Attachment no. 3

Summary of professional accomplishments

Molecular mechanisms of regulation of bacterial toxin-antitoxin systems in *Salmonella*

Grzegorz Jan Grabe

Intercollegiate Faculty of Biotechnology
University of Gdansk and Medical University of Gdansk

Gdansk 2025

1. Name

Grzegorz Jan Grabe

2. Diplomas, degrees conferred in specific areas of science or arts, including the name of the institution which conferred the degree, year of degree conferment, title of the PhD dissertation

2016 **Doctor of Philosophy**
Thesis: *The Salmonella SPI-2 Type III secretion system: Regulation of a substrate specificity switch and functional analysis of the SpvD effector*
Supervisor: Regius Professor David Holden
Imperial College London
London, United Kingdom

2011 **Master of Research**
Imperial College London
London, United Kingdom

2010 **Master of Science**
Intercollegiate Faculty of Biotechnology
University of Gdansk and Medical University of Gdansk
Gdansk, Poland

2007 **Bachelor of Science**
Intercollegiate Faculty of Biotechnology
University of Gdansk and Medical University of Gdansk
Gdansk, Poland

3. Information on employment in research institutes or faculties/departments or school of arts

2023 - now **Project leader**
Leader of POLONEZ BIS, OPUS, and EMBO IG research grants
Structural Biology Group of Professor Michał Szymański
Intercollegiate Faculty of Biotechnology
University of Gdansk and Medical University of Gdansk
Gdansk, Poland

2019 - 2022 **Postdoc**
Research group of Professor Sophie Helaine
Department of Microbiology
Harvard Medical School
Boston, USA

2016 - 2019 **Postdoc**
Research group of Professor Sophie Helaine
MRC Centre for Molecular Bacteriology and Infection (CMBI)
Imperial College London
London, United Kingdom

2011 - 2015 **PhD student**
Research group of Regius Professor David Holden
Wellcome Trust Molecular and Cellular Basis of Infection
MRC Centre for Molecular Bacteriology and Infection (CMBI)
Imperial College London
London, United Kingdom

2010 - 2011 **MRes student**
Wellcome Trust Molecular and Cellular Basis of Infection
Imperial College London
London, United Kingdom

2008 - 2010 **MSc student**
Intercollegiate Faculty of Biotechnology
University of Gdansk and Medical University of Gdansk
Gdansk, Poland
Research group of Professor Shohei Koide
University of Chicago
Chicago, USA

2007 - 2008 **Research assistant**
Research group of Doctor Jan van der Wolf
Plant Research International
Wageningen University and Research Centre
Wageningen, The Netherlands

4. Description of the achievements, set out in art. 219 para 1 point 2 of the Act

This application is based on the knowledge gained during my study of acetyltransferase toxin-antitoxin systems of *Salmonella enterica*. This work resulted in three research articles published in major scientific journals.

4.1. The title of the series of articles that meet the condition specified in Art. 219 section 1 point 2 point (b) of the Act

Molecular mechanisms of regulation of bacterial toxin-antitoxin systems in *Salmonella*

4.2. List of publications constituting the main achievement, detailing the candidate's contribution

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Research Article 1

Rycroft J.A., Gollan B., **Grabe G.J.**, Hall A., Cheverton A.M., Larrouy-Maumus G., Hare S.A., Helaine S., *Activity of acetyltransferase toxins involved in Salmonella persister formation during macrophage infection.* **Nature Communications** (2018) doi.org/10.1038/s41467-018-04472-6

Impact Factor (Journal Citation Reports 2023): **14.7**
Points awarded by the Ministry of Education and Science: **200**

Contribution of the applicant:

- Obtained toxin expression constructs used for protein purification
- Purified proteins required for biochemical and structural work
- Performed toxin pulldown experiments (Figure 4d)
- Crystallized and determined the structure of the TacT3 toxin (Figure 2c,d; PDB ID: 6G96)
- Determined thermostability of the TacT2 toxin variants (Figure S9)
- Performed and analyzed SEC-MALS experiment of the TacT3 toxin (Figure S6b)
- Generated Figures 2c-d, 3b, 4a,c-d and Supplementary figures S2, S5, S6, S9, and Supplementary tables S2 and S4
- Participated in experimental design
- Contributed to the writing of the manuscript

Research Article 2

Grabe G.J., Giorgio R.T., Hall A.M.J., Morgan R.M.L., Dubois L., Sisley T.A., Rycroft J.A., Hare S.A., Helaine S., *Auxiliary interfaces support the evolution of specific toxin-antitoxin pairing.* **Nature Chemical Biology** (2021) doi.org/10.1038/s41589-021-00862-y

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Impact Factor (Journal Citation Reports 2023): **13.0**
Points awarded by the Ministry of Education and Science: **200**

Contribution of the applicant:

- Obtained toxin and antitoxin expression constructs used for protein purification, toxicity, and neutralization assays
- Purified all proteins used in the study
- Performed protein pulldown and SEC-MALS experiments
- Crystallized and determined structures of TacA3ND-TacT3 (PDB ID: 7AK9), TacA1ND-TacT1 (PDB ID: 7AK8), and TacA2-TacT2 (PDB ID: 7AK7) toxin-antitoxin complexes
- Investigated the toxin-antitoxin interfaces

- Analyzed bioinformatically co-evolving toxin and antitoxin amino acids as well as phylogeny of related toxin variants
- Generated all figures and tables
- Co-initiated and co-orchestrated the project
- Co-wrote the manuscript with the last author
- Experimentally addressed reviewers' comments

Research Article 3

Grabe G.J.*, Giorgio R., Wieczór M., Gollan B., Sargen M., Orozco M., Hare S., Helaine S.*, *Molecular Stripping Underpins Derepression of a Toxin-Antitoxin System*. **Nature Structural and Molecular Biology** (2024) doi.org/10.1038/s41594-024-01253-2

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*Corresponding authors

Impact Factor (Journal Citation Reports 2023): **12.5**

Points awarded by the Ministry of Education and Science: **200**

Contribution of the applicant:

- Obtained toxin, antitoxin, and reporter expression constructs
- Purified all proteins used in the study
- Initiated and coordinated collaboration with Dr. M. Orozco and Dr. M. Wieczór (IRB Barcelona, Spain)
- Performed all reporter activity assays
- Analyzed proteins through SEC and SEC-MALS
- Crystallized and determined the structures of TacA1 antitoxin (PDB ID: 7ZG6) and TacA3-TacT3-DNA toxin-antitoxin-DNA complex (PDB ID: 7ZG5)
- Performed detailed investigation and analysis of obtained structures
- Generated all figures and tables
- Addressed reviewers' comments
- Co-wrote the article and co-orchestrated the project with the last author

Scientometrics for the three articles included in the series:

Total Impact Factor (Journal Citation Reports 2023): **40.2**

Total points awarded by the Ministry of Education and Science: **600**

4.3. Scientific aims of the works mentioned in point 4.2 and the obtained results

4.3.1 Introduction

Antibiotic treatment remains the default therapeutic approach for combating bacterial infections. However, prolonged infections often necessitate multiple antibiotic cycles, frequently resulting in antimicrobial resistance (**AMR**), characterized by bacterial insensitivity to antibiotics and continued growth. It is projected that by 2050, AMR will impose a significant burden on public health, contributing to an estimated 10 million deaths annually[1]. Although the exact mechanisms by which a sensitive bacterial population transitions to a resistant one are not fully understood, antibiotic recalcitrance (**AR**) - where bacteria enhance their survival upon antibiotic exposure - is considered a key factor. While AMR allows bacterial cells to grow despite the presence of antibiotics, in AR, bacteria do not divide but exhibit prolonged survival under antibiotic pressure. Increasing evidence suggests that **AR may facilitate AMR** by delaying cell death during treatment, thereby increasing the likelihood of mutations conferring antibiotic resistance[2,3]. AR can manifest across an entire bacterial population (**tolerance**) or within a subpopulation (**persister cells**) that survives antibiotic exposure. Persister cells represent a genetically antibiotic-sensitive subpopulation that enters a non-growing state, rendering them tolerant to antibiotics. Upon antibiotic withdrawal, these cells can resume growth, potentially leading to infection relapse[4,5]. The phenomenon of persister cells surviving penicillin treatment was first documented in the 1940s by Gladys Hobby[6] and Joseph Bigger[7]. Despite this early discovery, research on persisters stalled for four decades due to the lack of suitable tools for their study, the absence of a clear link to recurrent infections, and their transient nature. A breakthrough occurred in 1983 when Harris Moyed using a genetic screen identified an *Escherichia coli* gain-of-function *hipA7* mutant, exhibiting a 10,000-fold higher persister frequency than the wild-type strain[8].

Around the same time, two research groups discovered toxic and antitoxic genetic elements involved in plasmid maintenance in bacteria[9,10]. These elements, namely *hok-sok* and *ccdA-ccdB*, were later classified as **toxin-antitoxin** (hereafter **TA**) systems. Notably, the *hipA7* gain-of-function mutant discovered in 1983 by Moyed was subsequently recognized as a mutant of a *hipAB* TA module, linking AR to TA systems[11,12]. TA systems are small, typically bicistronic operons encoding a toxic protein (toxin) that disrupts vital bacterial processes and an antitoxin that neutralizes the toxin. Generally, the antitoxin gene precedes the toxin gene in the operon. There are eight types of TA systems (I-VIII) that have been identified to date, that differ in the nature of the antitoxin (protein or RNA) or the mechanism of toxin neutralization[13]. Among these, type II systems are particularly well-studied. In these systems, the antitoxin is a protein that directly interacts with the toxin forming a TA complex[14]. Many antitoxins, in addition to neutralizing their cognate toxin with neutralization domain, bind to the DNA operator within the TA promoter to **repress** transcription. However, under environmental stress, these systems often undergo transcriptional activation. This activation may be driven by proteolysis of the antitoxin pool, increasing levels of free toxin, which can act as TA **derepressor**[13]. Both reduction

in antitoxin pool and dissociation of a TA complex release the toxin which then targets essential bacterial processes, leading to cell growth arrest. Recent reports suggest that, beyond their role in bacterial persistence, TA systems also act as phage defense mechanism[15].

Salmonella enterica, a bacterial pathogen transmitted through contaminated food and water, infects nearly 15 million people annually worldwide, causing over 200,000 deaths[16]. This Gram-negative bacterium can cause non-systemic gastroenteritis limited to gastrointestinal tract or systemic typhoid fever and invasive salmonellosis, where bacteria disseminate via the blood stream to organs like the liver or spleen. With over 2,500 *Salmonella* serovars classified based on surface antigen composition[17], enteric (typhoid) fever is caused by human-restricted serovars such as **Typhi** and **Paratyphi**. In contrast, serovars causing gastroenteritis, such as **Typhimurium** and **Enteritidis**, exhibit a broad host range among vertebrates. These serovars cause gastritis or more severe bacteremia in immunocompromised patients, a condition frequently observed in sub-Saharan Africa, often leading to death[18].

The systemic spread of *Salmonella* is enabled by multiple virulence determinants that evolved over time, establishing it as a facultative intracellular pathogen. Key among these are *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2) which encode type III secretion systems (T3SS) that allow *Salmonella* to invade and replicate inside eukaryotic epithelial and macrophage cells[19]. These T3SSs deliver approximately 30 various so-called 'effector' proteins into the host cell that interfere with its signaling pathways, metabolism, or cell death[20]. To thrive within the host, *Salmonella* withstands various stressors, including reactive oxygen and nitrogen species, nutrient scarcity, acidic stress, and antimicrobial peptides. Many of these factors were shown to promote bacterial persister cell formation *in vitro*. Importantly, over a decade ago it was shown in an infection model that *Salmonella* internalization by macrophages leads to a 1,000 fold enrichment in persister cell subpopulation[21]. This phenomenon was dependent on fourteen type II TA systems, associating them with bacterial AR. At the time, three of these systems had no predicted function for the toxin element. In a subsequent study, one of them, later named as **TacT1**, was identified as an **acetyltransferase targeting amino acid charged tRNA molecules**[22].

Salmonella enterica serovar Typhimurium contains three predicted paralogous acetyltransferase TA systems: *tacAT1, tacAT2*, and *tacAT3*. Initially, it was unclear whether each toxin (TacT1, TacT2, and TacT3) was active or if they shared substrate specificity, how their respective antitoxins (TacA1, TacA2, and TacA3) neutralized and regulated them, and whether there was cross-neutralization or cross-regulation among these systems.

The primary aim of my research was to elucidate the molecular mechanisms underlying toxin neutralization and transcriptional regulation of *Salmonella tacAT* acetyltransferase TA systems. While studying neutralization, I discovered that multiplicity of TA interfaces and the resulting redundant toxin neutralization support the evolution of specificity of these systems. Additionally, I identified the molecular mechanism and allostery underlying the transcriptional activation of the *tacAT3* system. The three publications discussed below form the basis of my application for the habilitation degree.

4.3.2 Detailed description of the publications included in the achievement

Research Article 1

Rycroft J.A., Gollan B., **Grabe G.J.**, Hall A., Cheverton A.M., Larrouy-Maumus G., Hare S.A., Helaine S., *Activity of acetyltransferase toxins involved in salmonella persister formation during macrophage infection.*[23] **Nature Communications** (2018) doi.org/10.1038/s41467-018-04472-6

Introduction

Salmonella enterica serovars **Typhimurium** and **Enteritidis** cause relatively mild gastroenteritis in humans. However, in immunocompromised or malnourished patients, infection with these bacteria can lead to invasive **non-typhoidal salmonellosis (iNTS)**, a disease with biggest prevalence in sub-Saharan Africa. The mortality rate in this region can be as high as 50% in HIV-positive patients, significantly exceeding the mortality rate of typhoid fever[24]. Treatment of *Salmonella* infections relies on cephalosporin antibiotics; however, invasive strains are increasingly resistant to the first and second generation of this antibiotic. Moreover, the growing frequency of resistance to third-generation cephalosporins in Africa and Europe further complicates the therapy of infected patients[25,26]. Additionally, recurrent infections resulting from reinfection further complicate effective treatment[27].

Previous studies have shown that phagocytosis of *Salmonella enterica* serovar Typhimurium (here after referred to as **STm**) by macrophages significantly enriches a subpopulation of persister cells that do not divide and remain tolerant to antibiotic[21]. This persister cell increase was dependent on fourteen type II TA systems, which undergo transcriptional activation upon STm uptake by the macrophage. In 2016, one of these TA systems was found to encode an acetyltransferase toxin targeting amine group of amino acid-charged tRNA (aa-tRNA) molecules[22]. It was named **TacT**, which stands for **t**RNA **ac**etylating **t**oxin. This modification of the aa-tRNA molecules blocks amino acid transfer during protein synthesis leading to bacterial growth arrest. The toxic effect of TacT can be reversed by peptidyl-tRNA hydrolase (Pth), which removes the acetylated amino acid from tRNA, enabling reattachment of an unmodified amino acid to tRNA[22].

Aim of the study

The main goal of this work was to determine whether seven clinical *Salmonella enterica* strains (belonging to serovars Typhimurium and Enteritidis), isolated from blood samples of patient at Queen Elizabeth's Hospital in Malawi, form persister cells when internalized by human macrophages. We also investigated the TA distribution in these strains, focusing on the three acetyltransferase *tacAT1-3* systems. Moreover, we aimed to explore any functional

differences in paralogous TacT1-3 acetyltransferases in terms of toxin substrate specificity and activity.

Main findings

Since numerous infections are a result of clonal reinfection events, eight clinically relevant *Salmonella* strains (respectively, five Typhimurium and three Enteritidis) alongside reference strains were tested for persister cell formation upon internalization by human macrophages. All strains displayed a substantial **1,000 fold increase in persister cell fraction after macrophage uptake** when compared to the initial population, mirroring behavior seen in the STm laboratory reference strain. This confirmed that increased formation of antibiotic tolerant persister cells is a shared survival mechanism across clinical and reference *Salmonella* strains when confronted by immune cells.

We then analyzed the TA systems known to contribute to persister cell formation[21]. The clinical *Salmonella* isolates shared identical TA repertoires with their corresponding reference serovars, but differences emerged between the Enteritidis and Typhimurium strains. Serovar Enteritidis lacked two TA systems (*parDE* and *relBE1*). Additionally, Enteritidis and Typhimurium differed in the TacT2 toxin variant at position **29**, with Enteritidis carrying lysine (**$TacT2_{SEn}$**) and Typhimurium carrying glutamic acid (**$TacT2_{STm}$**). Three paralogous acetyltransferase TA systems (*tacAT1-3*) were present in both serovars, with TacT2 and TacT3 sharing respective 47% and 28% amino acid sequence identity with previously identified TacT1 toxin[22]. We tested whether the overexpression of ***$tacT2_{SEn}$*, *$tacT2_{STm}$***, and ***tacT3*** toxin genes promotes *in vitro* persister cell formation during exposure to cefotaxime antibiotic *in vitro*. Out of these, *$tacT2_{SEn}$* and *tacT3* toxins substantially induced persister fraction, an effect that was counteracted by co-expressing their corresponding antitoxins. The minimal effect of overexpressed *$tacT2_{STm}$* on persister cell formation suggested that the Glu29 polymorphism might affect the activity of TacT2 toxin.

To understand the structural relationship between acetyltransferase toxins, I determined the **crystal structure of TacT3 toxin at 1.5 Å resolution (Fig. 1)**. The dimeric acetyltransferase fold adopted by TacT3 protein bound to Ac-CoA cofactor was similar with that of TacT1 (PDB ID: 5FVJ), with the main difference being the presence of C-terminal α^T helix in TacT3 (Fig. 1). Using TacT1 as a template, structural models of $TacT2_{SEn}$ and $TacT2_{STm}$ were obtained. Both $TacT2_{SEn}$ and TacT3 toxins contained a substantial, positively charged region leading from the loop region of one monomer towards the active site of the other monomer. Mutating this region or a catalytic tyrosine (responsible for acetyl group transfer) abolished toxicity in *Salmonella* in TacT2 and TacT3, consistent with prior TacT1 findings[22]. Overexpression of *$tacT2_{STm}$*, however, did not inhibit growth of *Salmonella*, indicating that this toxin variant is inactive.

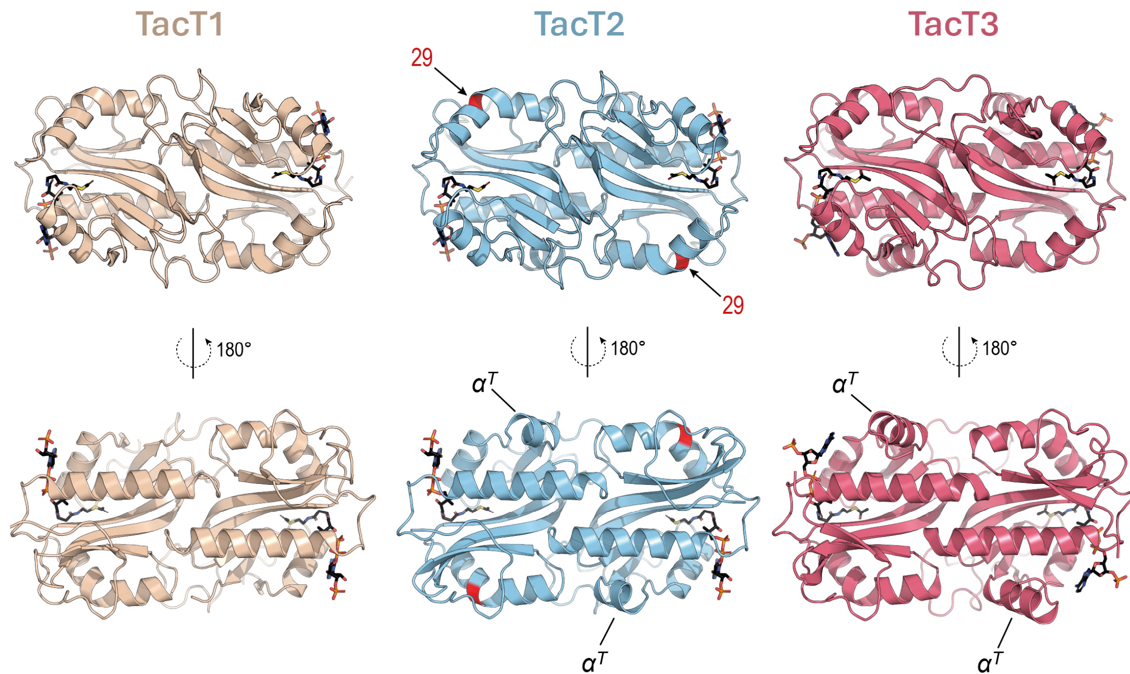

Figure 1. Three *Salmonella enterica* TacT1-3 acetyltransferases targeting aa-tRNA
Cartoon representation of front (top row) and back (bottom row) orientations of dimeric TacT1 (PDB ID: 5FVJ), TacT2 (model), and TacT3 (PDB ID: 6G96) aa-tRNA acetyltransferase toxins from *S. enterica*. Ac-CoA moieties are shown in black. Red region in the middle TacT2 model highlights a natural polymorphism at position 29. Additional C-terminal α^T helix present in TacT2 and TacT3 is shown with a black line.

In biochemical assays, purified TacT1, $TacT2_{STm}$, $TacT2_{SEn}$, and TacT3 all inhibited protein synthesis in a cell-free system and acetylated aa-tRNA molecules *in vitro*. Closer analysis of TacT2 variants revealed that the Lys29Glu polymorphism altered the surface charge distribution in toxin models and their theoretical isoelectric points (pI of 7.1 and 6.6 for $TacT2_{SEn}$ and $TacT2_{STm}$, respectively). *In vitro* acetyltransferase activity across a pH range (5.9–7.5) showed that $TacT2_{STm}$ was pH-sensitive, with a clear tRNA acetylation signal measured only at pH 7.5, whereas $TacT2_{SEn}$ was active across all tested pH conditions. The $TacT2_{SEn}$ toxin was also more stable in a thermostability experiment, which could be explained by stabilizing hydrogen bonds formed between a side chain of Lys29 and carbonyl oxygens of Thr15 and Phe17, which were absent in the $TacT2_{STm}$ model. Similar stabilization was seen in TacT1, where the corresponding Lys31 formed hydrogen bonds with Ala17 and Phe19. A TacT1 Lys31Glu mutation, mimicking the Glu29 variant in $TacT2_{STm}$, abolished TacT1 toxicity in *Salmonella*, underscoring the critical role of this region for TacT1 and TacT2 activity. A substrate-binding assays using catalytic mutants ($TacT2^{Y137F}$) revealed that $TacT2_{SEn}$ binds aa-tRNA more effectively than $TacT2_{STm}$, further corroborating its greater toxicity and *Salmonella* persister induction.

The substrate specificity of TacT1–3 toxins was determined in a cell-free translation assay by adding purified TacT1, $TacT2_{SEn}$, $TacT2_{STm}$, and TacT3 toxins with Ac-CoA. Acetylated aa-tRNA molecules were extracted, and the previously mentioned Pth hydrolase was used to release

modified amino acids for LC-MS analysis. The quantities of acetylated amino acids correlated well with the degree of acetylation observed *in vitro*. Of the twenty possible amino acids, twelve acetylated amino acids were detected in varying proportions, with glycine and leucine/isoleucine predominating.

Summary

We demonstrated that internalization of clinical *Salmonella* strains by human macrophage cells leads to over 1,000 fold enrichment in proportion of persister cells. The presence of persister cells is linked to recurrent infection and the ability of *Salmonella* to dramatically enrich its antibiotic-tolerant cell fraction suggests a type of an evolutionary adaptation in response to phagocytosis. This stress-induced non-growing subpopulation could constitute a reservoir for infection relapse as it was repeatedly shown that persister cells can resume growth.

Chromosomal type II TA systems affect persister formation in *Salmonella*. We showed that a single Glu29Lys polymorphism increases the activity of a TacT2 toxin and drives more robust persister cell formation. This increased toxin activity of $TacT2_{SEn}$ could possibly compensate for the lack of *parDE* and *relBE1* TA systems in Enteritidis serovar. At the time of this work's publication, both TacT2 and TacT3 represented a novel type of acetyltransferases modifying aa-tRNA[22,28].

Research Article 2

Grabe G.J., Giorgio R.T., Hall A.M.J., Morgan R.M.L., Dubois L., Sisley T.A., Rycroft J.A., Hare S.A., Helaine S., *Auxiliary interfaces support the evolution of specific toxin-antitoxin pairing.*[29]
Nature Chemical Biology (2021) doi.org/10.1038/s41589-021-00862-y

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Introduction

TA systems are stress response elements that promote bacterial survival. In the best-characterized type II systems, the antitoxin is a protein that controls the toxin through direct interaction (neutralization) and transcriptional repression. It consists of two key regions: a **neutralization domain (ND)**, which directly binds the toxin and blocks its activity, and a **DNA-binding domain (DBD)**, involved in transcriptional repression of the TA operon. This tight control over the toxin at both protein and transcriptional levels is critical, as unbound toxin can inhibit bacterial growth and lead to cell death. Many bacteria, including *Salmonella* Typhimurium (STm), carry paralogous TA systems in their chromosomes. While some toxins can be neutralized by noncognate antitoxins[30–32], the majority of paralogous TA systems do not cross-neutralize. This suggests that interference between TA systems is detrimental to the bacterial cell[33–36]. Interestingly, structural studies of various toxins in complex with their antitoxins revealed a single interaction surface which determines specificity and enables toxin neutralization. During the evolution of new specificity in TA complexes, the interaction between

these proteins must be maintained due to the toxic nature of the toxin. This led to the hypothesis that, in addition to the primary interface, TA systems may possess secondary interfaces that sustain toxin-antitoxin interactions, potentially supporting the evolution of new specificity in these complexes. The structure of a hexameric TA complex, with an A:T ratio of 2:1 and multiple TA interfaces observed, proved instrumental in exploring this hypothesis.

Aim of the study

The objective of this study was to identify the neutralization mechanism of the acetyltransferase toxins TacT1-3 in STm by their corresponding antitoxins TacA1-3 and to determine whether neutralization in specific to cognate TA pairs. An additional aim was to understand how paralogous *tacAT* systems evolve toward new specificity while maintaining interaction with the toxin.

Main findings

Previous work demonstrated that each TacA1, TacA2, and TacA3 antitoxins neutralize their cognate TacT1, TacT2, and TacT3 toxins[22,23]. Given their shared sequence similarity, we tested whether these antitoxins could neutralize noncognate toxins. In bacterial growth experiments, each antitoxin neutralized only its cognate toxin, and no cross-neutralization was observed, in line with previous findings on paralogous toxins from other TA families[33–38]. Through series of N-terminal truncations we then determined a minimal region (neutralization domain – ND) required for each antitoxin to counteract its toxin. All three **TacAND** antitoxins neutralized only their cognate TacT toxin, confirming that this region retains the specificity pattern of a full-length antitoxin.

To elucidate the molecular mechanism of neutralization and specificity in acetyltransferase TA systems, **I solved three crystal structures of TacT1-3 toxin complexes bound either to the ND domain of the antitoxin (tetrameric TacA1ND-TacT1 and TacA3ND-TacT3) or to the full-length antitoxin (hexameric TacA2-TacT2)**. In all complexes, the ND region of the antitoxins bound to the same area of the toxin and exhibited a similar structure, comprising α3 and α4 helices (Fig. 2). A key feature of this interaction was the disruption of the substrate aa-tRNA binding site (TBS), which is partially formed by a toxin loop. Specifically, the toxin loops in TacT1-3 were displaced from their original positions by the α3 helices of the TacA1-3 antitoxins (Fig. 2). Additionally, the C-terminal region of the antitoxin, including the α4 helix, bound near the acetyl-CoA binding pocket (ACP), a site critical for acetyltransferase enzymatic activity. This binding mode was similar to that observed in related antitoxins KacA and AtaR, suggesting a shared neutralization strategy based on blocking aa-tRNA binding and modification[28,39] (Fig. 2).

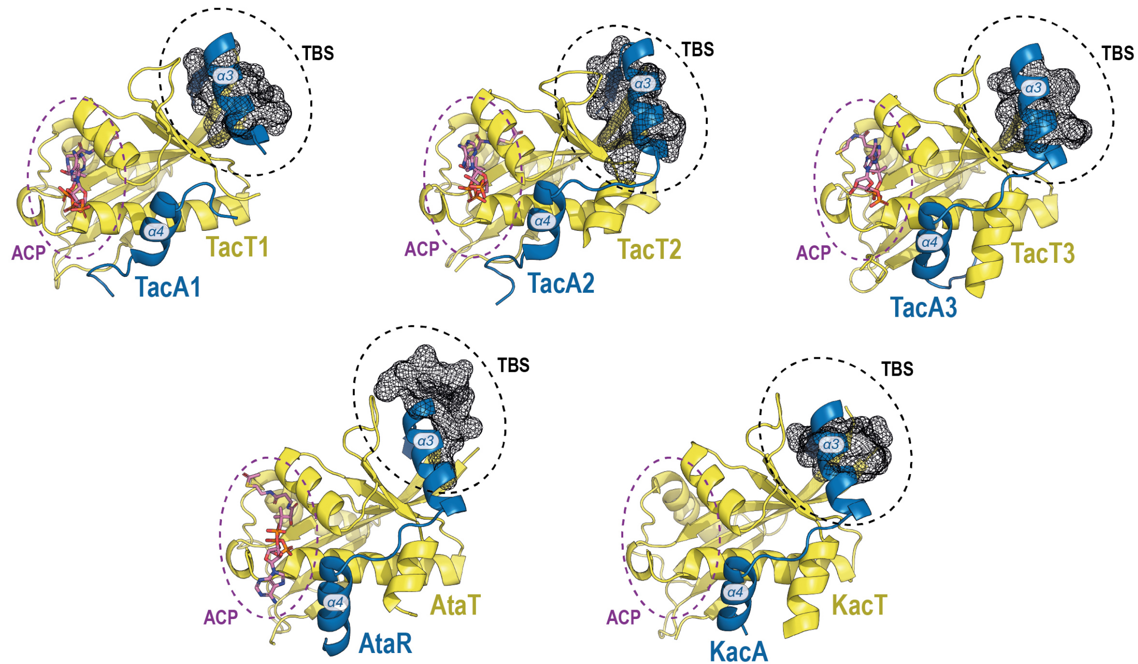

Figure 2. Acetyltransferase toxins in complex with their neutralizing antitoxin fragments
Crystal structures of TacT1, TacT2, TacT3, AtaT, and KacT toxins (yellow) in complex with their respective TacA1, TacA2, TacA3, AtaR i KacA antitoxins (blue). Toxin regions involved in aa-tRNA substrate binding (**TBS**; black dashed line) are displaced by antitoxin - for optimal displacement display, TBS region from the antitoxin-free toxin states composed of the loop element is represented as black mesh, which clearly overlaps with antitoxin helices α3. Additionally, all antitoxins bind in vicinity of Ac-CoA site (**ACP**; violet dashed lines) with their helices α4. Ac-CoA cofactors are shown as violet sticks. PDB IDs of neutralized toxins' states are given in brackets for each complex: TacA1-TacT1 (7AK8), TacA2-TacT2 (7AK7), TacA3-TacT3 (7AK9), AtaR-AtaT (6GTR), KacA-KacT (5ZGN). Black mesh TBS density was generated based on structures and model (given in brackets) of antitoxin-free toxins: TacT1 (5FVJ), TacT2 (Swiss model), TacT3 (6G96), AtaT (6GTP), KacT (5XUN).

Bacterial toxicity assays and pull-down experiments with tRNA confirmed the critical role of the TBS in toxin activity and substrate recognition. Unlike the unbound TacT3 toxin, TacT3 bound to TacA3ND did not interact with tRNA, underscoring the pivotal role of the TBS in tRNA binding. Beyond TBS disruption, all three antitoxins were found to contain lysine residues in their α4 helix, with side chains oriented toward the ACP. However, the minimal reduction in neutralization observed in TacA$^{ND,Lys/Ala}$ variants, where lysine was substituted with alanine, indicated that **TBS disruption is the dominant neutralization mechanism for TacAND**. The paralogous toxins TacT1-3 differed in the previously identified C-terminal $α^{T}$ helix, present in TacT2 and TacT3 but absent in TacT1[23]. Deletion of the $α^{T}$ helix in TacT3 did not eliminate toxicity, suggesting that its absence does not significantly impact toxin function. Notably, in the TacA3ND-TacT3 complex structure, the $α^{T}$ helix formed a local interface with the TacA3 antitoxin, constituting a significant portion of the overall TacA3ND-TacT3 interface. This suggests that this C-terminal element may serve as an interface add-on determining the specificity of the interaction[40]. Deletion of the $α^{T}$ helix significantly reduced neutralization by TacA3 without

enabling cross-neutralization by paralogous antitoxins, indicating that it is a determinant of TacA3-TacT3 interaction specificity.

Given that the ND domain in TacA antitoxins is critical for neutralization and determines the specificity of toxin interaction, there is a risk that the evolution of new specificity could lead to toxin release, resulting in cellular intoxication. Consequently, we investigated whether the evolution of new specificity in paralogous TA systems is supported by additional mechanisms. To this end, the structure of the hexameric TacA2-TacT2 complex, with an A:T ratio of 2:1, twice that observed in complexes of toxins with only the ND domains of antitoxins (TacA1ND-TacT1 and TacA3ND-TacT3), proved instrumental. This structure revealed **two TacA2 antitoxin dimers** formed by their N-terminal DNA-binding domains (**DBD**). Each of the four TacA2 antitoxin molecules interacted with two TacT2 toxin molecules, collectively covering 38% of the total TacT2 surface. This was facilitated by the presence of additional interfaces (beyond the primary ND-mediated interface), each formed by a separate antitoxin molecule. **In addition to the primary interface, termed P, which encompassed the ND-mediated interface observed in TacT-TacAND complexes, we identified secondary interfaces S, T, and Q**. The larger P and S interfaces were formed by the C-terminal regions of distinct TacA2 molecules. Surprisingly, the smaller T and Q interfaces were mediated by the α1 helix of the N-terminal DBD, a region through which the antitoxin binds DNA. Among all interfaces, the P interface exhibited the greatest sequence variability when homologous regions of the three TacA1-3 antitoxins were compared. Furthermore, the positioning of the S, T, and Q interfaces suggested that they provide additional neutralization beyond that conferred by the P interface. Functionally, the S and T interfaces further disrupted the tRNA-binding site (TBS), while the Q interface blocked the acetyl residue of the Ac-CoA cofactor. Moreover, the S interface disrupted the dimerization of the TacT2 toxin, a state essential for the activity of acetyltransferase toxins[22,23,41–43]. Using size-exclusion chromatography coupled with multiple-angle light scattering (SEC-MALS), we confirmed that the hexameric state is common to all three complexes (TacA1-TacT1, TacA2-TacT2, and TacA3-TacT3), suggesting that the additional S, T, and Q interfaces are also present in the TacA1-TacT1 and TacA3-TacT3 complexes. Accordingly, same hexameric state with multiple interfaces was later observed in the structure of the related acetyltransferase system, AtaR2-AtaT2, by an independent research group[44].

The identified diversity of interfaces revealed that TacA antitoxins are flexible proteins capable of adopting multiple conformations that redundantly neutralize TacT toxins at virtually all possible levels. Consequently, we hypothesized that the additional S, T, and Q interfaces support the evolution of new specificity in TA systems. Given that the P interface, with the most variable amino acid sequence, is the primary determinant of interaction specificity, we proposed that the S, T, and Q interfaces serve as a neutralization buffer during the evolution of new specificity, which may involve partial or significant disruption of the P interface. This hypothesis was tested by constructing a chimeric TacA$^{1\text{-}3}$ antitoxin, in which a part of region of TacA1 forming the P interface was replaced with the corresponding region from TacA3. The TacA$^{1\text{-}3}$ chimera robustly neutralized both TacT1 and TacT3 toxins, but not TacT2.

Substitution of a conserved aspartic acid (Asp) residue involved solely in the S interface with lysine (Asp-Lys) had negligible impact on neutralization in the context of a native P interface (TacA1$^{Asp\text{-}Lys}$-TacT1 and TacA3$^{Asp\text{-}Lys}$-TacT3). However, in the TacA$^{1\text{-}3}$ chimera with a disrupted P interface, the same substitution (TacA$^{1\text{-}3,\,Asp\text{-}Lys}$) significantly reduced neutralization efficiency for both toxins, confirming that the S interface acts as a neutralization buffer when the primary P interface is impaired. Similar results were obtained for mutations in the T and Q interfaces, confirming their role in supporting neutralization when the P interface is partially disrupted (Fig. 3).

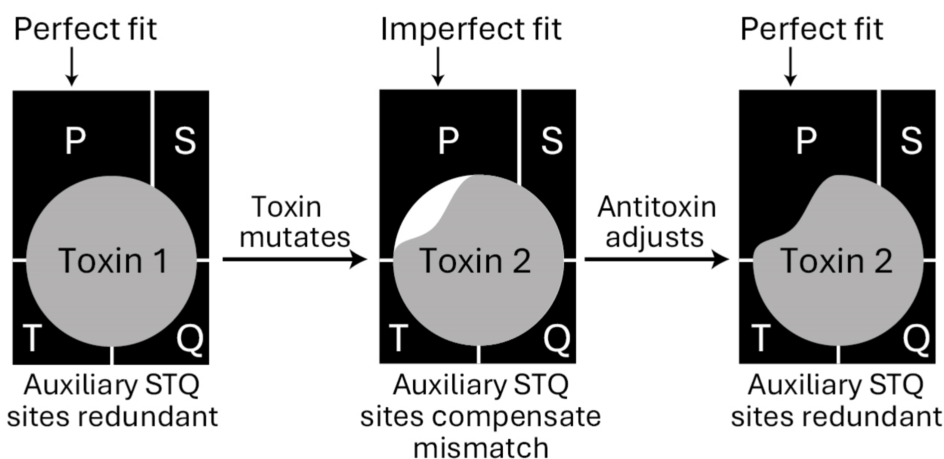

Figure 3. Auxiliary interfaces support the evolution of TA systems
Interface P acts as the main toxin neutralization site, with S, T and Q sites providing redundant neutralization. As the toxin mutates, the suboptimal fit of the P interface is compensated for by the additional S, T and Q interfaces, providing backup neutralization before adjustment of the P site and insulation of the evolving pair. Taken from Research Article 2.

Summary

In this study we elucidated how antitoxins specifically neutralize their cognate toxins in paralogous STm *tacAT* acetyltransferase systems. The primary mechanisms of neutralization of acetyltransferase toxins by antitoxins were identified and involved disrupting functions critical to toxin activity: (1) tRNA substrate binding (TBS), (2) Ac-CoA cofactor binding (ACP), and (3) the dimeric state of the toxin. It was discovered that antitoxins interact with acetyltransferase toxins through multiple interfaces, enabling redundant neutralization. These additional interfaces were shown to support neutralization, allowing divergence of the primary interface without necessitating toxin release and consequent cellular intoxication. This mechanism of “redundant neutralization” based on multiple interfaces could facilitate faster evolution of specificity in paralogous TA systems. Such accelerated evolution would not be feasible in TA systems reliant on a single interface.

This work was highlighted by Nature Chemical Biology article "**Evolving complex insulation**"[45] and in Science in Boston website in "**Publications of the week**" section (https://scienceinboston.com/wp-content/uploads/sites/6/2021/11/Volume-3.41-min.pdf).

Research Article 3

Grabe G.J.*, Giorgio R., Wieczór M., Gollan B., Sargen M., Orozco M., Hare S., Helaine S.*, *Molecular Stripping Underpins Derepression of a Toxin-Antitoxin System.*[46] **Nature structural and molecular biology** (2024) doi.org/10.1038/s41594-024-01253-2

*Corresponding authors

Introduction

Bacterial repressors regulate gene activity by binding to DNA sequences (known as operators) which overlap with promoter regions. The binding of a repressor to an operator prevents RNA polymerase from accessing the promoter, thereby inhibiting transcription initiation[47]. In this context, efficient transcription relies on a derepression step, during which the repressor dissociates from the operator due to structural rearrangements triggered by the binding or release of an inducer molecule[48,49]. Elucidating the molecular mechanisms responsible for repressor dissociation from the repressor-operator complex is challenging due to the dynamic nature of this process.

Type II bacterial toxin-antitoxin (TA) systems provide a convenient model for studying the molecular mechanisms underlying derepression. Type II TA systems are bicistronic operons encoding two proteins: a toxin, which disrupts essential cellular processes, and an antitoxin, which binds and neutralizes the toxin. In addition to toxin inhibition, the antitoxin also binds to an operator located within the TA promoter, enabling transcriptional autoregulation of the operon[13]. In many systems, the toxin:antitoxin (T:A) ratio significantly influences this regulation. At a low T:A ratio, the toxin cooperates with the antitoxin in repression, whereas at a high T:A ratio, the toxin acts as a derepressor and activator of transcription. This T:A ratio-dependent behavior of the toxin is referred to as **conditional cooperativity**[50,51]. Notably, at the time of this study, conformational changes associated with derepression in TA systems had not been described.

The chromosome of STm harbors three *tacAT1-3* TA modules, which encode acetyltransferase toxins (TacT1-3) that modify amino acid charged tRNA, and their corresponding antitoxins (TacA1-3). These antitoxins contain an N-terminal DNA-binding domain (DBD) and a C-terminal toxin-neutralizing domain (ND). Homologous acetyltransferase TA systems, *kacAT* and *ataRT*, have been described in *Klebsiella*[39] and *Escherichia*[43], respectively, where **hexameric** complexes with a **2T:4A** stoichiometry specifically bind the operator and regulate their own expression through a promoter derepression mechanism. However, the molecular mechanisms underlying the derepression process in these systems remained uncharacterized.

Aim of the study

The objective of this study was to elucidate how the *tacAT3* system regulates its transcription, with a particular focus on the molecular basis of promoter repression and the conformational changes required for its derepression.

Main results

Studies on the regulation of the *tacAT3* operon in *Salmonella* using transcriptional fusions and flow cytometry confirmed autoregulation of this system. The *tacAT3* promoter contained two potential operators, OP1 and OP2. It was established that OP1 was responsible for controlling *tacAT3* module expression, as only mutation of OP1 eliminated repression in wild-type *Salmonella* cells. Additionally, it was demonstrated that purified TacA3-TacT3 complex specifically binds the operator (OP1) and not its mutated form.

Toxins cooperate with antitoxins to enhance repression by bridging antitoxin dimers. Experiments confirmed that both TacA3 and TacT3 are essential for robust repression and operator binding. Structural modeling of the hexameric **4A:2T TacA3-TacT3 complex bound to the operator** revealed two TacA3 dimers engaging the major grooves of the DNA, involving amino acid residues within the DNA-binding site. Mutation of one of these residues abolished DNA binding and repression, confirming the critical role of this region in *tacAT3* promoter regulation. It was also observed that TacT3 toxins bridged two antitoxin dimers through primary (**P**) and secondary (**S**) interfaces (Fig. 4). This bridging suggested that the toxin enhances antitoxin-DNA binding strength through avidity, an effect previously described for other TA systems[52]. Furthermore, the toxin was found to orient its positively charged surface toward the minor grooves of the operator. This region, termed the **M site**, was experimentally validated as significant for DNA binding and promoter repression. Thus, beyond the avidity effect, the toxin itself contributes to promoter repression via its positively charged surface.

Although the toxin supports antitoxin-mediated promoter repression at a low T:A ratio, an increased toxin amount can destabilize the complex, leading to derepression. In DNA-binding experiments, cooperation between TacT3 and TacA3 in operator binding was observed at a low T:A ratio, whereas an excess of TacT3 disrupted operator binding. Notably, this disruption was accompanied by the formation of a higher molecular weight complex, potentially initiating dissociation from the operator. This is consistent with the observation that overexpression of *tacA3-tacT3* operon led to promoter derepression in a subset of stationary-phase bacterial population, possibly due to an excess of TacT3 toxin relative to the less stable TacA3 antitoxin. To elucidate the molecular basis of derepression and the release of the TacA3-TacT3 complex from the operator, I determined the crystal structure of the TacA3-TacT3 complex with the operator in the presence of additional TacT3 toxin, which led to formation of previously observed higher molecular weight complex. The crystal structure obtained revealed an **octameric TacA3-TacT3 complex bound to the operator at a 4A:4T stoichiometry**. In contrast to the hexameric complex, where each toxin molecule binds two antitoxin molecules via P and S

interfaces, in the octameric complex, each toxin molecule bound only one antitoxin molecule through either the **P** or **P'** interface (Fig. 4). Nevertheless, the connection between TacA3 dimers was maintained through toxin dimers interacting via **D dimerization interface** (Fig. 4). The D interface composed of TacT3 toxin dimer relied predominantly (43% of the surface) on a **loop element**, distinguishing it from the previously obtained toxin-only dimer (PDB ID: 6G96), where the same loop was surface-exposed and involved in binding of the charged tRNA substrate.

To verify the significance of the octameric state in *tacAT3* promoter regulation, a **TacT3^{Loop}** variant was created with substitutions in the loop region critical for the D dimerization interface sustaining the octameric complex. Importantly, this loop was not involved in formation of the hexameric TacA3-TacT3 repressor complex bound to the operator. Consistent with these observations, the TacA3-TacT3^{Loop} complex still formed a hexamer capable of binding the operator. It was also confirmed that the TacT3^{Loop} variant cooperated with the TacA3 antitoxin in operator binding, similarly to TacT3. However, unlike TacT3, an excess of TacT3^{Loop} in high T:A ratio did not initiate dissociation of the TacA3-TacT3^{Loop} complex from the operator. Moreover, in stationary-phase cells, overexpression of *tacA3-tacT3^{Loop}* resulted in stronger repression and, crucially, a lack of promoter derepression, in contrast to results obtained with *tacA3-tacT3*. **These findings support the hypothesis that the transition from a hexameric to an octameric complex is a key step in toxin-induced promoter derepression.**

To understand why octamer is required for *tacAT3* promoter derepression, the hexameric and octameric complexes were compared, revealing several significant differences. The additional two toxin molecules in the octamer led to a series of substantial conformational changes. The S interface, formed between the original toxin and antitoxin in the hexamer, was replaced by a dimeric D interface formed by two toxin molecules in the octamer. **The antitoxin, displaced from the S interface with the original toxin, formed a P' interface in the octamer, linking it to the additional toxin molecule** (Fig. 4). It was observed that, compared to the hexamer, the P interface in the octamer shifted, resulting in **loss of the M site**, which in the hexamer enhanced repression. Furthermore, superimposing both models on one TacA3 dimer revealed a displacement of the second antitoxin dimer, coinciding with an increased bending angle of the operator DNA. Additionally, the relative B-factor values in the operator region and the associated N-terminal regions of the antitoxins were significantly higher compared to the rest of the model than in the homologous hexameric KacA-KacT system, suggesting that the operator in the octameric TacA3-TacT3 complex was more dynamic. Multiple molecular dynamics-based simulations identified the formation of a local kink in the central region of the operator. These results qualified the proposal of a derepression model for the *tacAT3* system (Fig. 4).

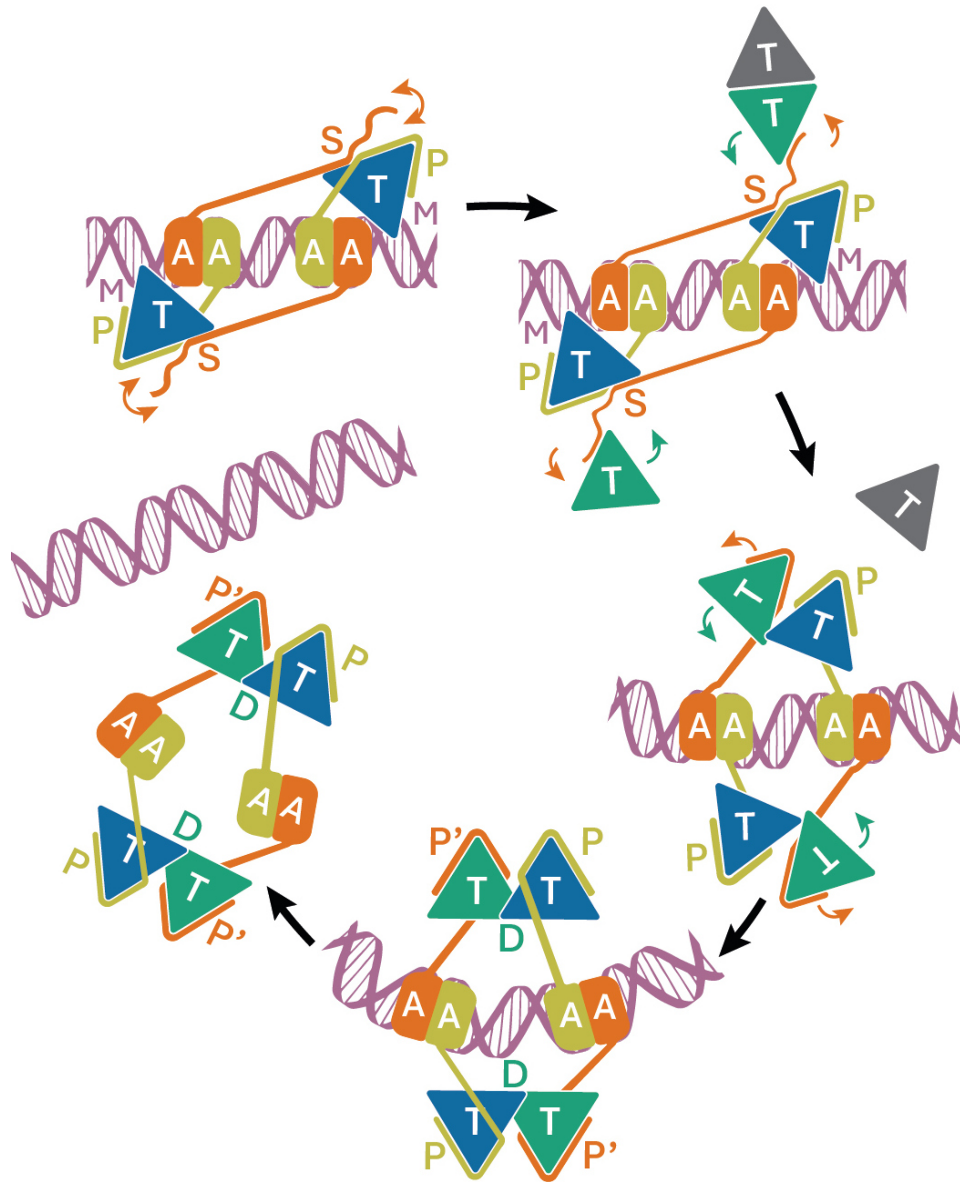

Figure 4. Derepression model of *tacAT3* system.
The hexameric TacA3–TacT3 complex establishes a repressed state on the operator DNA. Upon stress-induced release of additional cytosolic toxin, additional TacT3 monomers or dimers (green and gray) invade the hexameric complex via accessible C-terminal parts of the antitoxin molecules (orange) that form S interface with the toxin (blue). This initiates a cascade of allosteric rearrangements leading to molecular stripping of the repressor from the operator DNA, thus initiating operon transcription. A – antitoxin; T – toxin; P and P' – primary toxin-antitoxin interfaces; S – secondary toxin-antitoxin interface; M – toxin-operator minor groove region; D – toxin dimer interface; Taken from Research Article 3.

Summary

This study elucidated the molecular mechanism of *tacAT3* promoter regulation. It was demonstrated that at a high T:A ratio, derepression depends on the formation of a transitory octameric state, during which a cascade of conformational changes weakens the interaction

with DNA, enabling derepression (Fig. 4). A key element was the formation of the D interface, which displaces the S interface present in the hexameric complex and transforms it into a P' interface. The primary component facilitating the D interface formation is a toxin loop. Notably, the same loop is responsible for binding the substrate tRNA in the absence of the antitoxin[29], highlighting the functional plasticity of this element.

4.3.3 Outlook and future plans

Following the completion of my doctoral degree, I focused on analyzing the regulation of toxin-antitoxin (TA) stress response systems that support *Salmonella* survival during antibiotic exposure. My research concentrated on TA systems encoding acetyltransferase toxins that modify charged tRNA molecules. My structural biology work resulted in the determination of six crystal structures, capturing various states of these systems:

- ***Toxin-only*** - TacT3 acetyltransferase (PDB ID: 6G96)
- ***Antitoxin-only*** - TacA1 antitoxin (PDB ID: 7ZG6)
- ***Antitoxin-toxin complexes*** - $TacA1^{ND}$-TacT1 (PDB ID: 7AK8), TacA2-TacT2 (PDB ID: 7AK7), $TacA3^{ND}$-TacT3 (PDB ID: 7AK9)
- ***Operator-bound toxin-antitoxin complex*** - TacA3-TacT3-DNA (PDB ID: 7ZG5)

These structures, combined with biochemical and cellular experimental results, deepened our understanding of the function and diversity of TacT toxins. Analysis of the TA complex revealed multiple toxin-antitoxin interfaces within a single toxin, ensuring multi-layered and redundant neutralization. We demonstrated that the presence of additional toxin interfaces supports the evolution of specificity in paralogous TA systems, acting as a buffer to maintain toxin-antitoxin interactions during evolutionary changes in the primary interface. Furthermore, the octameric TA complex bound to the operator proved critical for understanding the molecular switch responsible for transcriptional activation of this system. Toxin loop was essential for forming the octameric state, and its mutation enabled the creation of "super-repressor" complexes insensitive to high T:A ratio, underscoring the octamer's role in transcriptional regulation.

My current research, supported by POLONEZ BIS, EMBO Installation Grant, and OPUS grants, focuses on analyzing the molecular mechanisms underlying stress response and *Salmonella* virulence. I am expanding my studies on toxin-antitoxin (TA) systems to include those with reversed operon organization, where the toxin gene precedes the antitoxin gene. I am also investigating the evolution of specificity in toxin-antitoxin interactions. Moreover, we are exploring whether TA systems, beyond autoregulation, influence the expression of other genes critical for bacterial physiology. Additionally, I am analyzing the control of other transcriptional factors, with a particular emphasis on virulence regulators. Understanding these mechanisms may have biotechnological applications, such as designing small therapeutic molecules to disrupt processes essential for *Salmonella* survival and pathogenicity.

Beyond TA systems, my research encompasses *Salmonella* effector proteins that manipulate host cellular processes via the type III secretion system (T3SS), including the ubiquitin-proteasome system (UPS), which is crucial for eukaryotic cell proteostasis. The results of these studies may enable the development of biotechnological strategies to interfere with *Salmonella* virulence and contribute to designing therapies that modulate UPS function in human diseases.

4.4. Other achievements since obtaining a PhD degree

During my postdoctoral training in the research group of Professor Sophie Helaine, we published a review article that discussed the state of the literature demonstrating a link between recurrent infections and antibiotic-tolerant persister cells. This work traced the history and development of persister cell research, assessed its current state (at the time), and outlined future research directions. It summarized the existing literature on the formation, survival, and regrowth of persister cells, as well as strategies employed to combat them. The article also analyzed the connections between bacterial antibiotic tolerance and antibiotic resistance.

- Gollan B.*, **Grabe G.***, Michaux C.*, Helaine S., *Bacterial persisters and infection: past, present, and progressing*. **Annual Review of Microbiology** (2019) doi.org/10.1146/annurev-micro-020518-115650
  *Equal contribution
  Impact Factor (Journal Citation Reports 2023): **8.5**
  Points awarded by the Ministry of Education and Science: **200**

In addition to my work on acetyltransferase TA systems, I collaborated with the research group of Dr. Anna Barnard (Imperial College London, United Kingdom) to investigate the bacterial type II Phd-Doc TA system. In this system, the Doc toxin is a kinase that phosphorylates the elongation factor EF-Tu, leading to translation inhibition, while Phd is the antitoxin that binds and neutralizes Doc. Various peptides based on the Phd sequence were evaluated for their ability to inhibit Doc kinase activity *in vitro*, their binding affinities were determined, and their capacity to counteract the toxic effects of Doc in bacterial cells was assessed. These studies resulted in two scientific publications.

- de Castro G.V., Worm D.J., **Grabe G.J.**, Rowan F.C., Haggerty L., de la Lastra A.L., Popescu O., Helaine S., and Barnard A., *Characterization of the Key Determinants of Phd Antitoxin Mediated Doc Toxin Inactivation in Salmonella.* **ACS Chemical Biology** (2022) doi.org/10.1021/acschembio.2c00276
  Impact Factor (Journal Citation Reports 2023) = **3.5**
  Points awarded by the Ministry of Education and Science: **100**

- Worm D.J., **Grabe G.J.**, de Castro G.V., Rabinovich S., Warm I., Isherwood K., Helaine S., and Barnard A., *Stapled Phd Peptides Inhibit Doc Toxin Induced Growth Arrest in Salmonella*. **ACS Chemical Biology** (2023) doi.org/10.1021/acschembio.3c00411
  Impact Factor (Journal Citation Reports 2023) = **3.5**
  Points awarded by the Ministry of Education and Science: **100**

During my stay in Boston, I collaborated with Dr. Benjamin Springstein from Professor Ann Hochschild's laboratory on a project utilizing a bacterial two-hybrid (B2H) system to map the intraviral interactome of sixteen SARS-CoV-2 proteins. The detected interactions were sensitive to disruptive mutations, confirming the utility of the B2H system in analyzing interacting viral protein pairs. Furthermore, modification of this platform enabled the detection of interactions dependent on disulfide bonds.

- Springstein B.L., Deighan P., **Grabe G.J.**, Hochschild A., *A bacterial cell-based assay to study SARS-CoV2 protein-protein interactions*. **mBio** (2021) doi.org/10.1128/mBio.02936-21
  Impact Factor (Journal Citation Reports 2023) = **5.1**
  Points awarded by the Ministry of Education and Science: **140**

5. Presentation of significant scientific or artistic activity carried out at more than one university, scientific or cultural institution, especially at foreign institutions

I have always been keen on any international scientific experience. Already at the age of seventeen, I attended the Summer Research School organized by Uppsala University (Sweden). After completing my bachelor's degree at the Intercollegiate Faculty of Biotechnology (IFB) in Gdansk, I undertook a year-long internship in the research group of Dr. Jan van der Wolf at Plant Research International, Wageningen University (Netherlands), where I studied bacterial plant pathogens. This work contributed to three scientific publications[53–55].

During the second year of my master's studies at IFB, I conducted a project in the laboratory of Professor Shohei Koide at the University of Chicago (USA) as part of a scientific collaboration (currently supported by the Fulbright Foundation). There, I acquired skills in protein purification, characterization, crystallization, and the phage display technique. This enabled me to generate binding proteins (monobodies) specific to the SH2 domain of the Bcr-Abl tyrosine kinase, a causative factor in chronic myelogenous leukemia. My work resulted in a publication demonstrating the inhibition of Bcr-Abl kinase activity by these binding proteins both *in vitro* and in cells, highlighting the SH2-kinase interface as a potential pharmaceutical target[56].

After obtaining my master's degree, I was awarded a prestigious Wellcome Trust Molecular and Cellular Basis of Infection scholarship for combined master's (MRes) and doctoral studies, which I pursued at Imperial College London (United Kingdom). During my MRes, I participated in three laboratory rotations, one of which, conducted in the group of Professor Steve Matthews and focused on bacterial curli fiber systems, led to authorship in a scientific publication[57]. My doctoral research, conducted in the laboratory of Regius Professor David Holden, focused on

determinants of *Salmonella* pathogenesis, including the type III secretion system (T3SS) and the SpvD effector protein[58–60]. Part of this work was carried out in collaboration with the laboratory of Professor David Komander at the Laboratory of Molecular Biology in Cambridge (United Kingdom), resulting in a published research article[61]. Additionally, I established a long-term scientific collaboration between the Holden group and the Mass Spectrometry Laboratory at the Institute of Biochemistry and Biophysics in Warsaw (Poland), which led to several publications[60,62,63].

For my postdoctoral training at Imperial College London (United Kingdom) and Harvard Medical School (USA), I joined the laboratory of Professor Sophie Helaine, focusing on the *tacAT1-3* TA modules in *Salmonella enterica*. These studies contributed to understanding: (i) the role of *tacAT1-3* systems in bacterial survival under antibiotic exposure[23], (ii) the mechanisms of neutralization and evolution of specificity in these systems[29], and (iii) the transcriptional regulation of the *tacAT3* system[46]. While investigating the transcriptional regulation of *tacAT3,* I initiated a collaboration with Dr. Modesto Orozco and Dr. Miłosz Wieczór from the Institute for Research in Biomedicine in Barcelona (Spain), which, through the application of molecular simulations, elucidated and visualized the derepression mechanism of this TA system. I also collaborated with Dr. Anna Barnard at Imperial College London (United Kingdom), where we studied the neutralization dynamics of the Doc kinase toxin in the Phd-Doc TA system, resulting in two scientific publications[64,65].

In 2023, I leveraged my international scientific experience to secure the POLONEZ BIS grant [“Dynamics and evolution of specificity in toxin-antitoxin complexes”; budget 1,130,821 PLN], aimed at Polish scientists abroad that want to return to Poland. This enabled me to lead my first independent research project on the dynamics of TA systems at the IFB, the institution where I took my initial steps in science. In 2024, I was awarded an OPUS grant [“Investigation of the impact of *Salmonella* bacteria on the ubiquitin-proteasome system”; budget 3,818,680 PLN] to study the interactions between *Salmonella* and the eukaryotic ubiquitin-proteasome system. In 2025, I became a beneficiary of an EMBO Installation Grant [“Investigation of *Salmonella* pathogenicity and survival”; budget 50,000 EUR annually for 3-5 years], which supports the establishment of an independent research group. The funding secured over the past two years effectively enables me to address complex questions in basic science. Achieving habilitation degree will allow me to become a fully independent researcher, enabling the supervision of doctoral students and guiding their development. Moreover, I am motivated to contribute to the advancement of IFB and strengthen its position as a leading research institution.

6. Presentation of teaching and organizational achievements as well as achievements in popularization of science or art.

In 2024, I delivered two public lectures: “*Salmonella enterica* – pathogenesis and survival” (20th March 2024) for high school students participating in the “Basics of Oncogenesis” knowledge competition, and “Combating the wedding bug – *Salmonella* pathogenesis

mechanisms and contemporary challenges in antibiotic therapy" (18th December 2024) as part of the Gdansk University of the Third Age seminar series. During my time at Imperial College London, I participated in Imperial Open Days, an event aimed at popularizing science among the public and presenting research projects conducted by various research groups. Additionally, in 2019, I contributed to the organization of the EMBO "Toxin-Antitoxin Systems" workshop in Windsor, England.

Throughout my doctoral and postdoctoral career at Imperial College London and Harvard Medical School, I supervised a total of ten master's and early-stage doctoral (rotation) students, several of whom chose to continue their research in the group I represented. Over the past one and a half years, I have supervised a research assistant on the POLONEZ BIS project. Furthermore, I established scientific collaborations with the research groups of Dr. Charlotte Michaux (University of Rennes, France), Professor Wojciech Bal (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Poland), and the biotechnology company Acteryon (Gdansk, Poland). I currently lead a dynamic team consisting of two postdoctoral researchers, one doctoral student, and one research assistant.

7. Apart from information set out in 1-6 above, the applicant may include other information about his professional career, which he deems important

7.1. Research grants obtained

- **2025 EMBO Installation Grant**
  "*Investigation of Salmonella pathogenicity and survival*"
  Budget: 150 000 – 250 000 EUR
  Intercollegiate Faculty of Biotechnology, Poland
- **2024 OPUS26 research grant funded by Polish National Science Centre**
  "*Exploring the Impact of Salmonella on the Ubiquitin-Proteasome System*"
  Budget: 3 818 680 PLN
  Intercollegiate Faculty of Biotechnology, Poland
- **2023 POLONEZ BIS 2 research grant co-funded by the National Science Centre and the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement no. 945339**
  "Dynamics and evolution of specificity of TA complexes"
  Budget: 1 130 821 PLN
  Intercollegiate Faculty of Biotechnology, Poland

7.2. Awards and scholarships

- 2022 **Poster award** - Boston Bacterial Meeting, Boston, USA
- 2018 **Poster award** - EMBO Workshop: Bacterial persistence and antimicrobial

therapy. Ascona, Switzerland

- 2011 **Outstanding Wellcome Trust MRes Student Award** - Centenary Prize for MRes Molecular and Cellular Basis of Infection, Imperial College London, United Kingdom
- 2010 **Wellcome Trust Molecular and Cellular Basis of Infection MRes and PhD studentship** at Imperial College London, united Kingdom

I declare that I have not previously applied for the degree of habilitation.

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Applicant's signature

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