fraction contained both proteins, indicating transient or nonspecific interaction.

Further experiments were conducted with XzGyrBA and CszB attempting to reconstitute an eventual weak complex. Previous native PAGE studies with the homologue QnrA have reported complex formation of the PRP with the gyrase holoenzyme, GyrB, and GyrA (Tran et al., 2005). Our results from the native PAGE with XzGyrBA and CszB have not shown any band shift that would indicate complex formation. However, the above-mentioned experiments used specific antibodies to identify bands containing QnrA, while our experiments relied on visible band shifts after Coomassie staining. It is possible that the size difference of the constituents makes this method too insensitive without using antibodies. To visualize potential

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weak interactions between XzGyrBA and CszB, a TSA was performed showing no influence of CszB on XzGyrBA's stability or vice versa. Finally, an EMSA was performed to investigate the influence of CszB on DNA binding by XzGyrBA. Even though the effect is very small, CszB slightly decreases the ability of XzGyrBA to bind DNA, which is consistent with data collected for other PRPs (Tran et al., 2005) (Mazurek et al., 2021).

Together, all these results suggest that XzGyrBA and CszB don't form a stable complex. The reason for this most likely lies in the construct design of XzGyrBA, which included deletion of the N-terminal ATPase domain of GyrB to enhance crystallisation properties (Blower et al., 2016). The hypothesis that the ATPase domain might be essential for complex formation is supported by the co-crystal structure of the related PRP MfpA in complex with the ATPase domain of GyrB (Feng et al., 2021).

Due to CszB's structural and phylogenetic closeness to Qnr1B (Zuo, 2024), it is likely that they exhibit a comparable mode of action (Liang et al., 2025). The current model for QnrB1's interaction with gyrase is a stabilizing interaction with the N-terminal ATPase domain of GyrB (Mazurek et al., 2021) and a more transient interaction with the tower domain in GyrA, modulated by the loops protruding from QnrB1 (Xiong et al., 2011) (C. Chen et al., 2021), which are also present in CszB.

Several AlphaFold predictions were created to estimate the interaction of CszB with our fusion construct XzGyrBA and the native complex: The predictions of XzGyrBA with CszB show that the PRP is modelled close to the DNA gate of the truncated fusion construct (**Figure 24A**). This arrangement resembles the G-segment mimic model that was proposed in 2005 by Hedge et al. when the first PRP's structure was solved and modelled with a GyrA construct (Hegde et al., 2005). However, our observations during purification and biochemical characterisation did not provide evidence for a stable complex formation that would support the G-segment mimic model. Instead, we observed potential non-specific association during purification of His₆-MBP-tagged XzGyrBA and a slight decrease in DNA binding caused by CszB in the EMSA that could be explained through non-specific interaction of CszB with the DNA-gate of XzGyrBA or a transient, loop-mediated association with the tower domain of GyrA, as proposed for QnrB1 (Xiong et al., 2011) (C. Chen et al., 2021). Consequently, the data speaks against the G-segment mimic model (Hegde et al., 2005), in which case we would have expected a stable complex formation with our fusion model at the DNA-gate.

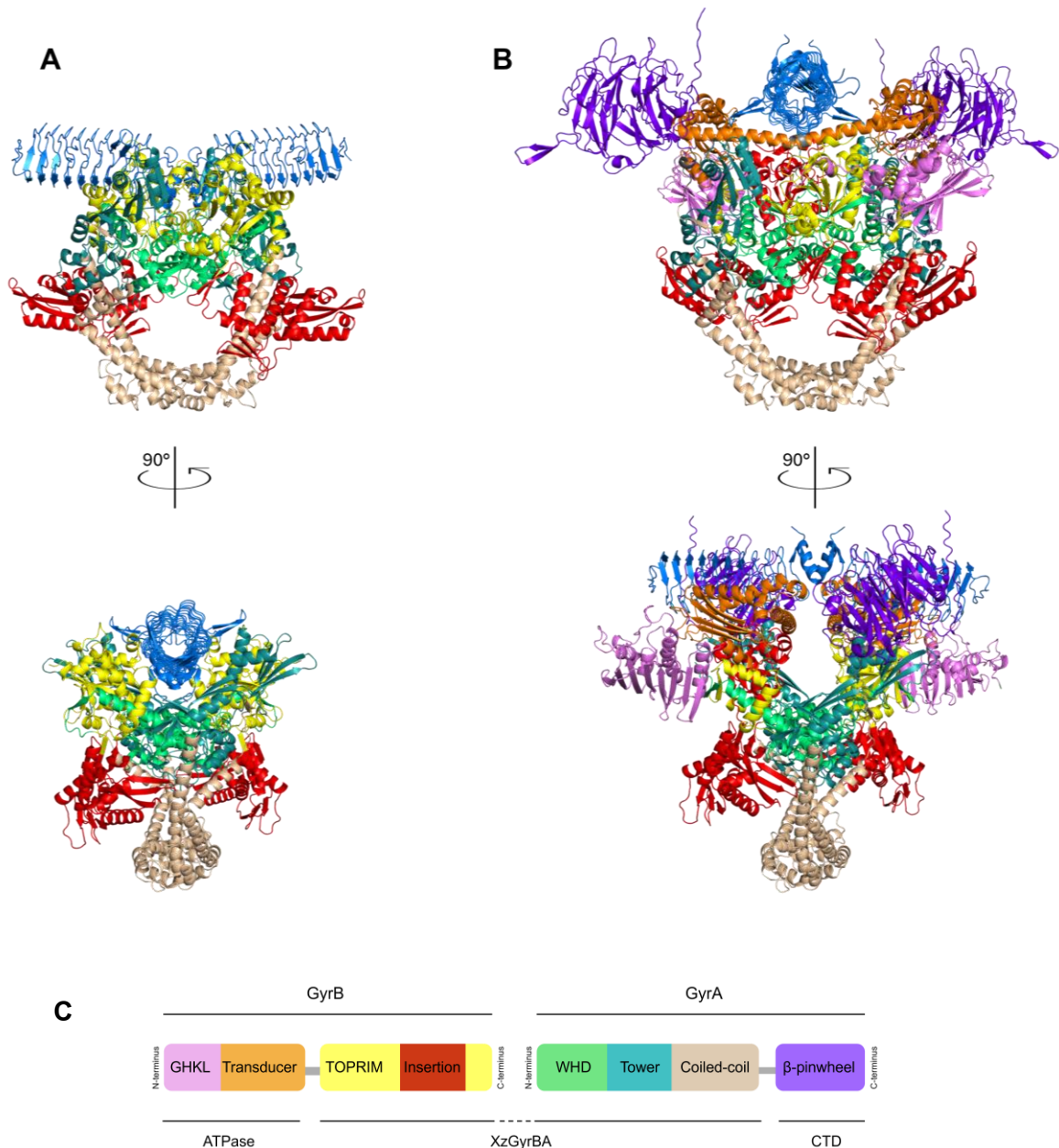


Figure 24: **A:** AlphaFold model (Abramson et al., 2024) of the dimerized fusion protein XzGyrBA complexed with a CszB dimer. **B:** AlphaFold model (Abramson et al., 2024) of the native *X. szentirmaii* gyrase heterocomplex bound to a CszB dimer. **C:** Domain structure of *X. szentirmaii* gyrase indicating GyrA, GyrB, and XzGyrBA.

In the AlphaFold prediction of the native gyrase (**Figure 24B**), the PRP dimer is modelled much further outside the complex along the N-terminal ATPase domain of GyrB and rotated by approximately 90°. This prediction corresponds strongly to the T-segment mimic model, which was proposed more recently and proposes a main interaction of the PRP with the ATPase domain of GyrB (Feng et al., 2021) (Vetting et al., 2011). The lack of complex formation of CszB with the truncated fusion construct XzGyrBA is consistent with the T-segment mimic

model, suggesting that the GyrB ATPase domain is essential for complex formation (Mazurek et al., 2021) (Feng et al., 2021).

4.1.4 Outlook for CszB-Gyrase Studies

To obtain a co-crystal structure of CszB with *X. szentirmaii* gyrase, a co-purification strategy will be developed with the N-terminal ATPase domain of *X. szentirmaii* after Feng et al. (Feng et al., 2021). Simultaneously, cryo-EM strategies with the *X. szentirmaii* gyrase complex will be explored according to Broeck et al (Broeck et al., 2019). With this approach, it might be possible to obtain a complete gyrase structure, bound to CszB and DNA, to get valuable insights into their interaction, including a potential loop-mediated interaction with the GyrA tower domain (C. Chen et al., 2021).

Bioactivity assays have already shown that while CszB protects most efficiently against Csz, it also protects against the FQs ciprofloxacin and, to a small extent, nalidixic acid, while it has no influence on aminocoumarins (Zuo, 2024). Since the target of Csz is *M. tuberculosis*, it would be interesting to see if the plasmid-mediated resistance proteins MfpA and MfpB are able to rescue *M. tuberculosis* gyrases from Csz like they do from FQs. Regarding the non-overlapping mutations that were observed in gyrases to confer resistance to Csz or to FQs (Liang et al., 2025) it would be interesting to know if plasmid-mediated quinolone resistance through PRPs affects Csz.

4.2 Interaction of Thiomarinol A with IleRS Variants

4.2.2 Thiomarinol A binds significantly weaker to PmIleRS2 than IleRS1 Variants

Although thiomarinol A and mupirocin share remarkable structural similarities and both target IleRS, their mechanism of action most likely doesn't completely overlap. Previous *in vitro* studies with thiomarinol A only focused on the mupirocin-susceptible variant SalleRS1 (R. A. Johnson et al., 2021), although the inhibitor remains potent against the high-level mupirocin-resistant IleRS2 variant MupA (Fukuda et al., 2011).

In this research, the influence of thiomarinol A was tested on the mupirocin-susceptible variants, SalleRS1 and PmIleRS1, and the mupirocin-resistant variant, PmIleRS2. Our results from the TSA reflect how much thiomarinol A's binding interaction differs between IleRS1 and IleRS2. While the melting temperatures of SalleRS1 and PmIleRS1 increased by 33.5 and 35 °C, the thermal shift for PmIleRS2 was only +7 °C upon ligand binding.

4 Discussion

Our results are comparable to previous thermal shift analysis via CD melts for SalleRS1, showing an increase in melting temperature by $30,9 \pm 0,9$ °C (R. A. Johnson et al., 2021). However, these results are the first biophysical quantification of thiomarinol A's interaction with an IleRS2 variant compared to IleRS1 variants.

4.2.1 Preliminary 3.5 Å Cryo-EM Structure of PmIleRS1 with Thiomarinol A

The main goal of this project was to produce a structure of IleRS bound to thiomarinol A, elucidating its mode of inhibition and how it differs from mupirocin (Nakama et al., 2001). During this research, different protein crystals of SalleRS1 and PmIleRS1 were tested but showed little to no diffraction. An attempt to visualize the co-complexed PmIleRS1 via cryo-EM, however, resulted in a promising preliminary structure at 3.5 Å. Overlaying the model with a mupirocin-bound crystal structure of PmIleRS1 (Brkic et al., 2023) showed that thiomarinol A doesn't cause major structural changes in the protein backbone. Minor divergence in the curvature of the protein might be a result of crystal packing distortion. This observation is consistent with circular dichroism (CD) studies of SalleRS1 bound to thiomarinol A, showing no structural difference to the apo-protein (R. A. Johnson et al., 2021). Studies comparing mupirocin-bound and apo-IleRS also show that even though residues in the active site are remodelled upon binding, the backbone of the protein shows no major structural changes (X. Chen et al., 2024). The current data is consistent with previous studies, claiming that complex formation with thiomarinol A doesn't cause major structural changes (R. A. Johnson et al., 2021) (X. Chen et al., 2024).

4.2.3 Outlook for Thiomarinol A-IleRS Studies

Further studies will be necessary to improve the resolution of the PmIleRS1 cryo-EM structure and be able to estimate the mode of interaction between thiomarinol A and the protein's active site. In the next step, a high-resolution dataset will be collected to push the resolution low enough to visualize the inhibitor and structural details in the active site. The results will be compared to mupirocin (X. Chen et al., 2024) and the influence of the zinc-chelating DTP-moiety will be further investigated.

The results of the TSA have underlined how much the interaction of thiomarinol differs between IleRS1 and IleRS2 variants. The structural basis of mupirocin resistance in IleRS2 results from a steric clash in the active site preventing hydrogen-bond formation and stabilisation of mupirocin with the active site (Chung et al., 2020) (Brkic et al., 2023). To investigate how thiomarinol A overcomes this high-level mupirocin resistance, we will attempt to obtain a thiomarinol A-bound structure of PmIleRS2 and compare it to the thiomarinol A-bound structure of PmIleRS1. Furthermore, corresponding structures of *S. aureus* SalleRS1 and the

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IleRS2 variants MupA and MupB will be pursued to investigate the binding interaction in a clinically relevant target. The activity of thiomarinol A against MupA has only been studied *in vivo*, the MupB IleRS2 variant has not been evaluated yet (R. A. Johnson et al., 2021).

Some IleRS2 variants have developed an additional mupirocin resistance mechanism, termed “hyper-resistance”, which comes from a non-canonical HXGH motif (Brkic et al., 2023). In canonical HXGH motifs, the first histidine is essential to stabilise the transition state of the amino acid activation step, and mutation of the residue usually severely compromises its catalytic ability (Borgford et al., 1987). In some IleRS2 variants, however, the first and third residues are swapped (GXHH), resulting in a severe steric clash of mupirocin with the histidine in position 3, increasing the K_i value in mutants around 430-fold. This adaptation is exclusive for IleRS2 variants since IleRS1 variants lose activity after said mutation (Brkic et al., 2023). In the longer run of this project, it might be interesting to recreate a hyper-resistant IleRS2 variant according to Brkic et al. (Brkic et al., 2023), harbouring the non-canonical GXHH motif to investigate if thiomarinol A also remains active against this resistance mechanism.

Thiomarinol A' high potency against mupirocin-resistant MRSA (R. A. Johnson et al., 2021) showcases the potential of hybrid antibiotics in future drug development. However, it is unclear if the NP is a suitable drug candidate. Thiomarinol A is able to chelate zinc ions in *S. aureus* (R. A. Johnson et al., 2021) like other DTPs that are toxic to human cell lines (Chan et al., 2017). *In vivo* studies have shown that the cytotoxic concentration of thiomarinol A to human cell lines is only 7-fold the MIC necessary against MupA (R. A. Johnson et al., 2021). To estimate how much the potential antibiotic candidate targets the eukaryotic IleRS, it might be interesting to assess the thermal stabilisation of eukaryotic IleRS through thiomarinol A binding compared to bacterial IleRS variants.

5 Abbreviations

A280	UV absorbance at $\lambda = 280$ nm
aaRS	Aminoacyl-tRNA synthetases
Amp	Ampicillin
AMR	Antimicrobial resistance
APS	Ammonium persulfate
BGC	Biosynthetic gene clusters
bp	Base pairs
BRD	Breakage-reunion domain
CD	Circular dichroism
Cryo-EM	Cryogenic electron microscopy
Csz	Chloroszentirazine
CszA	Flavin-dependent tryptophan halogenase chloroszentirazine A
CszB	Resistance protein chloroszentirazine B
CszC	Non-ribosomal peptide synthetase chloroszentirazine C
CszD	Carbamoyltransferase chloroszentirazine D
CszE	Hypothetical protein chloroszentirazine E
CszF	Multidrug resistance protein chloroszentirazine F
CszG	Racemase chloroszentirazine G
CTD	C-terminal domain
CV	Column volumes
ddH₂O	Double distilled water
DESY	Deutsches Elektronen-Synchrotron
DMSO	Dimethylsulfoxide-d ₆
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	Deoxynucleotide triphosphate
DTP	Dithiolopyrrolone
DTT	Dithiothreitol
E. coli	Escherichia coli
EMSA	Electrophoretic mobility shift assay
ESI-MS	Electrospray ionization mass spectrometry
FS	Fine screen
for	Forward
FQ	Fluoroquinolones
G-segment	Gate segment
GyrA	DNA gyrase subunit A
GyrB	DNA gyrase subunit B

5 Abbreviations

His6	Hexahistidine
^{HL}MR	High-level mupirocin resistance
Ile-AMP	Isoleucyl-adenylate
IleRS	Isoleucyl-tRNA synthetase
IMAC	Immobilized-metal affinity chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
<i>K. pneumoniae</i>	<i>Klebsiella pneumoniae</i>
KLD	Kinase-Ligase-DpnI
LB	Lysogeny broth
^{LL}MR	Low-level mupirocin resistance
LUCA	Last universal common ancestor
<i>M. smegmatis</i>	<i>Mycobacterium smegmatis</i>
<i>M. tuberculosis</i>	<i>Mycobacterium tuberculosis</i>
MALDI-MS	Matrix-assisted laser desorption ionization mass-spectroscopy
mAU	Milli absorbance unit
MCS	Multiple cloning site
MIC	Minimum inhibitory concentration
MPD	2-methyl-2,4-pentenediol
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
Ni-NTA	Nickel nitrilotriacetic acid
NP	Natural products
NRPS	Non-ribosomal peptide synthetase
OD	Optical density
<i>P. megaterium</i>	<i>Priestia megaterium</i>
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PmIleRS1	<i>Priestia megaterium</i> Isoleucyl-tRNA synthetase 1
PmIleRS2	<i>Priestia megaterium</i> Isoleucyl-tRNA synthetase 2
PmIleRS2ct	<i>Priestia megaterium</i> Isoleucyl-tRNA synthetase 2 c-terminally truncated
POI	Protein of interest
PRP	Pentapeptide repeat protein
r.m.s.d.	Root-mean-square deviation
rev	Reverse
RFU	Relative fluorescence units
rpm	Revolutions per minute
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
SalleRS1	<i>Staphylococcus aureus</i> Isoleucyl-tRNA synthetase 1
SDS	Sodium dodecyl sulfate

5 Abbreviations

SEC	Size exclusion chromatography
SOC	Super optimal broth
SUMO	Small ubiquitin-like modifier
TA	Thiomarinol A
TAE	Tris acetate EDTA
TB	Tuberculosis
TBE	Tris borate EDTA
TEMED	Tetramethylethylenediamine
TOPRIM	Topoisomerase-primase
Tris	Tris(hydroxymethyl)aminomethane
t-RNA	Transfer RNA
t-RNA^{Ile}	Transfer RNA for isoleucine
TSA	Thermal shift assay
T-segment	Transported-segment
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
WHD	Winged-helix domain
<i>X. szentirmaii</i>	<i>Xenorhabdus szentirmaii</i>
XDS	X-ray Detector Software
XzGyrBA	<i>Xenorhabdus szentirmaii</i> DNA Gyrase fusion-protein

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7 Supplementary Data

7.1 Vector Maps

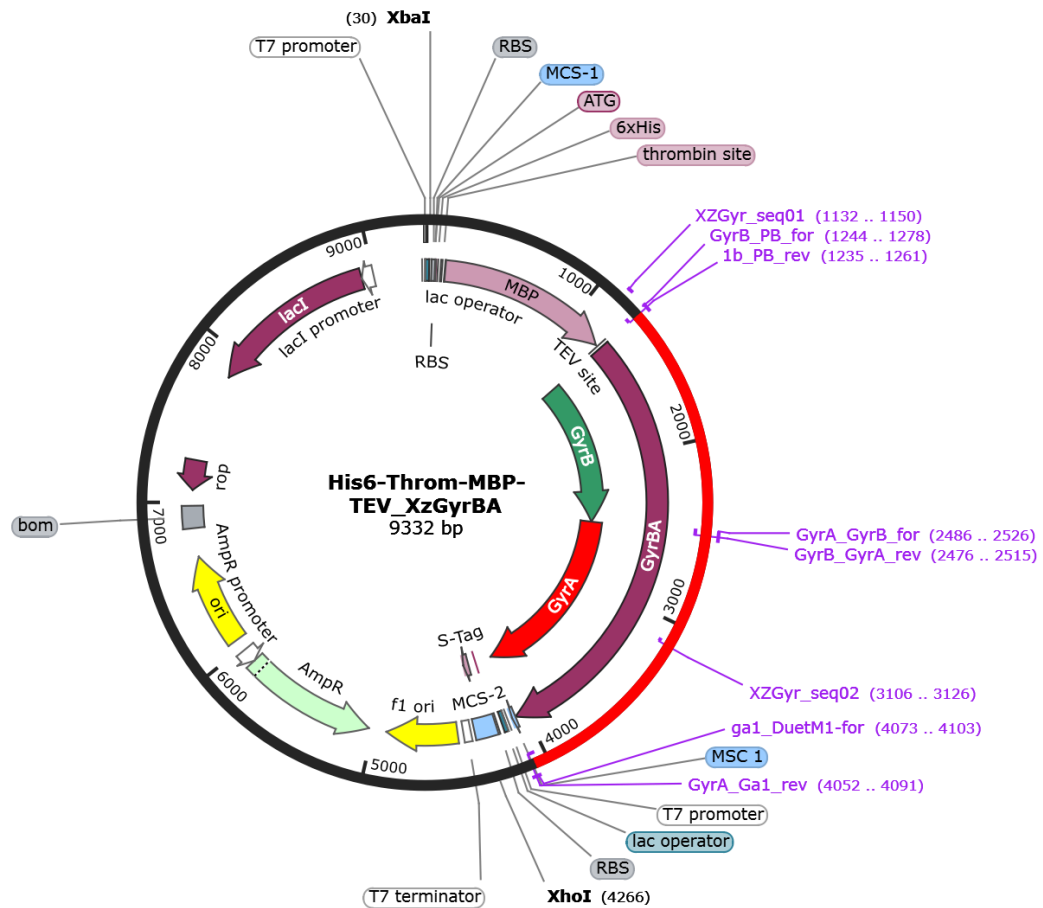


Figure S1: Vector map of His₆-Throm-MBP-TEV_XzGyrBA. Exported from SnapGene.

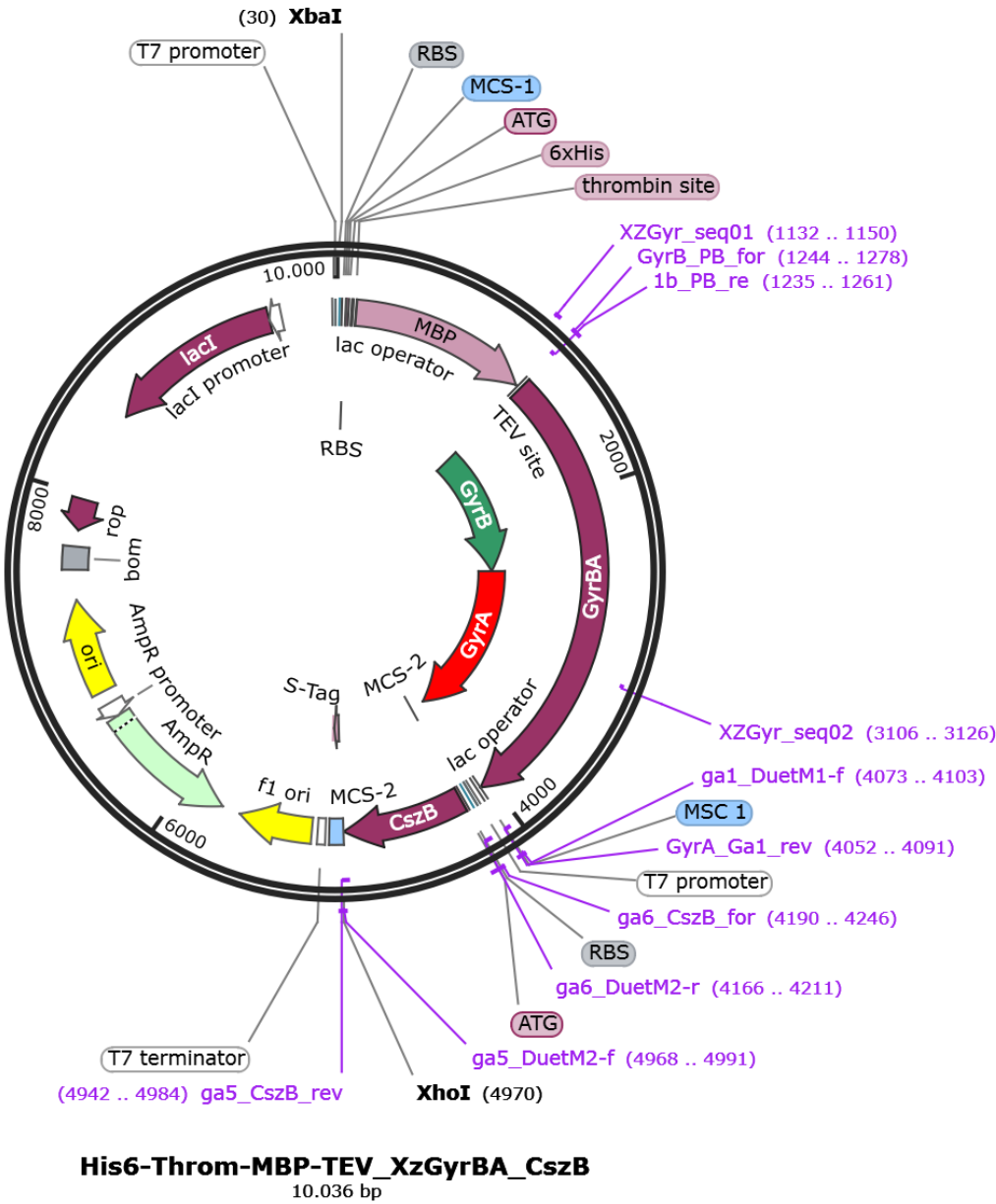


Figure S2: Vector map of His₆-Throm-MBP-TEV_XzGyrBA_CszB. Exported from SnapGene.

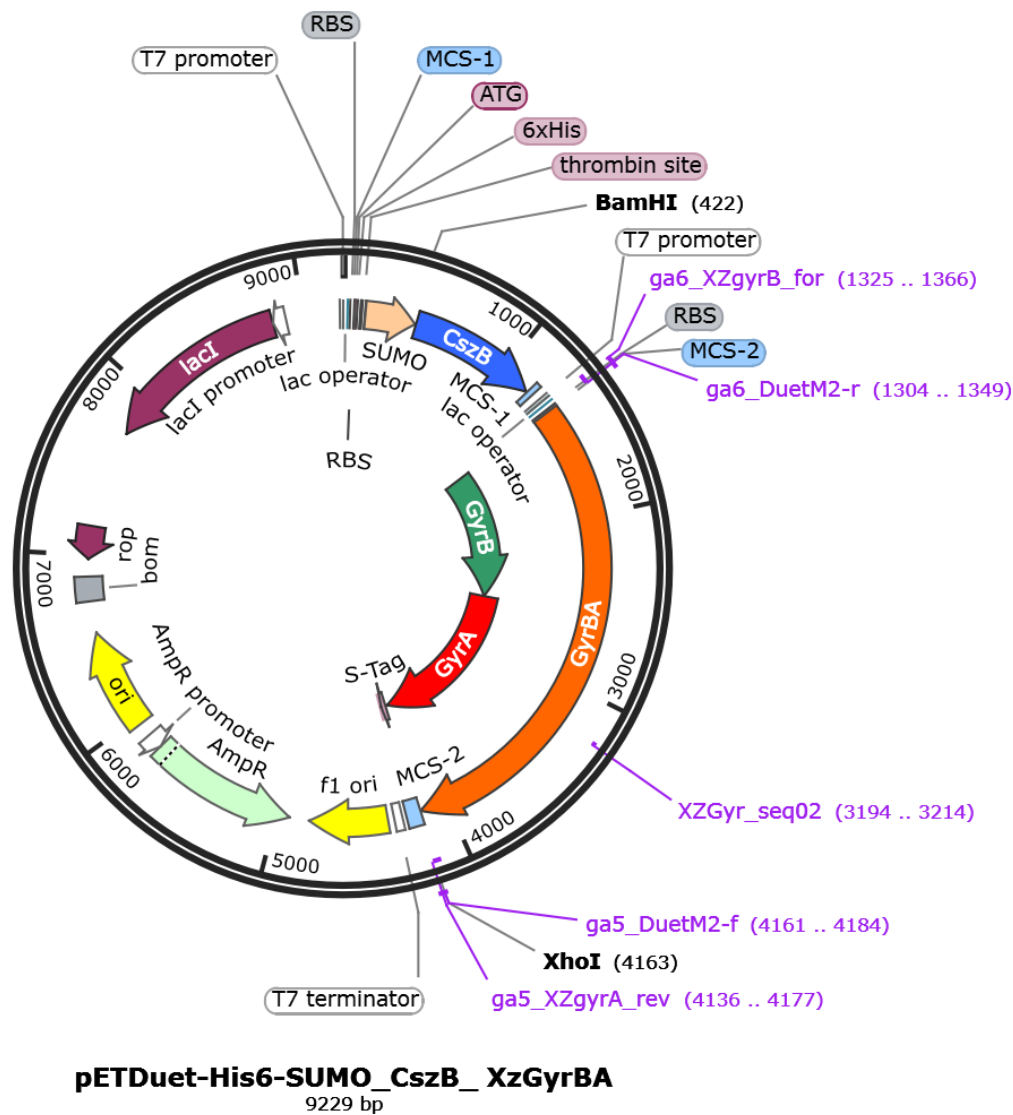


Figure S3: Vector map of pETDuet-His6-SUMO_CszB_XzGyrBA. Exported from SnapGene.

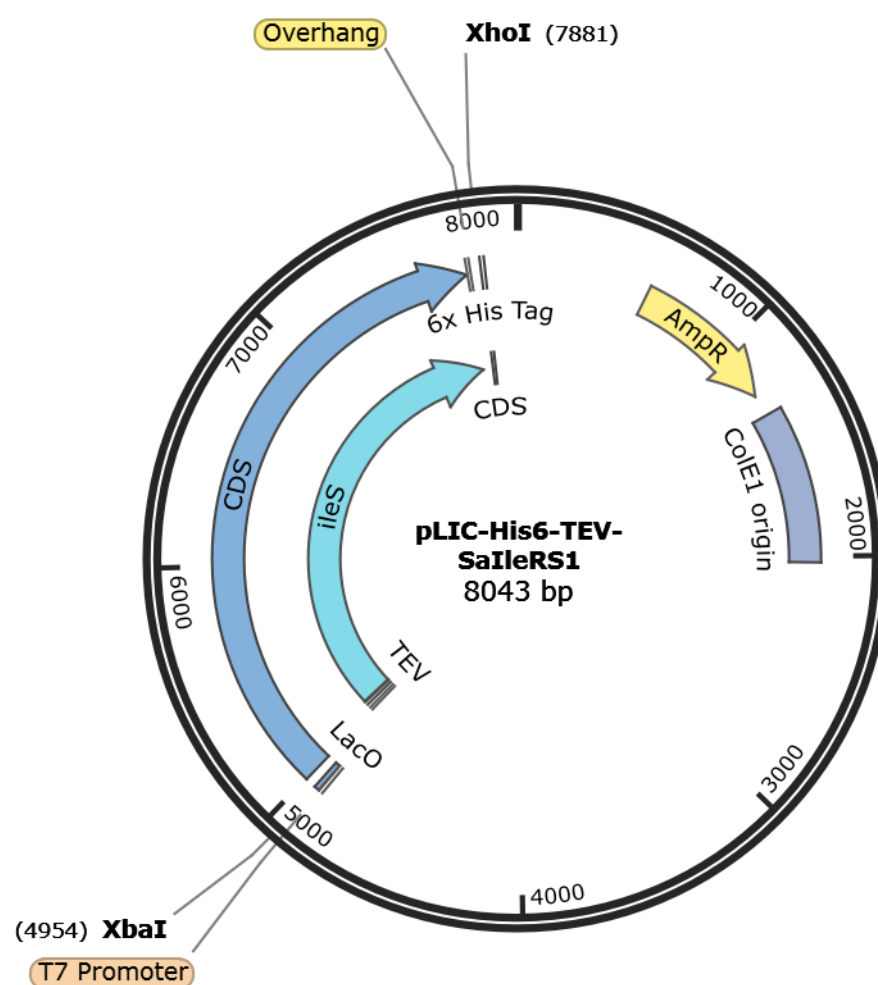


Figure S4: Vector map of pLIC-His₆-TEV-SaIleRS1. Exported from SnapGene.

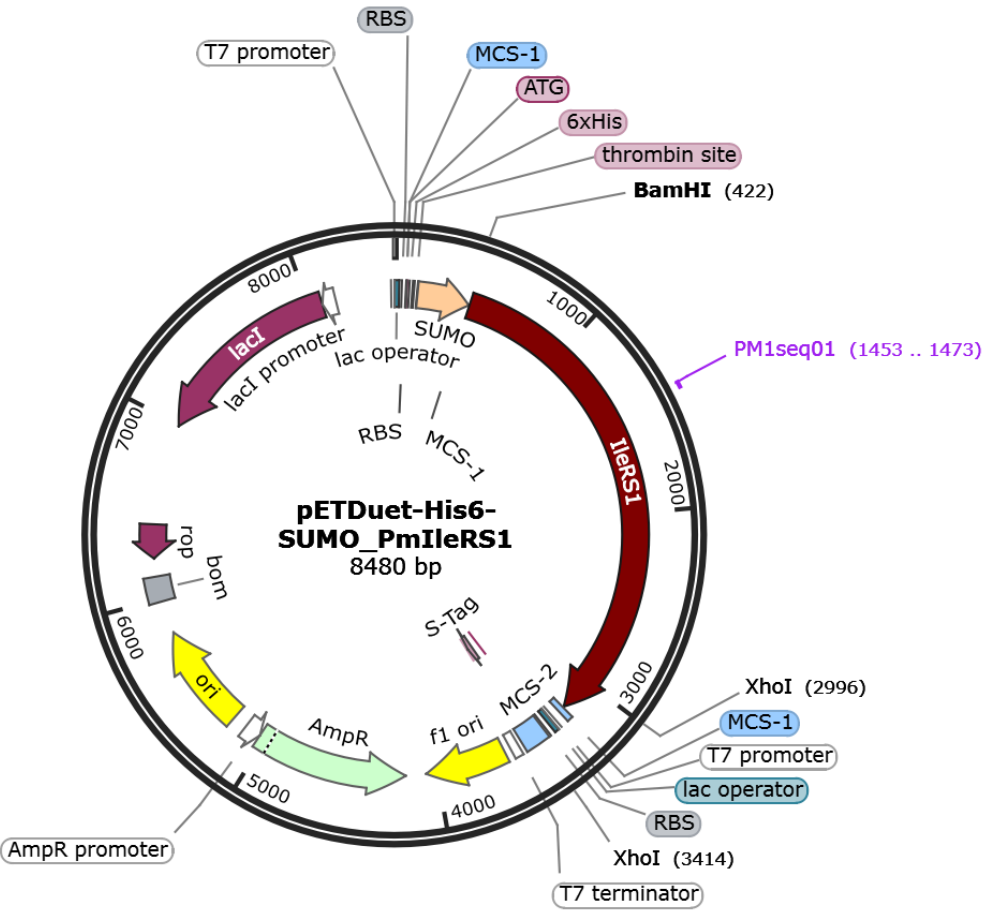


Figure S5: Vector map of pETDuet-His6-SUMO_PmIleRS1. Exported from SnapGene.

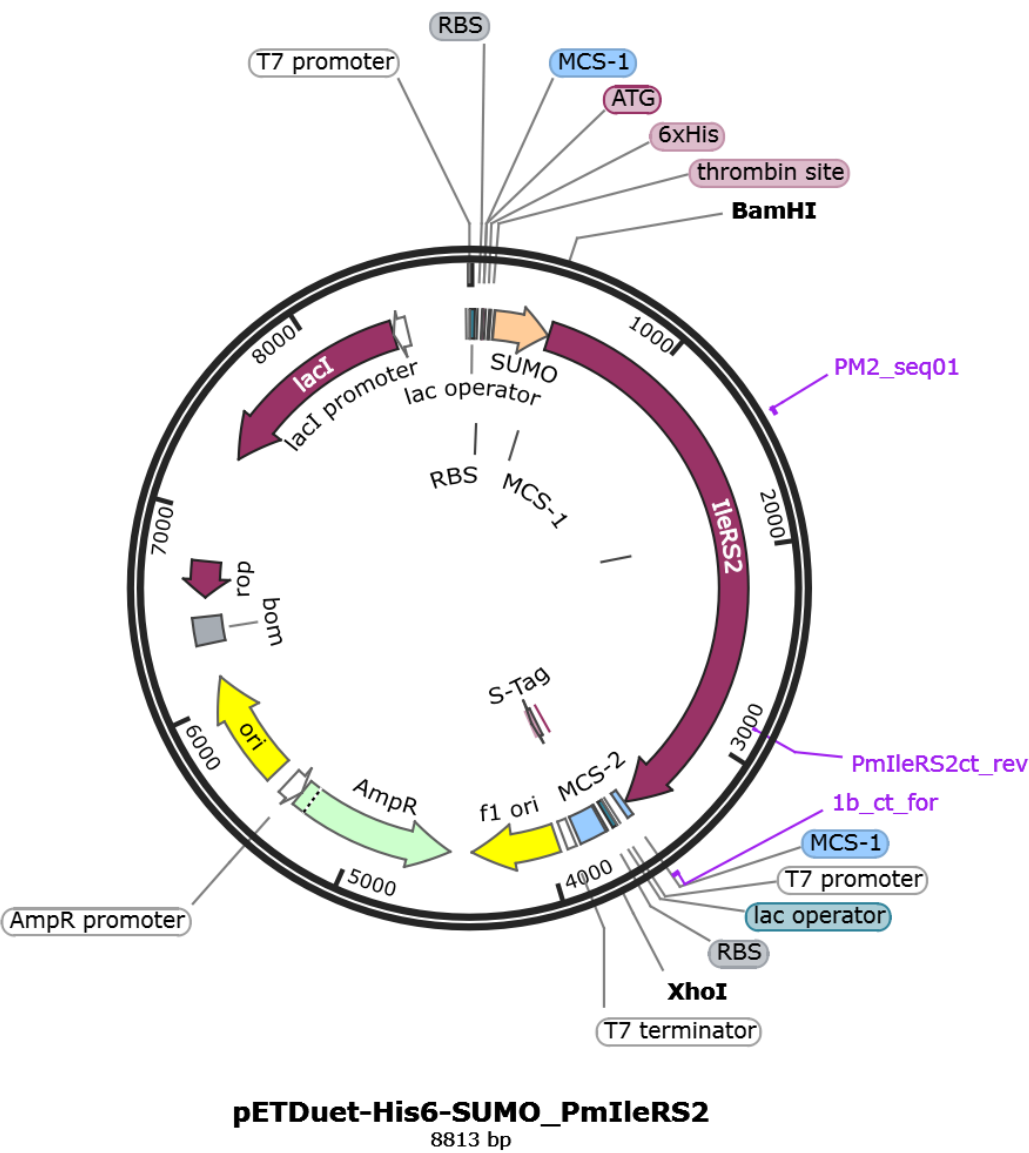


Figure S6: Vector map of pETDuet-His₆-SUMO_PmIleRS2. Exported from SnapGene.

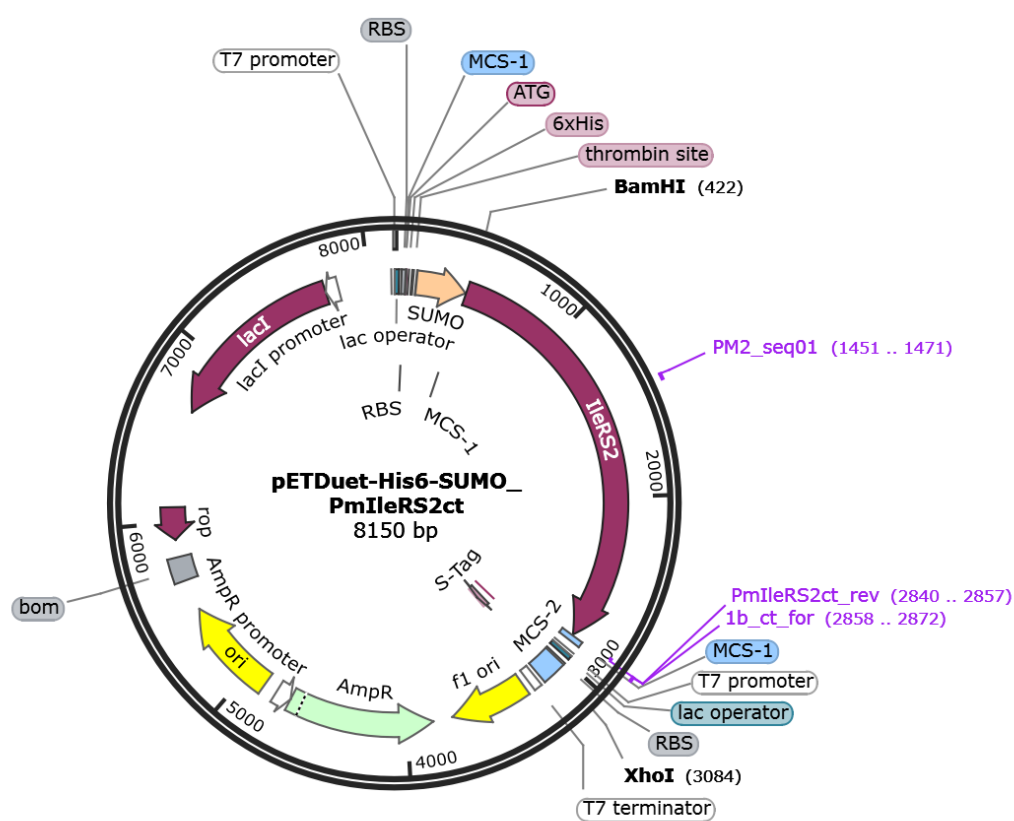


Figure S7: Vector map of pETDuet-His₆-SUMO_PmIleRS2ct. Exported from SnapGene.

7.2 Protein Analysis

7.2.1 Analytical SEC

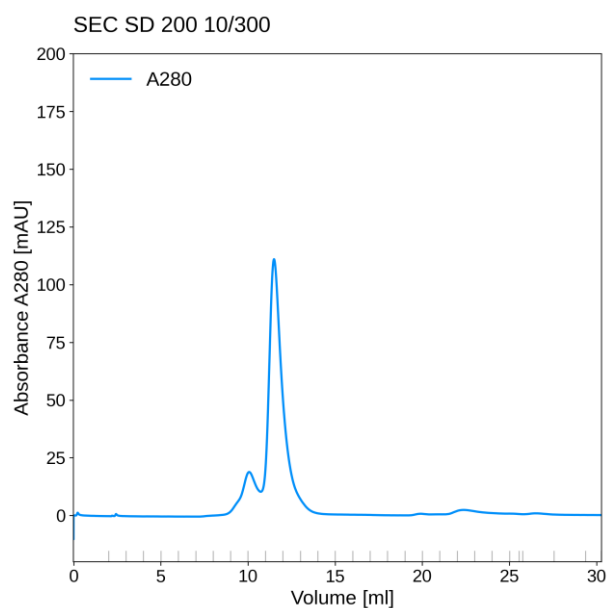


Figure S8: Analytical SEC chromatogram of XzGyrBA. Peak at 11.5 mL.

7.2.2 ESI-MS Spectra

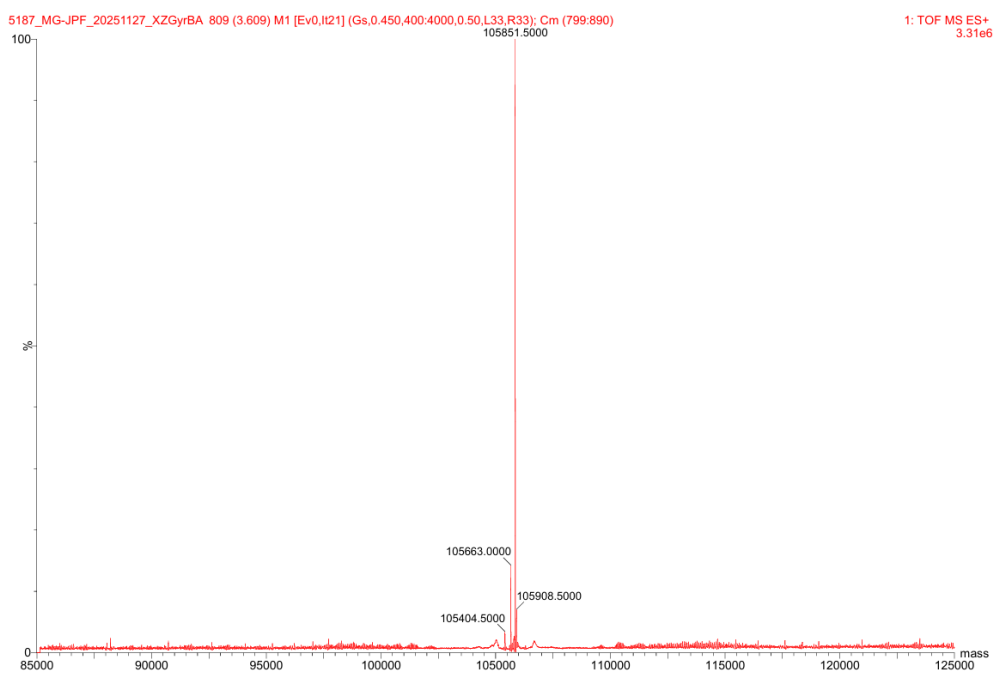


Figure S9: ESI-MS spectrum of XzGyrBA directly after purification. MW = 105851.90 g/mol.

7 Supplementary Data

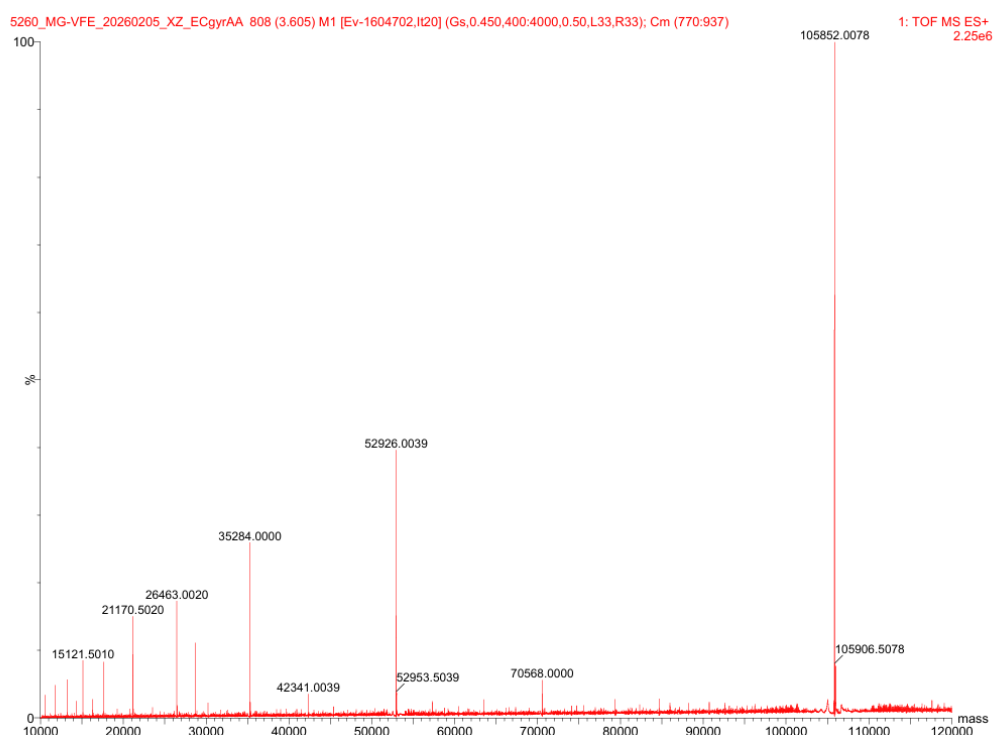


Figure S10: ESI-MS spectrum of freshly thawed XzGyrBA. MW = 105851.90 g/mol.

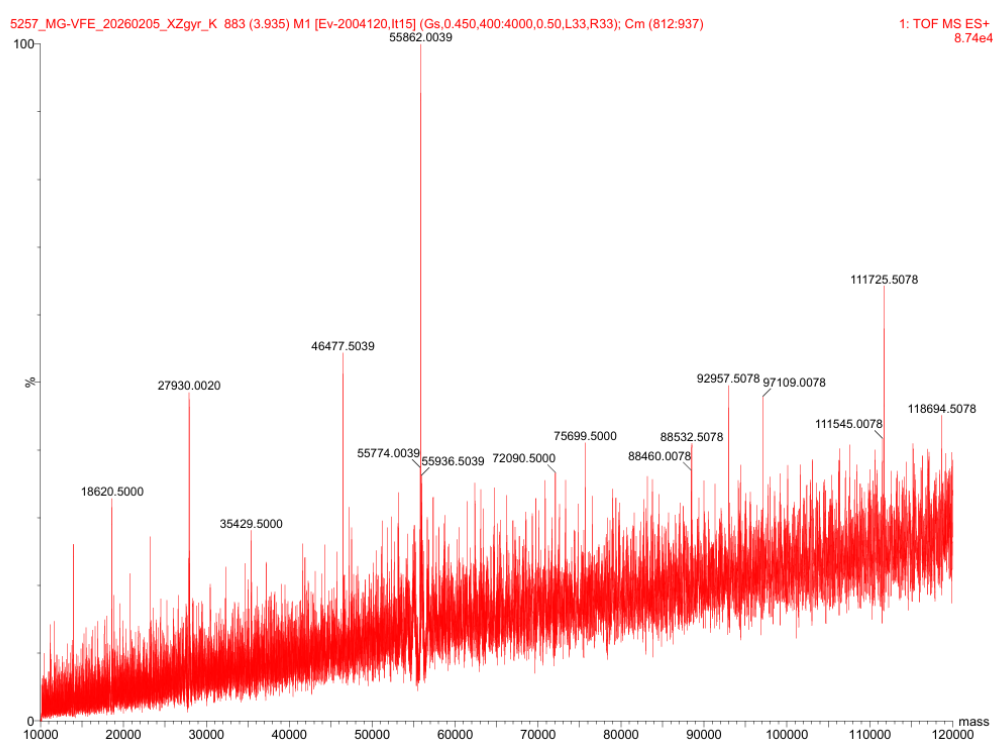


Figure S11: ESI-MS spectrum of degraded XzGyrBA from dissolved protein crystal.
 MW of XzGyrBA = 105851.90 g/mol, MW of XzGyrBA₄₄₁₋₉₃₇ = 55862.10 g/mol

7 Supplementary Data

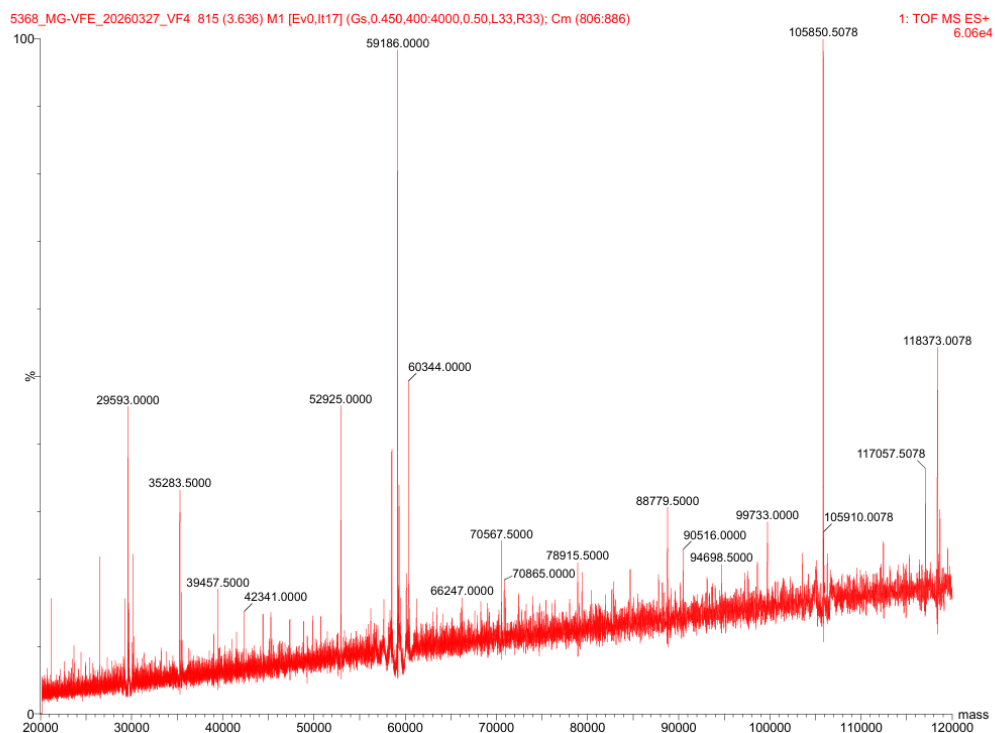
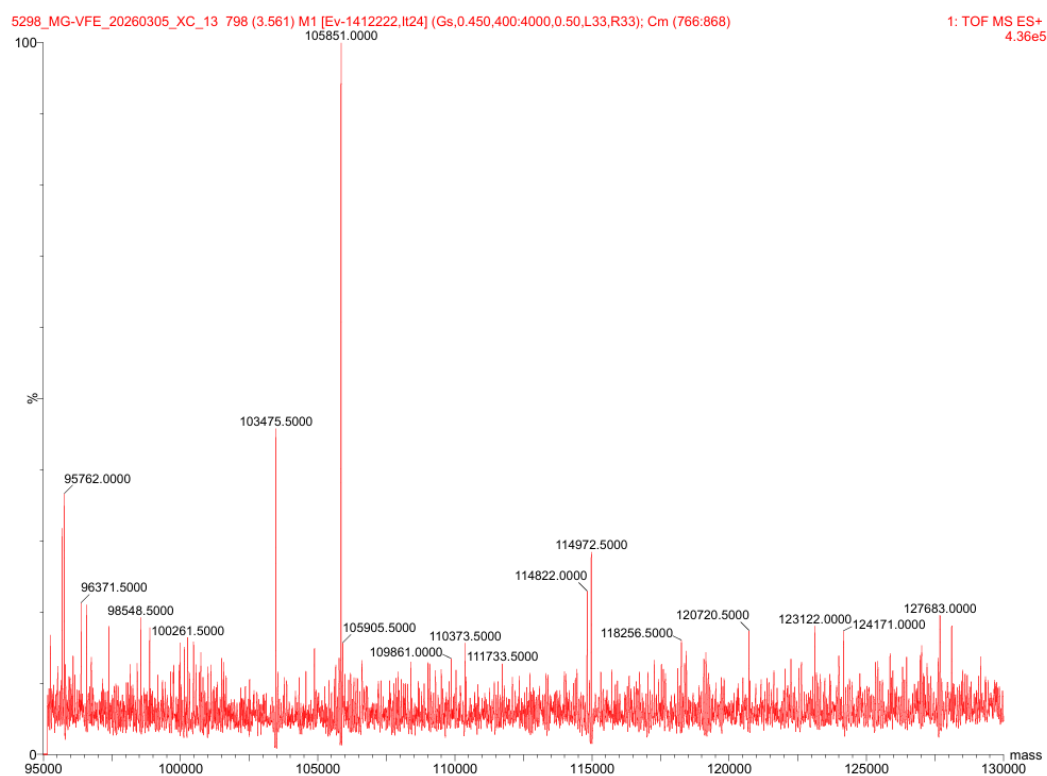


Figure S12: ESI-MS spectrum of partially degraded XzGyrBA after 14 days of incubation at 4 °C.
MW = 105851.90 g/mol.

7 Supplementary Data

A



B

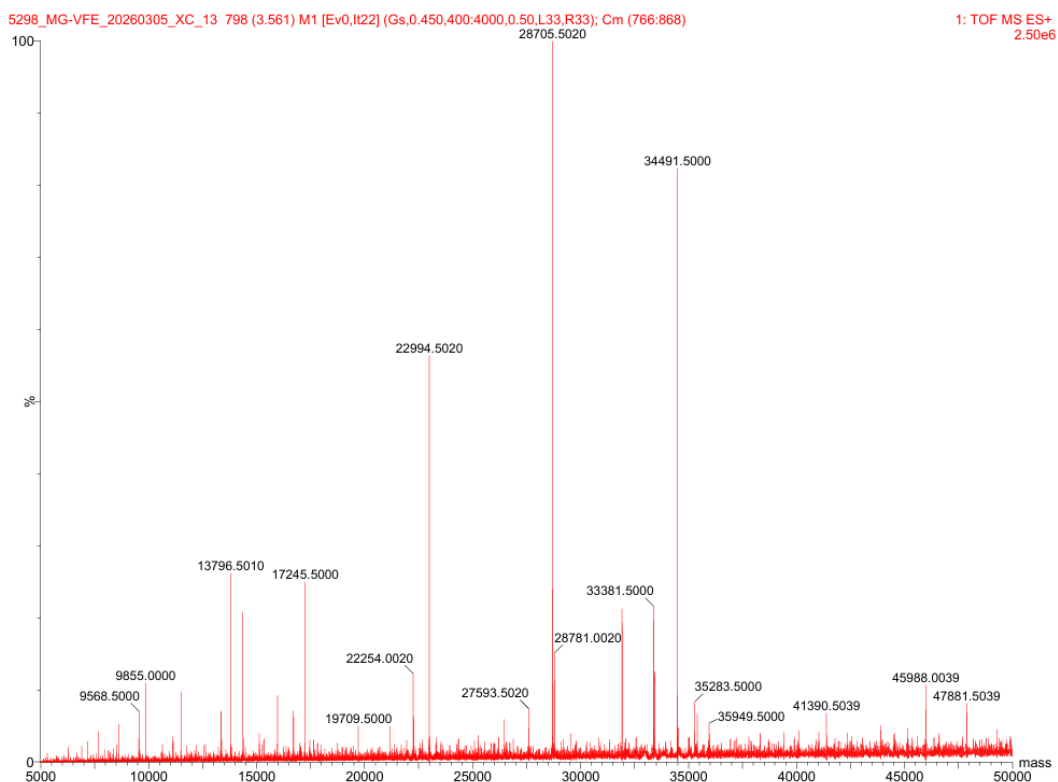


Figure S13: ESI-MS spectra of the same sample over different ranges containing XzGyrBA and CszB. MW XzGyrBA = 105851.90 g/mol, MW CszB = 28706.38 g/mol.

7 Supplementary Data

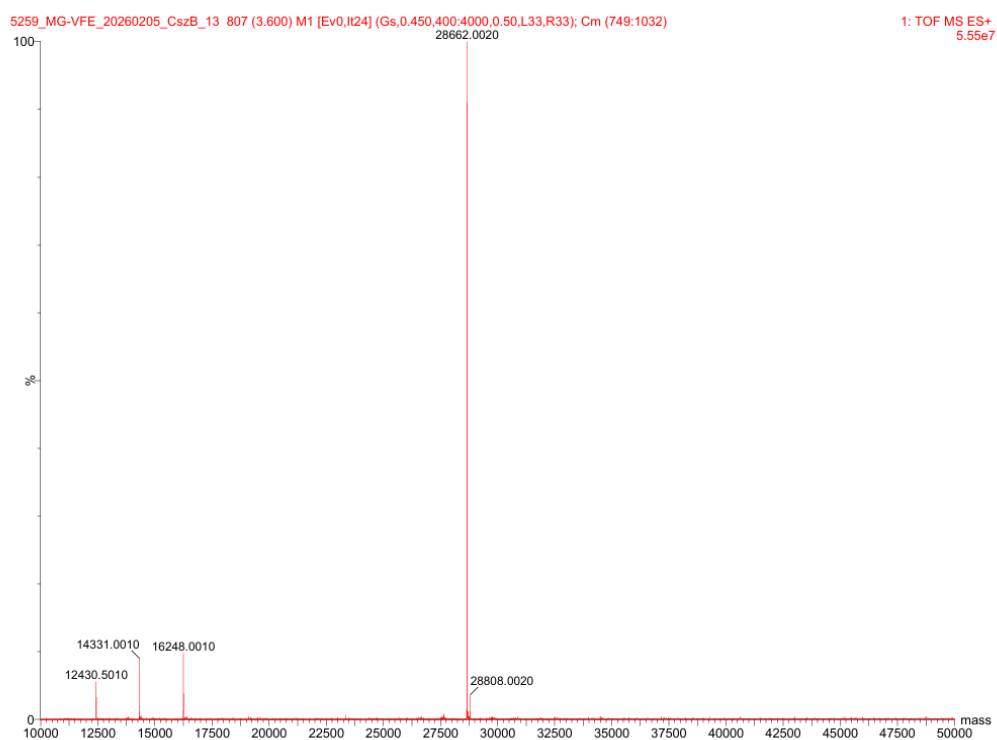


Figure S14: ESI-MS spectrum of CszB. MW = 28662.27 g/mol.



Figure S15: ESI-MS spectrum of SallerS1. MW = 105157.80 g/mol.

7 Supplementary Data

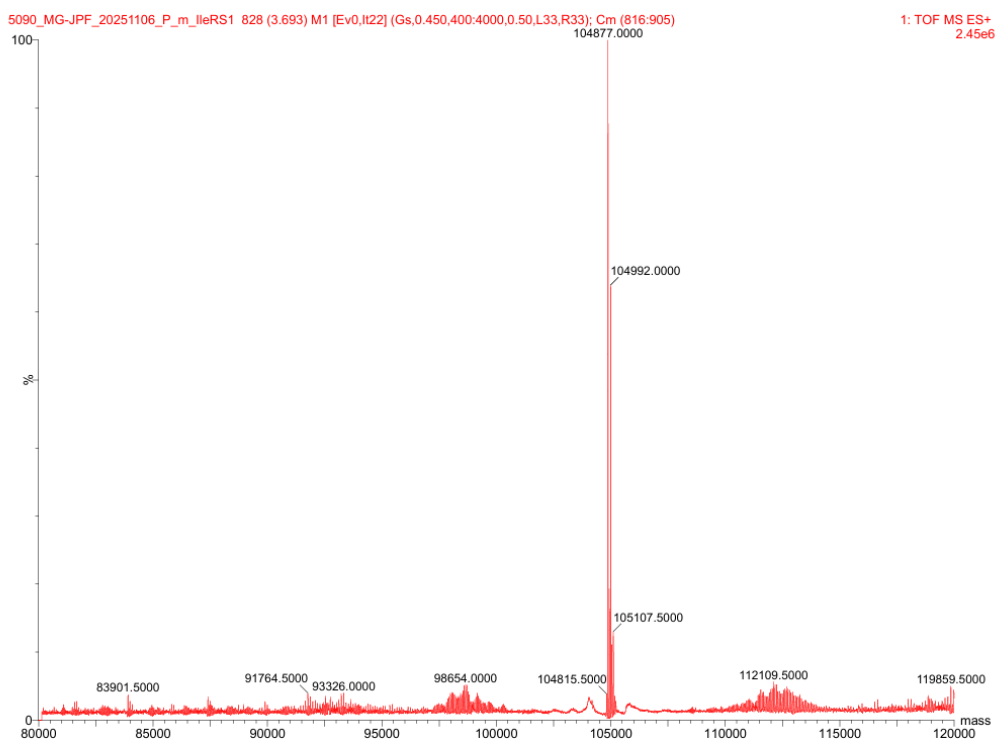


Figure S16: ESI-MS spectrum of PmIleRS1. MW = 104875.58 g/mol.

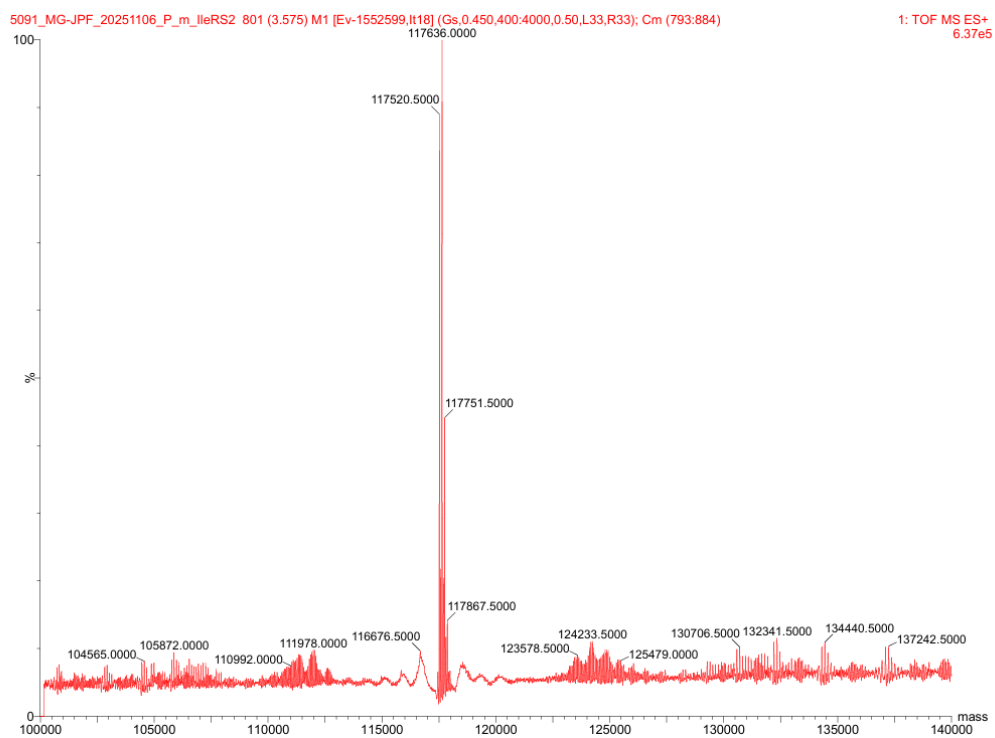


Figure S17: ESI-MS spectrum of PmIleRS2. MW = 117520.54 g/mol.

7 Supplementary Data

7.2.3 Thermal Shift Analysis

	1	2	3	4	5	6
A	47 MES pH6.0 100mM NaCl	46,5 MES pH6.0 100mM NaCl	46 MES pH6.0 100mM NaCl	9,5 MES pH6.0 250mM NaCl	48,5 MES pH6.0 250mM NaCl	48,5 MES pH6.0 250mM NaCl
B	46 HEPES pH7.0 100mM NaCl	46 HEPES pH7.0 100mM NaCl	46 HEPES pH7.0 100mM NaCl	49 HEPES pH7.0 250mM NaCl	48,5 HEPES pH7.0 250mM NaCl	49 HEPES pH7.0 250mM NaCl
C	46 TRIS pH8.0 100mM NaCl	46,5 TRIS pH8.0 100mM NaCl	46,5 TRIS pH8.0 100mM NaCl	48 TRIS pH8.0 250mM NaCl	48 TRIS pH8.0 250mM NaCl	48 TRIS pH8.0 250mM NaCl
D	45 BICINE pH9.0 100mM NaCl	44,5 BICINE pH9.0 100mM NaCl	45 BICINE pH9.0 100mM NaCl	47 BICINE pH9.0 250mM NaCl	47 BICINE pH9.0 250mM NaCl	47 BICINE pH9.0 250mM NaCl
E	43 MES pH6.0 100mM NaCl 10% Glycerin	50 MES pH6.0 100mM NaCl 10% Glycerin	50,5 MES pH6.0 100mM NaCl 10% Glycerin	52 MES pH6.0 250mM NaCl 10% Glycerin	52 MES pH6.0 250mM NaCl 10% Glycerin	52 MES pH6.0 250mM NaCl 10% Glycerin
F	49 HEPES pH7.0 100mM NaCl 10% Glycerin	49 HEPES pH7.0 100mM NaCl 10% Glycerin	49 HEPES pH7.0 100mM NaCl 10% Glycerin	51,5 HEPES pH7.0 250mM NaCl 10% Glycerin	51,5 HEPES pH7.0 250mM NaCl 10% Glycerin	51,5 HEPES pH7.0 250mM NaCl 10% Glycerin
G	49 TRIS pH8.0 100mM NaCl 10% Glycerin	49,5 TRIS pH8.0 100mM NaCl 10% Glycerin	49,5 TRIS pH8.0 100mM NaCl 10% Glycerin	51 TRIS pH8.0 250mM NaCl 10% Glycerin	51 TRIS pH8.0 250mM NaCl 10% Glycerin	51 TRIS pH8.0 250mM NaCl 10% Glycerin
H	46,5 BICINE pH9.0 100mM NaCl 10% Glycerin	None BICINE pH9.0 100mM NaCl 10% Glycerin	None BICINE pH9.0 100mM NaCl 10% Glycerin	49,5 BICINE pH9.0 250mM NaCl 10% Glycerin	66,5 BICINE pH9.0 250mM NaCl 10% Glycerin	51,5 BICINE pH9.0 250mM NaCl 10% Glycerin

	7	8	9	10	11	12
A	50,5 MES pH6.0 500mM NaCl	50 MES pH6.0 500mM NaCl	50 MES pH6.0 500mM NaCl	51 MES pH6.0 750mM NaCl	51,5 MES pH6.0 750mM NaCl	50,5 MES pH6.0 750mM NaCl
B	50,5 HEPES pH7.0 500mM NaCl	51 HEPES pH7.0 500mM NaCl	50,5 HEPES pH7.0 500mM NaCl	51,5 HEPES pH7.0 750mM NaCl	52 HEPES pH7.0 750mM NaCl	53 HEPES pH7.0 750mM NaCl
C	50,5 TRIS pH8.0 500mM NaCl	50 TRIS pH8.0 500mM NaCl	50 TRIS pH8.0 500mM NaCl	51 TRIS pH8.0 750mM NaCl	51 TRIS pH8.0 750mM NaCl	53,5 TRIS pH8.0 750mM NaCl
D	49 BICINE pH9.0 500mM NaCl	49 BICINE pH9.0 500mM NaCl	49 BICINE pH9.0 500mM NaCl	50,5 BICINE pH9.0 750mM NaCl	51 BICINE pH9.0 750mM NaCl	52 BICINE pH9.0 750mM NaCl
E	53 MES pH6.0 500mM NaCl 10% Glycerin	53 MES pH6.0 500mM NaCl 10% Glycerin	53 MES pH6.0 500mM NaCl 10% Glycerin	54,5 MES pH6.0 750mM NaCl 10% Glycerin	54 MES pH6.0 750mM NaCl 10% Glycerin	57,5 MES pH6.0 750mM NaCl 10% Glycerin
F	54 HEPES pH7.0 500mM NaCl 10% Glycerin	53,5 HEPES pH7.0 500mM NaCl 10% Glycerin	53,5 HEPES pH7.0 500mM NaCl 10% Glycerin	55 HEPES pH7.0 750mM NaCl 10% Glycerin	55 HEPES pH7.0 750mM NaCl 10% Glycerin	56 HEPES pH7.0 750mM NaCl 10% Glycerin
G	54,5 TRIS pH8.0 500mM NaCl 10% Glycerin	53 TRIS pH8.0 500mM NaCl 10% Glycerin	53 TRIS pH8.0 500mM NaCl 10% Glycerin	54,5 TRIS pH8.0 750mM NaCl 10% Glycerin	54,5 TRIS pH8.0 750mM NaCl 10% Glycerin	54,5 TRIS pH8.0 750mM NaCl 10% Glycerin
H	54,5 BICINE pH9.0 500mM NaCl 10% Glycerin	53,5 BICINE pH9.0 500mM NaCl 10% Glycerin	52 BICINE pH9.0 500mM NaCl 10% Glycerin	53 BICINE pH9.0 750mM NaCl 10% Glycerin	53 BICINE pH9.0 750mM NaCl 10% Glycerin	53 BICINE pH9.0 750mM NaCl 10% Glycerin

Figure S18: Heatmap of thermal shift assay testing the influence of different buffers on the thermal stability of CszB. Top: melting temperature. Bottom: Buffer composition.

7.3 X-ray Diffraction Data

<i>XzGyrBA</i>	
Crystal parameters	
Space group	P1
Cell constants	a= 92.0 Å b= 92.2 Å c= 95.8 Å α = 106.1 ° β =107.5 ° γ =113.5 °
Molecules / AU ^a	4
Data collection	
Beam line	P13, DESY
Wavelength (Å)	0.97620
Resolution range (Å) ^b	81.0-1.7 (1.8-1.7)
No. observations	937365
No. unique reflections ^c	262315
Completeness (%) ^b	96.9 (95.9)
R _{merge} (%) ^{b, d}	5.6 (76.0)
I/σ (I) ^b	12.0 (1.6)
Refinement (REFMAC5)	
Resolution range (Å)	30-1.7
No. refl. working set	249171
No. refl. test set	13114
No. non hydrogen	16000
Solvent (H ₂ O)	1097
R _{work} /R _{free} (%) ^e	17.4/19.8
r.m.s.d. bond (Å) / angle (°) ^f	0.002/1.208
Average B-factor (Å ²)	31.4
Ramachandran Plot (%) ^g	97.7/2.2/0.1

^[a] Asymmetric unit

^[b] The values in parentheses for resolution range, completeness, R_{merge} and I/σ (I) correspond to the highest resolution shell

^[c] Data reduction was carried out with XDS and from a single crystal.
Friedel pairs were treated as identical reflections

^[d] $R_{\text{merge}}(I) = \frac{\sum_{hkl} \sum_j |I(hkl)_j - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_j I(hkl)_j}$, where $I(hkl)_j$ is the j^{th} measurement of the intensity of reflection hkl and $\langle I(hkl) \rangle$ is the average intensity

^[e] $R = \frac{\sum_{hkl} ||F_{\text{obs}}| - |F_{\text{calc}}||}{\sum_{hkl} |F_{\text{obs}}|}$, where R_{free} is calculated without a sigma cut off for a randomly chosen 5% of reflections, which were not used for structure refinement, and R_{work} is calculated for the remaining reflections

^[f] Deviations from ideal bond lengths/angles

^[g] Percentage of residues in favored / allowed / outlier region