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A matter of life or death

Regulation of type I toxin-antitoxin systems in bacteria

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Abstract

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Bacteria live in constantly changing environments and face diverse stresses, including bacteriophage attacks. To survive, they must rapidly adjust their physiology through precise gene regulation. Post-transcriptional control is a central player to this adaptation, as it dictates the translation and degradation of transcripts. RNA structural elements, and small non-coding RNAs (sRNAs), often together with RNA-binding proteins, serve as key regulators of messenger RNAs (mRNAs). Among the most tightly controlled transcripts are those of type I toxin-antitoxin (T1TA) systems. Here, a self-acting toxin mRNA is produced in an inactive form, and its cognate sRNA antitoxin inhibits translation of the active transcript. Despite their importance, many regulatory and functional aspects of these systems remain unclear. This thesis aims to characterize the post-transcriptional regulation of the recently identified *Salmonella* T1TA system *timP/ timR* and to examine the role of chromosomally encoded T1TA systems in *Escherichia coli* during bacteriophage infection.

We first show that the *Salmonella timP* mRNA, unlike many type I toxin transcripts, does not undergo enzymatic processing. Instead, it is regulated through a non-canonical mechanism of 'structural processing' The active mRNA adopts a pseudoknot structure essential for translation initiation, and the antitoxin sRNA TimR binds preferentially to this active conformation, destabilizing the pseudoknot and thereby inhibiting translation. We further demonstrate that the pseudoknot enables a long-range interaction that exposes the Shine-Dalgarno sequence. An alternative interaction prevents pseudoknot formation, maintains sequestration of the Shine-Dalgarno sequence in a stable stem-loop, and inhibits translation. Finally, we show that a T1TA system encoded within an *Escherichia coli* cryptic prophage promotes bacteriophage infection in cooperation with a major bacterial immune system, revealing an unexpected role for T1TA systems in host-phage interaction.

Overall, this thesis broadens our understanding of RNA-mediated regulation by uncovering a structural RNA switch that governs toxin expression and revealing an alternative mechanism of translation initiation in bacteria. Furthermore, it expands the functional repertoire of T1TA systems by demonstrating that they can influence host-bacteriophage interactions in previously unrecognized ways.

Keywords: Gene regulation, post-transcriptional regulation, RNA-mediated control, timPR, structural switch, ralAR

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To the ones that keep dreaming.

«*Όσες κι αν χτίζουν φυλακές, κι αν ο κλοιός στενεύει,*
ο νους μας είναι αληταριό που όλο θα δραπετεύει»
Αερικό, Θανάσης Παπακωνσταντίνου

'No matter how many prisons they build, even if the noose tightens,
our mind is a trickster that will always escape'
From the song 'Aeriko', Thanasis Papakonstantinou

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **Eleftheraki, A.**, Holmqvist, E. (2024) An RNA pseudoknot mediates toxin translation and antitoxin inhibition. *Proceedings of the National Academy of Sciences of the United States of America*, 121(27): e2403063121.

II. **Eleftheraki, A.**, Escalera-Maurer, A., Hien, E.D.M., Vircilio, A., Conangle, M., Tourasse, N.J., Le Rhun, A., Holmqvist, E. (2026) An RNA structural switch controlling bacterial toxin translation. *Nucleic Acids Research,* 54(6): gkag240.

III. **Eleftheraki, A.**, Hiller. T., Petzold, S., Schreiber, V., Holmqvist, E. (2026) A prophage encoded toxin mediates phage infection. *Manuscript.*

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Abbreviations

Abi	Abortive infection
Acr	Anti-CRISPR
ATP	Adenosine triphosphate
Cas	CRISPR-associated
CDS	Coding sequence
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
FASTBAC	Functional analysis of toxin-antitoxin systems in bacteria
FMN	Flavin Mononucleotide
GTP	Guanosine triphosphate
H-NS	Histone-like Nucleoid Structuring protein
mRNA	Messenger RNA
ORF	Open reading frame
PAPI	Poly(A) polymerase I
PARIS	Phage anti-restriction-induced system
RBD	RNA-binding domain
RBP	RNA-binding protein
RBS	Ribosome binding site
RM	Restriction-modification
RNA	Ribonucleic acid
RNAP	RNA polymerase
rRNA	Ribosomal RNA
SD	Shine-Dalgarno
sRNA	Small non-coding RNA
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
TA	Toxin-antitoxin
TIR	Translation initiation region
tRNA	Transfer RNA
uORF	Upstream open reading frame
UTR	Untranslated region

Sammanfattning på svenska

Bakterier är encelliga prokaryota organismer som lever i varierande och ständigt föränderliga miljöer. De är avgörande för vår omgivning och för människors hälsa, men under vissa omständigheter kan de utgöra en hälsorisk. *Escherichia coli* (*E. coli*) är en av de mest studerade bakterierna och kan leva i många olika miljöer, bland annat i människans tarm, där den vanligtvis är harmlös och ofta nyttig. Patogena former av *E. coli* kan däremot orsaka urinvägsinfektioner och matförgiftning. På liknande sätt kan *Salmonella* orsaka matförgiftning och i allvarligare fall blodinfektioner såsom sepsis. Eftersom bakterier utsätts för ett brett spektrum av förhållanden både inuti och utanför sina värdar, måste de kunna anpassa sig snabbt för att överleva.

Bakterier anpassar sig till miljöförändringar genom genuttryck, processen där informationen i en gen omvandlas till en funktionell produkt, antingen RNA eller protein. Det första steget, transkription, kopierar DNA till RNA. Om genen kodar för ett protein kallas RNA:t för budbärar-RNA (mRNA); annars blir det ett icke-kodande RNA. Efter transkription kan mRNA översättas till protein, medan icke-kodande RNA ofta har regulatoriska funktioner. Eftersom produktion av RNA och protein kräver mycket energi, reglerar bakterier noggrant när och hur gener uttrycks. Vissa gener uttrycks kontinuerligt, medan andra aktiveras endast under specifika förhållanden. På samma sätt översätts vissa mRNA omedelbart, även under pågående transkription, medan andra förblir icke-översatta. Denna avhandling fokuserar på regulatoriska processer som verkar på RNA-nivå, mellan produktionen av RNA och dess översättning till protein.

Bakterier använder flera strategier för att reglera mRNA-uttryck. En av de viktigaste involverar små icke-kodande RNA (sRNA), som kan binda till mRNA och påverka hur snabbt de översätts eller bryts ned, och därmed hur mycket protein som produceras. Denna reglering stöds ofta av RNA-bindande proteiner (RBP), som antingen kan hjälpa sRNA eller verka oberoende. Dessa samordnade interaktioner hjälper bakterier att överleva stress. En viktig grupp stressregulatorer är toxin–antitoxin-system (TA-system). TA-system kodar för ett självverkande toxin som vanligtvis leder till celldöd, samt ett antitoxin som på olika sätt skyddar cellen från toxinet. TA-system betraktades tidigare främst som självdestruktiva element, men är numera kända för att ha bredare

funktioner, bland annat vid bakteriell infektion. En djupare förståelse för regulatoriska RNA och TA-system är fokus i artiklarna I, II och III.

Artikel I undersöker hur *Salmonella*-toxinet TimP, som ingår i ett typ I TA-system, översätts – en process som måste kontrolleras noggrant för att förhindra oavsiktlig toxinproduktion. Vi upptäckte att toxinets mRNA kan anta två olika strukturer. Endast en av dessa, som innehåller en särskild RNA-struktur kallad pseudoknut, gör det möjligt för cellens proteinmaskineri att läsa av mRNA:t och producera TimP. Den alternativa strukturen håller toxinet ”avstängt”. Vi fann att mRNA:t naturligt växlar mellan dessa två strukturer. Ett sRNA, TimR, binder specifikt till den ”på-aktiverade” strukturen och tvingar den tillbaka till ”av-läget”, vilket blockerar toxinproduktionen. Till skillnad från många andra typ I TA-system som är beroende av proteinbaserade enzymer för att aktivera eller inaktivera mRNA, styrs detta system helt av RNA-veckning. Detta avslöjar ett nytt och mycket precist sätt för bakterier att förhindra skadlig och oavsiktlig toxinproduktion.

Artikel II bygger vidare på resultaten från artikel I och undersöker hur cellen avgör när TimP ska produceras. Även om vi visste att mRNA:t kunde anta ”på”- och ”av”-strukturer var startpunkten för proteinproduktion dold, även i ”på”-formen. Med hjälp av flera avancerade metoder, inklusive storskalig mutationsanalys, upptäckte vi att *timP*-RNA styrs av en strukturell omkopplare som involverar långväga interaktioner inom RNA-molekylen. I ”på”-tillståndet förs två avlägsna regioner, inklusive pseudoknuten, samman för att helt exponera translationsstarten. I ”av”-tillståndet förhindrar en konkurrerande interaktion pseudoknuten från att bildas och håller startpunkten dold. Denna strukturella omorganisation avgör när translation kan börja. Resultaten avslöjar en oväntat komplex nivå av RNA-baserad reglering och breddar vår förståelse av hur bakterier bestämmer när specifika proteiner ska produceras.

I artikel III undersökte vi om flera typ I TA-system i *E. coli* påverkar infektion av bakteriofager (fager). Eftersom typ I-toxiner alltid finns närvarande som inaktiva mRNA som snabbt kan aktiveras, är de intressanta kandidater för snabba antivirala svar. Detta gör dem potentiellt viktiga komponenter i bakteriens försvar mot virusangripare som kallas bakteriofager. Bland de system vi testade var det endast ett som visade en tydlig effekt på faginfektion. Vi kunde spåra denna effekt till proteinet RalR, som oväntat främjade faginfektion snarare än att skydda bakteriepopulationen, vilket TA-system vanligtvis gör. Vi fann också att RalR-produktionen ökar specifikt under faginfektion, vilket tyder på att det ingår i ett reglerat svar på virusintrång. Slutligen visade vi att denna effekt är beroende av EcoKI, ett av *E. colis* viktigaste försvarssystem. Sammanlagt avslöjar våra resultat en överraskande ny roll för

typ I TA-system i interaktioner mellan bakterier och fager och utvidgar deras kända funktioner bortom stressrespons.

Sammanfattningsvis omfattar denna avhandling studier av RNA-medierad reglering i bakterier och de biologiska funktionerna hos typ I-toxiner. De upptäckter som presenteras här belyser vikten av strikt reglering av toxinexpression för att undvika oavsiktlig toxinproduktion och efterföljande celldöd. Vi har också utökat den kända funktionsrepertoaren för typ I-toxiner och visat att dessa system har roller bortom traditionella stressreaktioner. Tillsammans pekar dessa resultat på spännande möjligheter för framtida forskning som syftar till att ytterligare kartlägga komplexiteten i RNA-baserad genreglering och hur dessa mekanismer kan användas i utvecklingen av nya antimikrobiella strategier.

Περίληψη στα Ελληνικά

Τα βακτήρια είναι μονοκύτταροι προκαρυωτικοί οργανισμοί που κατοικούν σε ποικίλα και συνεχώς μεταβαλλόμενα περιβάλλοντα. Είναι απαραίτητα για το περιβάλλον μας και για την ανθρώπινη υγεία, αλλά υπό ορισμένες συνθήκες μπορούν να αποτελέσουν κίνδυνο για την υγεία. Το βακτήριο *Escherichia coli* (*E. coli*) είναι ένα από τα πιο καλά μελετημένα βακτήρια και μπορεί να ζει σε πολλά διαφορετικά περιβάλλοντα, συμπεριλαμβανομένου του ανθρώπινου εντέρου, όπου συνήθως είναι αβλαβές και συχνά ωφέλιμο. Ωστόσο, παθογόνα στελέχη της *E. coli* μπορούν να προκαλέσουν λοιμώξεις του ουροποιητικού συστήματος και τροφιμογενείς ασθένειες. Παρομοίως, η *Salmonella* μπορεί να προκαλέσει τροφική δηλητηρίαση και, σε πιο σοβαρές περιπτώσεις, σηψαιμία. Επειδή τα βακτήρια εκτίθενται σε ένα ευρύ φάσμα συνθηκών τόσο μέσα όσο και έξω από τους ξενιστές τους, πρέπει να μπορούν να προσαρμόζονται γρήγορα για να επιβιώσουν.

Τα βακτήρια προσαρμόζονται σε περιβαλλοντικές αλλαγές μέσω της γονιδιακής έκφρασης, της διαδικασίας με την οποία η πληροφορία που είναι αποθηκευμένη σε ένα γονίδιο μετατρέπεται σε λειτουργικό προϊόν, είτε RNA είτε πρωτεΐνη. Το πρώτο βήμα, η μεταγραφή, αντιγράφει το DNA σε RNA. Αν το γονίδιο κωδικοποιεί πρωτεΐνη, το RNA ονομάζεται αγγελιαφόρο RNA (mRNA) διαφορετικά, μετατρέπεται σε μη κωδικό RNA. Μετά τη μεταγραφή, το mRNA μπορεί να μεταφραστεί σε πρωτεΐνη, ενώ τα μη-κωδικά RNA έχουν συχνά ρυθμιστικούς ρόλους. Επειδή η παραγωγή RNA και πρωτεΐνης απαιτεί πολλή ενέργεια, τα βακτήρια ελέγχουν αυστηρά το πότε και πώς εκφράζονται τα γονίδια. Ορισμένα γονίδια εκφράζονται συνεχώς, ενώ άλλα ενεργοποιούνται μόνο υπό συγκεκριμένες συνθήκες. Ομοίως, κάποια mRNA μεταφράζονται άμεσα, ακόμη και κατά τη διάρκεια της μεταγραφής, ενώ άλλα παραμένουν αμετάφραστα. Η παρούσα διατριβή επικεντρώνεται σε ρυθμιστικές διαδικασίες που δρουν σε επίπεδο RNA, μεταξύ της παραγωγής RNA και της μετάφρασής του σε πρωτεΐνη.

Τα βακτήρια χρησιμοποιούν διάφορες στρατηγικές για να ρυθμίζουν την έκφραση των mRNA. Μία από τις πιο σημαντικές περιλαμβάνει τα μικρά μη-κωδικά RNA (sRNA), τα οποία μπορούν να δεσμεύουν mRNA και να επηρεάζουν πόσο γρήγορα αυτά μεταφράζονται ή καταστρέφονται, και συνεπώς πόση πρωτεΐνη παράγεται. Αυτή η ρύθμιση συχνά υποστηρίζεται

από πρωτεΐνες που προσδένουν RNA, οι οποίες μπορούν είτε να βοηθούν τα sRNA είτε να λειτουργούν ανεξάρτητα. Αυτές οι συντονισμένες αλληλεπιδράσεις βοηθούν τα βακτήρια να επιβιώνουν σε συνθήκες στρες. Μια ομάδα γονιδίων με σημαντικό ρόλο υπό στρες είναι τα συστήματα τοξίνης-αντιτοξίνης (ΤΑ). Τα συστήματα ΤΑ κωδικοποιούν μια τοξίνη που προκαλεί θάνατο του ίδιου του κυττάρου και μια αντιτοξίνη που προστατεύει το κύτταρο από την τοξίνη. Ενώ κάποτε θεωρούνταν κυρίως αυτοκαταστροφικά στοιχεία, σήμερα είναι γνωστό ότι διαδραματίζουν ευρύτερους ρόλους, συμπεριλαμβανομένων αυτών κατά τη διάρκεια βακτηριακών λοιμώξεων. Μια βαθύτερη κατανόηση των ρυθμιστικών RNA και των συστημάτων ΤΑ αποτελεί αντικείμενο των Άρθρων Ι, ΙΙ και ΙΙΙ.

Το Άρθρο Ι εξετάζει πώς μεταφράζεται η τοξίνη TimP της *Salmonella*, η οποία ανήκει στα σύστηματα ΤΑ τύπου Ι. Η μετάφραση αυτών των τοξινών πρέπει να ρυθμίζεται αυστηρά ώστε να αποτρέπεται η τυχαία παραγωγή τους. Ανακαλύψαμε ότι το mRNA της τοξίνης μπορεί να υιοθετήσει δύο διαφορετικές δομές. Μόνο μία από αυτές, η οποία περιλαμβάνει μια ειδική δομή RNA που ονομάζεται ψευδοκόμβος, επιτρέπει στο κυτταρικό μεταφραστικό μηχανισμό να διαβάσει το mRNA και να παράγει TimP. Η εναλλακτική δομή διατηρεί την τοξίνη «ανενεργή». Βρήκαμε ότι το mRNA μεταπίπτει φυσικά μεταξύ αυτών των δύο δομών. Ένα sRNA, το TimR, δεσμεύεται ειδικά στην «ενεργή» δομή και την επαναφέρει στην «ανενεργή» μορφή, μπλοκάροντας την παραγωγή τοξίνης. Σε αντίθεση με πολλά άλλα συστήματα ΤΑ τύπου Ι που βασίζονται σε πρωτεϊνικά ένζυμα για την ενεργοποίηση ή απενεργοποίηση mRNA, αυτό το σύστημα εξαρτάται αποκλειστικά από την αναδίπλωση του mRNA. Αυτό αποκαλύπτει έναν νέο και ιδιαίτερα ακριβή μηχανισμό με τον οποίο τα βακτήρια αποτρέπουν την επιβλαβή και ακούσια παραγωγή τοξίνης.

Το Άρθρο ΙΙ επεκτείνει τα ευρήματα του Άρθρου Ι διερευνώντας πώς το κύτταρο αποφασίζει πότε θα παράγει την TimP. Παρόλο που γνωρίζαμε ότι το mRNA μπορούσε να σχηματίσει «ενεργές» και «ανενεργές» δομές, το σημείο έναρξης της μετάφρασης παρέμενε κρυμμένο ακόμη και στη «ενεργή» μορφή. Με τη χρήση προηγμένων μεθόδων, συμπεριλαμβανομένης μεγάλης κλίμακας ανάλυσης μεταλλάξεων, ανακαλύψαμε ότι το mRNA *timP* ρυθμίζεται από έναν δομικό διακόπτη που περιλαμβάνει αλληλεπιδράσεις μεγάλου εύρους μέσα στο μόριο του RNA. Στην «ενεργή» κατάσταση, δύο απομακρυσμένες περιοχές RNA, συμπεριλαμβανομένου του ψευδοκόμβου, έρχονται κοντά ώστε να εκθέσουν πλήρως το σημείο έναρξης της μετάφρασης. Στην «ανενεργή» κατάσταση, μια ανταγωνιστική αλληλεπίδραση εμποδίζει τον σχηματισμό του ψευδοκόμβου και διατηρεί την αλληλουχία στην οποία ξεκινάει η μετάφραση κρυμμένη. Αυτή η δομική

αναδιάταξη καθορίζει το πότε μπορεί να αρχίσει η μετάφραση. Τα αποτελέσματα αποκαλύπτουν ένα απροσδόκητα πολύπλοκο επίπεδο ρύθμισης που βασίζεται αποκλειστικά στη δομή του RNA και διευρύνουν την κατανόησή μας για το πώς τα βακτήρια αποφασίζουν πότε να παράγουν συγκεκριμένες πρωτεΐνες.

Στο Άρθρο ΙΙΙ μελετήσαμε αν διάφορα συστήματα ΤΑ τύπου Ι τα οποία βρίσκονται στο γονιδίωμα της *E. coli* επηρεάζουν τη μόλυνση από βακτηριοφάγους (φάγους). Επειδή οι τοξίνες τύπου Ι είναι συνήθως παρούσες ως ανενεργά mRNA που μπορούν να ενεργοποιηθούν γρήγορα, αποτελούν υποψήφιους παράγοντες για ταχεία αντιιική απόκριση. Αυτό τις καθιστά ενδιαφέροντες πιθανούς μηχανισμούς άμυνας έναντι των φάγων. Από τα συστήματα που εξετάσαμε, μόνο ένα έδειξε ξεκάθαρο αποτέλεσμα στη μόλυνση από φάγους. Εντοπίσαμε ότι η πρωτεΐνη RalR, αντί να προστατεύει τον βακτηριακό πληθυσμό, όπως συνήθως συμβαίνει στα ΤΑ συστήματα, προήγαγε τη μόλυνση από φάγους. Επιπλέον, διαπιστώσαμε ότι η παραγωγή της RalR αυξάνεται ειδικά κατά τη διάρκεια της φαγικής μόλυνσης, γεγονός που υποδηλώνει ότι αποτελεί μέρος μιας ρυθμισμένης απόκρισης στην είσοδο του ιού. Τέλος, δείξαμε ότι αυτό το φαινόμενο εξαρτάται από το EcoKI, ένα από τα κύρια αμυντικά συστήματα της *E. coli*. Συνολικά, τα αποτελέσματά μας αποκαλύπτουν έναν νέο και απροσδόκητο ρόλο για τα συστήματα ΤΑ τύπου Ι στη διαμόρφωση των αλληλεπιδράσεων μεταξύ βακτηρίων και φάγων, επεκτείνοντας τις γνωστές λειτουργίες τους πέρα από την απόκριση σε στρες.

Συνοψίζοντας, η διατριβή αυτή περιλαμβάνει μελέτες σχετικά με τη ρύθμιση μέσω RNA στα βακτήρια και τις βιολογικές λειτουργίες των τοξινών τύπου Ι. Τα ευρήματα που παρουσιάζονται εδώ υπογραμμίζουν τη σημασία της αυστηρής ρύθμισης της έκφρασης των τοξινών για την αποφυγή ακούσιας παραγωγής τους και επακόλουθου κυτταρικού θανάτου. Επιπλέον, επεκτείνουμε το γνωστό λειτουργικό εύρος των τοξινών τύπου Ι, δείχνοντας ότι τα συστήματα αυτά έχουν ρόλους πέρα από τις παραδοσιακές αποκρίσεις στο στρες. Συλλογικά, τα αποτελέσματα αυτά ανοίγουν νέους και ενδιαφέροντες δρόμους για μελλοντική έρευνα που στοχεύει στην περαιτέρω κατανόηση της πολυπλοκότητας της ρύθμισης των γονιδίων μέσω RNA και στη διερεύνηση του πώς αυτοί οι μηχανισμοί μπορούν να αξιοποιηθούν στην ανάπτυξη νέων αντιμικροβιακών στρατηγικών.

Introduction

Bacteria are ubiquitous prokaryotic organisms living mainly unicellular life, in contrast to human and plant cells. The gold standard of bacterial classification is the Gram staining, a technique in which the outcome depends on the structural composition of the bacterial cell wall. Bacteria with a thick peptidoglycan layer retain the dye and are classified as Gram-positive, whereas those with a thin peptidoglycan layer and an outer membrane lose the dye and are classified as Gram-negative (Bartholomew & Mittwer, 1952). Two well-studied Gram-negative model organisms are *Escherichia coli* (*E. coli*) and *Salmonella enterica* serovar Typhimurium. *E. coli* is a commensal, opportunistic bacterium living in different environments, including the human gut, generally conferring beneficial traits and rarely causing disease. However, several pathogenic variants of *E. coli* are involved in conditions ranging from urinary tract infections to gastrointestinal disease in both humans and animals (reviewed in (Alhadlaq et al., 2024)). *Salmonella enterica* (hereafter *Salmonella*, unless otherwise specified) on the other hand, is one of the most common foodborne pathogens affecting humans and animals, and it includes more than 2600 serovars, distinguished primarily by their surface antigens. The serovar Typhimurium belongs to the non-typhoidal serovars and is a major contributor to foodborne diseases worldwide. Its pathogenicity relies on a diverse repertoire of virulence mechanisms, many of which are encoded by genes located within genomic regions called *Salmonella* pathogenicity islands. Additional virulence factors, such as the pSLT virulence plasmid, biofilm-associated proteins, flagella, and adhesins also enhance its ability to colonize and invade host tissues (reviewed in (Fàbrega & Vila, 2013; Jajere, 2019)). All three papers presented in this thesis use the laboratory strains *E. coli* MG1655 and *Salmonella enterica* serovar Typhimurium SL1344 as model organisms.

Bacteria live in ever-changing environments, facing a plethora of hazards, including temperature and pH shifts, as well as attacks by bacterial viruses (bacteriophages or phages) and the immune system of their hosts. Among the mechanisms that help bacteria withstand environmental stress are toxin-antitoxin (TA) systems. TA systems are traditionally viewed as 'suicide' elements that allow the survival of the bacterial population under extreme conditions. However, many TA systems have been reported to have implications in

biofilm formation and bacterial persistence, supporting the virulent phenotype (reviewed in (Sonika et al., 2023)). TA systems and especially type I TA systems are the focus of Papers I, II, and III.

An essential feature of bacteria is their ability to modulate gene expression in response to environmental signals, allowing them to adapt to both favorable and stress conditions. Although early studies emphasized transcriptional regulation as the main mechanism of bacterial gene control, it is now clear that the regulatory landscape is far more complex. Gene expression can be modulated at multiple levels, including transcription, post-transcriptional processing, translation, and post-translational modification.

All these regulatory mechanisms differ from those found in eukaryotes, making them good antibiotic targets. However, bacteria have also evolved a wide array of antimicrobial resistance mechanisms. They acquire genetic mutations that allow for survival in the presence of antibiotics that they were previously susceptible to. As a result, antibiotic resistance has become a global health crisis. With an estimated 5 million deaths per year associated with antimicrobial resistance in 2019 alone, it is increasingly described as a 'silent pandemic' (reviewed in (GBD 2021 Antimicrobial Resistance Collaborators, 2024; Ho et al., 2025; Hutchings et al., 2019)). A variety of research strategies aim to address the global antibiotic resistance crisis. These include studies on collateral sensitivity, where resistance to one antibiotic would result in sensitivity to another antibiotic (Chauhan et al., 2025), combinational therapy and use of phages to battle resistant pathogenic bacteria (reviewed in (Schrader et al., 2020)).

In addition to acquiring genetic mutations, for example through horizontal gene transfer, phenomena such as antibiotic persistence and antibiotic perseverance may further increase the likelihood of developing resistance. During antibiotic persistence, a small subset of genetically susceptible bacteria survives antibiotic treatment by adopting a transient dormant or slow-growing state. These 'persister' cells can withstand otherwise lethal antibiotic exposure without being resistant and subsequently resume growth once the antibiotic pressure is removed (reviewed in (Cotten & Davis, 2023; Eisenreich et al., 2022; Ronneau et al., 2021)). In contrast, antibiotic perseverance describes a scenario in which a subpopulation continues to grow despite being genetically identical to the susceptible population, similar to persister cells (Brandis et al., 2023). Nevertheless, in both cases the risk of acquiring genetic mutations leading to antibiotic resistance is increased. Ultimately, a deep and comprehensive understanding of the bacterial physiology and regulatory mechanisms is a valuable ally in the battle against antibiotic resistance (Allander et al., 2024, 2025; González-López et al., 2025; Rajer et al., 2022; Roghanian et al., 2021).

Gene regulation in bacteria

Bacteria live in ever-changing environments, facing multiple challenges. For example, pathogenic bacteria, such as *Salmonella*, encounter extreme environmental changes during host infection (reviewed in (Pradhan & Devi Negi, 2019)). In order to adapt, bacteria need to alter their cellular functions. This is mainly achieved *via* gene regulation. Gene regulation is the process by which the flow of the genetic information is controlled. According to the central dogma, the genetic information is stored in deoxyribonucleic acid (DNA), which is transcribed into messenger ribonucleic acid (mRNA). The mRNA is then translated by ribosomes into proteins that carry out numerous cellular functions (Figure 1). Notably, reverse transcription, the process by which an RNA molecule is converted into a DNA molecule has also been observed in bacteria (reviewed in (Zimmerly & Wu, 2015)). Because protein synthesis is energetically costly, gene expression is tightly regulated for energy and resources conservation. Gene regulation also allows for rapid adaptation to various environmental stresses, such as nutrient depletion, temperature or pH changes, or phage infection, by activating or repressing specific genes as needed. In addition, some cellular functions require the coordinated action of multiple cellular components. In this case, gene regulation ensures that these components are expressed simultaneously and at appropriate levels.

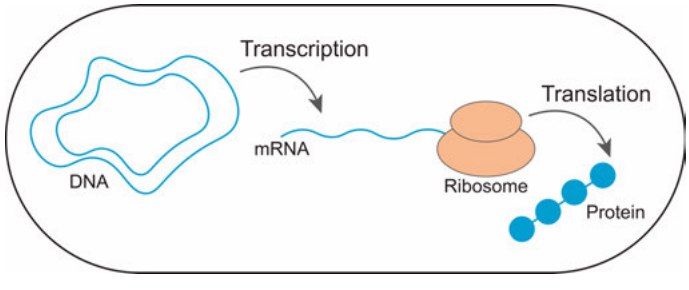

Figure 1: Schematic representation of the central dogma of genetic information flow in bacteria. The DNA is transcribed into mRNA, which is then translated by ribosomes into proteins.

All levels of gene expression, transcriptional, post-transcriptional, translational, and post-translational are strongly regulated. Transcriptional control is essential for determining which genes are transcribed under certain circumstances. It mainly includes the activity of different transcription factors (TFs), that regulate the initiation and rate of transcription. On the other hand, post-transcriptional control occurs at the mRNA level, where translation initiation and mRNA stability are regulated. Here, RNA-binding proteins (RBPs), small

non-coding RNAs (sRNAs), and RNA structural elements have central roles. Furthermore, control at the level of translation is generally describing the elongation and termination rates and efficiency. Finally, post-translational regulation determines whether a protein is modified as well as its degradation rate, modulating its function and abundancy. The focus of this thesis is mainly on post-transcriptional control. Hence, this layer of regulation will be explored more in depth.

Transcriptional control

Bacterial transcription is mainly regulated at the level of initiation, with additional layers of control acting at the elongation and termination steps. The key elements governing transcription initiation include the promoter sequence, which typically consists of the -10 and -35 hexameric recognition sites, and the transcription initiation site. RNA polymerase (RNAP) is a multi-subunit enzyme capable of binding DNA non-specifically. Hence, specific promoter recognition requires the loading of sigma (σ) factors. The binding of the holoenzyme (RNAP and associated σ factor) on the promoter represents the first transcription event.

Bacteria encode several different σ factors, each directing RNAP to a distinct set of genes. For example, in *Salmonella* and *E. coli* the dominant σ^{70} regulates the expression of most housekeeping genes during exponential growth (reviewed in (Busby & Browning, 2024; Saecker et al., 2011)). In contrast, the alternative σ factor σ^{S}, also known as σ^{38}, allows for transcriptional response to a plethora of stresses, controlling about 10% of *E. coli* genes by redirecting RNAP to their promoters. Notably, *rpoS*, encoding σ^{S}, that is having a key role during stationary growth phase, is itself under tight and multilayered regulation (reviewed in (Bouillet et al., 2024; Handler & Kirkpatrick, 2024)). Thus, σ factor-dependent recruitment of RNAP to promoters represents a central control point in bacterial transcription.

Building on this core mechanism, TFs provide an additional regulatory layer. TFs are DNA-binding proteins that directly target transcription initiation regions to control the rate of initiation. They are generally classified into two categories, global regulators that target a large set of genes, and local regulators, that target a specific gene (reviewed in (Browning et al., 2019)). For instance, LexA, is a global transcriptional repressor that silences SOS genes under favorable growth conditions. Upon DNA damage, the SOS response functions as an emergency repair system that induces the expression of genes involved in DNA repair, mutagenesis, and survival. Upon SOS response, LexA undergoes degradation, leading to de-repression of its target genes (reviewed in (Butala et al., 2009; Maslowska et al., 2019). Another example is the Histone-like Nucleoid Structuring protein (H-NS), responsible for transcriptional repression of hundreds of genes involved e.g. in virulence and

metabolism in *E. coli* and *Salmonella.* H-NS binding on its target genes leads to chromatin structural changes and repression of transcription initiation (reviewed in (Purohit, 2025)). In contrast, the *lac* operon, involved in the lactose metabolism of *E. coli,* serves as a classical example of local transcription regulation. Here, the TF LacI represses the expression of the operon by binding to a region overlapping with the promoter, the operator, and thereby preventing RNAP binding (reviewed in (Browning et al., 2019; Wilson et al., 2007)). TFs may also interact directly with the RNAP, before exhibiting their regulatory effect. For instance, DksA targets the initiating RNAP, instead of the promoter region, inhibiting rRNA transcription upon nutrient starvation (B. J. Paul et al., 2004). Interestingly, TFs can also function as activators by either binding on the promoter region and reshaping the local DNA architecture or interacting directly with RNAP to facilitate its recruitment (reviewed in (Kompaniiets et al., 2024)). AraC is a well-studied example of a TF with dual regulatory role. In the absence of arabinose, AraC occupies two distant regulatory sites, resulting in DNA looping that prevents RNAP access to the arabinose operon, essential in arabinose metabolism. When arabinose binds to AraC, it induces a protein conformational change that resolves the DNA loop and promotes RNAP recruitment, thereby converting AraC into an activator (Lee et al., 1987; Schleif, 2010).

Transcriptional pausing during elongation is also an important regulatory step in gene expression. RNAP can sense various regulatory signals, including nascent RNA structures, providing a time window necessary for regulatory decisions. For example, a transcriptional pause may enable the binding of a signaling molecule on a riboswitch aptamer (see 'Regulatory structures') or allow for loading of the ribosome and subsequent translation initiation. Four types of transcriptional pauses have been identified so far: elemental pauses that are sequence-dependent, backtrack pauses that can be triggered by DNA damage, nucleotide misincorporation or physical roadblocks, hairpin-stabilized pauses that often involve secondary structures like hairpins, or pseudoknots, and regulatory-stabilized pauses where TFs bind RNAP and modulate its kinetics (reviewed in (J. Y. Kang et al., 2019; Landick, 2021)). Importantly, several TFs directly influence RNAP during elongation. For example, the Gre proteins stimulate rapid transcript cleavage, thereby resolving long backtracking pauses, and allowing transcription to resume (Marr & Roberts, 2000; Sekine et al., 2015). On the contrary, other factors, such as NusA, enhance pausing by interacting with hairpin-stabilized paused complexes. NusA contacts both the RNAP and the nascent RNA hairpin, increasing pause stability and thus providing additional time for RNA folding, and regulatory decisions. NusA also promotes intrinsic termination by stabilizing terminator hairpins (X. Guo et al., 2018; Pan et al., 1999). Interestingly, although the TF DksA is best known for its role in transcription initiation, as described earlier,

it can also modulate elongation *via* direct binding to RNAP. However, the underlying mechanisms remain to be elucidated (Tehranchi et al., 2010; Y. Zhang et al., 2014). Transcriptional pausing plays a crucial role in ensuring correct co-transcriptional RNA folding, which in turn enables both transcriptional and post-transcriptional regulation. For instance, pausing can allow for proper termination, promote sequestration of the translation initiation region (TIR) to prevent translation during transcription, or facilitate the formation of regulatory RNA structures that permit co-transcriptional translation.

Post-translational control

Post-translational regulation allows for rapid adaptation to environmental changes without spending time and energy for *de novo* protein synthesis. Such regulation can act through several distinct mechanisms, including protein modification, allosteric regulation, proteolytic degradation, and modulation of protein folding. This way, cells can fine-tune protein function, and stability with high precision.

In bacteria, post-translational modifications affect a relatively small fraction of proteins compared to eukaryotes, and the modified proteins typically exhibit low, sub-stoichiometric modification levels (reviewed in (Macek et al., 2019)). Most of these modifications are reversible, with histidine phosphorylation being a widespread example (Potel et al., 2018). One well-characterized illustration of post-translational control as a rapid response to stress is the PhoP/ PhoQ two-component system of Gram-negative bacteria. PhoQ is a histidine kinase which detects environmental signals *via* its periplasmic sensory domain, while its transmembrane region links this domain to cytosolic regions responsible for downstream signaling. PhoQ gets activated by autophosphorylation on a histidine residue upon sensing of various cues, such as low pH or Mg^{2+} concentrations, indicative of host invasion. PhoP is subsequently phosphorylated by PhoQ at an aspartate residue, and acts as a transcriptional regulator, mainly involved in virulence (reviewed in (Omelaniuk et al., 2026)). The regulation of this two-component system is a nice example of rapid response that results in alterations of the bacterial transcriptional landscape.

Allosteric regulation, which refers to conformational changes upon effector ligand binding, is another part of the rapid bacterial response to stress. One well-characterized example is the LacI repressor of the *lac* operon. As described earlier, LacI binds to the operon's operator inhibiting RNAP binding. However, under nutrient permissive conditions, the *lac* operon is expressed. This switch is partially mediated by allosteric changes of the repressor upon binding of allolactose, an isomer of lactose. Therefore, rapid adaptation to nutrient availability and efficient lactose metabolism is achieved (reviewed in

(Wilson et al., 2007)). Similarly, binding of the alarmone (p)ppGpp, the key molecule of nutrient starvation, induces conformational changes as part of the bacterial stringent response, e.g. during amino-acid starvation. For example, (p)ppGpp binding to RNAP in *E. coli* is proposed to trigger structural alterations that reshape the transcriptional landscape. A major component of this response is reduction of ribosome synthesis (reviewed in (Hauryliuk et al., 2015)). More recent findings demonstrate that (p)ppGpp acts together with the TF DksA, allosterically potentiating its function, when in complex with RNAP, and redirecting transcription towards survival genes (Molodtsov et al., 2018). Additionally, alarmone binding can induce conformational changes within the same enzyme, as in the case of RelA (see 'Regulatory RNAs'). In the absence of (p)ppGpp, the alarmone-synthesis domain remains inhibited by the adjacent hydrolysis domain. Upon binding of (p)ppGpp, however, a conformational switch relieves this inhibition, activates the synthesis domain, and establishes a positive feedback loop in *E. coli* (Roghanian et al., 2021). On the other hand, allosteric activation can be a result of protein-protein interaction as in the case of HspQ in *Yersinia pestis*. Here, HspQ, that is also conserved in *Salmonella*, allosterically activates the Lon protease, enhancing its unfoldase activity (Puri & Karzai, 2017; Yeom & Groisman, 2019). Similarly, the heat-shock protein LarA allosterically activates Lon in *Caulobacter crescentus* (Omnus et al., 2023; H.-J. Wang et al., 2025). These regulatory shifts can reshape bacterial physiology, influencing processes such as starvation responses, biofilm formation, and virulence.

Proteolytic degradation is a key post-translational regulatory step, responsible for removing misfolded or non-functional proteins, and for selectively degrading key regulatory proteins. In bacteria, protein degradation is primarily carried out by ATP-dependent proteases. Because protein degradation is irreversible, multiple checkpoints ensure that proteolysis occurs with appropriate specificity and timing (reviewed in (Mahmoud & Chien, 2018)). One major system is the Clp protease machinery, found in both bacteria and mitochondria. The ClpX ATPase associates with the ClpP protease, and the ClpXP complex facilitates the unfolding and subsequent degradation of the target protein substrates. Importantly, loss of ClpXP in bacteria leads to various deficient phenotypes, such as impaired stress responses, biofilm formation, or virulence (reviewed in (Aljghami et al., 2022; Illigmann et al., 2021)). Recent work has further shown that deletion of ClpXP in *Streptococcus pneumoniae* causes abnormal cell wall organization and increased autolysis, expanding the regulatory repertoire of the system (Mebus et al., 2025). Another key ATP-dependent protease is Lon, conserved among prokaryotes and eukaryotes. Bacterial Lon protease governs a plethora of essential cellular processes, including

biofilm formation, proteotoxic stress response, transcription, and DNA replication (Akar et al., 2023; Ching et al., 2019; Kallazhi et al., 2025; Zeinert et al., 2020). In *E. coli,* Lon assembles into hexameric or dodecameric complexes, with the larger dodecameric form restricting access of larger proteins, adding an extra layer of selectivity (Vieux et al., 2013). While Lon does not recognize a universally conserved substrate motif, the presence of specific degradation tags can target proteins for Lon-dependent degradation (Cragan et al., 2025; Gur & Sauer, 2008a). However, Lon is well known for removing misfolded proteins by recognizing exposed hydrophobic and aromatic residues (Gur & Sauer, 2008b). Interestingly, it was recently shown that it can also act post-mortem in *E. coli*, where Lon protease released from dead cells catabolizes co-released proteins to small peptide nutrients, potentially benefiting the kin-cells (Gibson et al., 2025).

Protein folding is essential for proteins to avoid degradation and achieve their functional three-dimensional structures. Despite the notion that folding is solely inherent to the amino-acid sequence characteristics, it can be mediated by chaperones. The ATP-dependent bacterial chaperone GroEL, together with its co-factor GroES, is a well-studied example of molecular chaperones assisting post-translational protein folding. Unfolded or partially folded polypeptides with exposed hydrophobic regions bind to GroEL. Upon ATP binding, GroES associates with GroEL, capping the complex and encapsulating the substrate within a protected 'folding' cavity. Inside this isolated, hydrophilic chamber, the substrate protein can fold without the risk of aggregation. Following ATP hydrolysis, GroES dissociates, and the folded, or partially folded protein is released. This allows for proper protein function, reducing the likelihood of toxic misfolding events (reviewed in (Dupuy & Collet, 2024; Hayer-Hartl et al., 2016)).

Post-transcriptional regulation

Despite the initial assumptions that gene regulation is mainly performed at the level of transcription, it is now evident that post-transcriptional control is an essential and major step. Bacterial post-transcriptional regulation mainly affects translation initiation or mRNA degradation. Thanks to the rapid technological advances, particularly high-throughput sequencing, a plethora of elements involved in post-transcriptional control have been discovered during the past few decades, including regulatory structures, sRNAs, and RBPs (reviewed in (Hör et al., 2018)). In the past few years, complex regulatory networks have been identified for the previously assumed 'simple' bacteria. These networks have been shown to function in a highly dynamic and context-dependent manner, enabling bacteria not only to adjust expression levels

with great precision but also to rapidly reprogram cellular physiology under stress. This expanding view highlights post-transcriptional regulation as a central hub in bacterial adaptation and survival. Interestingly, accumulating evidence highlights that post-transcriptional regulation may be interconnected with transcriptional and post-translational control, creating a robust, yet flexible regulatory structure.

Translation initiation

Unlike eukaryotes where transcription and translation are physically separated in the nucleus and cytoplasm, in bacteria these two processes occur in the same cellular compartment. Therefore, co-transcriptional translation, where translation initiates at an mRNA undergoing transcription, is a common phenomenon (reviewed in (Woodgate & Zenkin, 2023)). However, translation can also start post-transcriptionally, for instance if *cis*- or *trans*-acting regulatory elements prohibit co-transcriptional ribosome binding. Nevertheless, in both cases translation initiation is the rate-limiting step of protein synthesis and thereby a major determinant of protein production in bacteria (Laursen et al., 2005). For the last 50 years, the canonical model of translation initiation dominates the field. Here, initiation is driven by the direct recruitment of the free small ribosomal subunit, 30S, to the canonical ribosome binding site (RBS) of the mRNA. The canonical RBS consists of the Shine-Dalgarno (SD) sequence and the start codon, forming the translation initiation region (TIR). Initiation is driven by base-pairing between the SD sequence and its complementary anti-SD motif located at the 3'-end of the 16S rRNA within the 30S subunit (Figure 2) (Gualerzi & Pon, 2015; Shine & Dalgarno, 1974). This interaction facilitates the binding of the initiator transfer RNA (tRNA), fmet-tRNA^{fmet}, to the start codon at the P-site of 30S, forming the 30S pre-initiation complex (Gualerzi & Pon, 2015; Shine & Dalgarno, 1974; Steitz & Jakes, 1975). The initiation factors IF1, IF2, and IF3 are also recruited to stabilize the developing complex and facilitate the start of translation. Once the 30S pre-initiation complex is assembled, IF1 and IF3 dissociate. The 50S ribosomal subunit then joins the complex, and IF2 remains bound until it has correctly positioned fmet-tRNA^{fmet} at the P-site of the initiation 70S complex. Subsequently, the first elongation event is marked by the formation of an 'initiation dipeptide' which occurs when the elongation factor EF-Tu delivers the aminoacyl-tRNA corresponding to the second codon to the A-site (reviewed in (Gualerzi & Pon, 2015)).

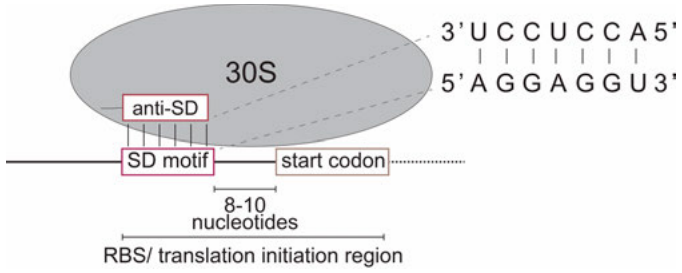

Figure 2: Graphical representation of translation initiation in bacteria. The TIR includes the SD motif approximately 8-10 nucleotides upstream of the start codon. In the canonical, SD-dependent model, translation initiation relies on the base-pairing between an accessible SD sequence within the mRNA's TIR region and the anti-SD sequence of the 16S rRNA in the 30S subunit.

However, it has become increasingly evident that alternative modes of 30S recruitment are frequently used. A growing body of evidence demonstrates that mRNA elements outside the TIR can play key roles in guiding 30S binding (see 'Regulatory structures') (Andreeva et al., 2018; Boni, 2006; Darfeuille et al., 2007; Duval et al., 2013; Jagodnik et al., 2017; Masachis et al., 2019; Skorski et al., 2006; Thisted et al., 1995). Additionally, comparative genomic studies have revealed that many bacteria, including important human pathogens, rely far less on SD-mediated initiation than previously thought (Hockenberry et al., 2018; Jha et al., 2021; McNutt et al., 2021; Nakagawa et al., 2010). Indeed, approximately one fourth of all sequenced bacterial genes lack an SD motif (Omotajo et al., 2015). One striking example are the leaderless mRNAs that can directly recruit the full ribosomal complex, 70S, and initiate translation (reviewed in (Beck & Moll, 2018)).

Beyond mRNA features, ribosomes themselves contribute to translation initiation. Although traditionally viewed as homogeneous entities (Schluenzen et al., 2000; Wimberly et al., 2000), 30S subunits are now known to contain sub-stoichiometric or conditionally assembled ribosomal proteins, potentially giving rise to sub-populations with distinct functional properties. Notably, ribosomal proteins such as S1 can act both on and off the ribosome influencing initiation (Delvillani et al., 2011; Deusser et al., 1974; Deusser & Wittmann, 1972; Moll et al., 1998; Romilly et al., 2019; Van Duin & Van Knippenberg, 1974). Moreover, variations in rRNA modification patterns have been proposed to modulate SD recognition and initiation efficiency, further expanding the regulatory repertoire (reviewed in (Shen et al., 2025)).

Regulatory structures

Bacterial mRNAs fold co-transcriptionally into secondary and tertiary structures such as stem-loops, hairpins, and pseudoknots, creating highly dynamic molecules that continuously refold during their short lifetimes (Mustoe et al., 2018). This intrinsic structural flexibility enables rapid regulatory responses. Shifts in RNA conformation, both co-transcriptionally and post-transcriptionally, can occur within milliseconds, allowing translation and decay to be adjusted quickly in response to changes in nutrient availability, temperature, or other environmental cues. In contrast to eukaryotic transcripts, bacterial mRNAs are uncapped, short-lived, and directly exposed to the translation and degradation machinery in the cytosol. These features make RNA structure a central determinant of both rapid adaptation and fine-tuning of gene expression. Within the transcript, distinct regions contribute differently to regulation. The 5' untranslated region (UTR) is a major regulatory hub, with variable length and structural complexity that controls translation initiation. Notably, growing evidence highlights the presence of *cis*-acting regulatory elements on bacterial 5'UTRs. The open reading frame (ORF), although generally less structured than UTRs, can still influence ribosome movement and mRNA stability. Finally, the 3'UTR, once considered functionally minimal in bacteria, is now recognized as an important element in modulating mRNA stability and interactions with RBPs (see 'RNA-binding proteins') (reviewed in (Chiaruttini & Guillier, 2020)).

Effect on translation initiation

RNA structural elements have a key regulatory role in translation initiation. Research findings demonstrate that stem-loop structures located within the ORF can actively promote translation initiation (Figure 3A) (Jagodnik et al., 2017; Paulus et al., 2004). For instance, in the case of the *E. coli fepA* mRNA, involved in iron uptake, this internal stem-loop is proposed to serve as a 'starting block' that helps position the 30S subunit for proper initiation (Jagodnik et al., 2017). A similar mechanism appears to operate in *bamA* mRNA, which encodes an essential outer-membrane protein involved in β-barrel assembly. Here, the ORF-located stem-loop may assist in correctly positioning the 30S subunit at the start codon, compensating for the unusually long distance between the SD motif and the start codon (Jagodnik et al., 2017). Interestingly, stem-loop structures within the ORF can also enhance translation initiation, potentially by preventing inhibitory long-range interactions between the SD motif and downstream regions, thereby maintaining SD accessibility (Paulus et al., 2004). However, in many mRNAs, the TIR is instead sequestered within stable stem-loop structures, requiring the deployment of alternative mechanisms to expose the SD sequence and enable initiation. This is the subject of Papers I and II.

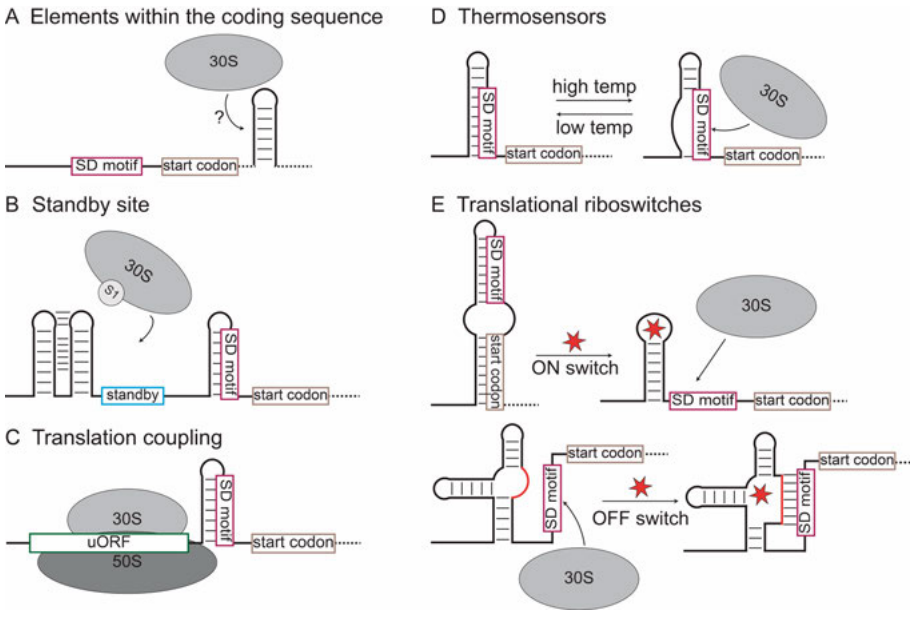

Figure 3: Effect of RNA structural elements on bacterial translation initiation. A) Structural elements located within the coding sequence can promote initiation, even when the RBS is largely unstructured. B) When the SD sequence is sequestered in a stem-loop, upstream pseudoknot structures can facilitate the recruitment of 30S at an adjacent standby site. C) Translation coupling to an upstream open reading frame (uORF) can promote initiation by helping unfold an otherwise sequestered RBS. D) Temperature-dependent structural changes can modulate the exposure of the RBS, thereby activating translation at higher temperatures. E) In translational riboswitches, ligand binding regulates access to the TIR. Ligand binding may either expose the TIR (ON state) or occlude it (OFF state).

Intramolecular structures within the mRNA can strongly hinder translation by restricting access to the TIR. Since formation of the 30S pre-initiation complex requires an unfolded RBS, the rate of initiation is inversely correlated with the stability of SD-sequestering hairpin structures (de Smit & van Duin, 1990). However, the time during which such hairpins transiently unfold is far too brief to allow for 30S binding to the RBS directly from solution. Yet, translation is observed at high rates. This paradox was solved by the standby model (Figure 3B), which was first showcased in the phage MS2 coat protein. Here, the 30S subunit initially binds non-specifically to ribosome standby sites, single-stranded regions positioned adjacent to the inhibitory hairpin, allowing the ribosome to relocate rapidly to the RBS when it transiently unfolds (De Smit & Van Duin, 1993, 2003). Over the years, the definition of standby sites has become more complicated, but here the term refers specifically to sites required to overcome inhibitory RNA structures. Standby sites have been identified in multiple bacterial mRNAs, including the *E. coli* type I toxin *tisB*

(Darfeuille et al., 2007; Vogel et al., 2004), and the *iroN* mRNA in *Salmonell*a and *E. coli*, which encodes the outer-membrane receptor of Fe^{3+}-bound salmochelin (Balbontín et al., 2016). These standby elements typically consist of 5' single-stranded sequences that can reach up to 18 nucleotides in length (Sterk et al., 2018; Studer & Joseph, 2006). In the case of *tisB*, a nearby RNA pseudoknot is recognized by ribosomal protein S1, which guides the 30S subunit to the single-stranded standby region (Figure 3B). Disruption of the pseudoknot structure abolishes S1/ 30S binding and initiation, whereas compensatory mutations that restore pseudoknot base-pairing rescue translation and toxicity, demonstrating that structure, not sequence, underlies the function (Romilly et al., 2019, 2020). The helicase-like activity of S1 (Duval et al., 2013; Lund et al., 2020; Qu et al., 2012) then assists unfolding of the previously occluded RBS, enabling translation initiation, and thereby explaining the long-distance standby site (approximately 110 nucleotides upstream of the TIR) (Romilly et al., 2019, 2020). Consistent with this, many bacterial pseudoknots show high affinity for S1 (Duval et al., 2013; Lund et al., 2020; Qu et al., 2012). Similar 5' pseudoknots are predicted in other type I TA systems, suggesting that this may be a more general strategy for translating highly structured mRNAs (Romilly et al., 2020).

Another mechanism to overcome regulatory structures is through translation coupling, where translation of a downstream ORF is tightly linked to the translation of an upstream ORF (uORF). This phenomenon is common in bacterial operons, where it ensures coordinated expression of proteins that function together, irrespectively of SD-accessibility. In overlapping ORFs, translation initiation can also be regulated by the translation rate of the uORF (reviewed in (K. M. Brown & Wade, 2025)). A recent study in *Staphylococcus aureus* highlights that slow decoding of a rare codon can cause translation pausing of the uORF and physical occlusion of the TIR of the downstream gene (Kohl et al., 2026). However, a full discussion of translation coupling falls outside the scope of this thesis. Therefore, only the cases where translation coupling assists the unfolding of inhibitory intramolecular structures and thereby promotes access to the downstream TIR (Figure 3C), are discussed here. A classic example is the expression of RepA, a protein essential for replication of the plasmid R1. The RBS of *repA* is sequestered in a stable stem-loop structure and cannot be accessed directly by the 30S subunit. Translation of the uORF *tap* disrupts this inhibitory structure, thereby unmasking the *repA* RBS and allowing initiation (Blomberg et al., 1992; E. G. Wagner et al., 1987). A similar mechanism has been observed for the type I toxin *hok*. Here, the *hok* TIR is structurally inaccessible, preventing direct 30S loading. During elongation of the uORF *mok*, translating ribosomes progressively disrupt local

RNA hairpins, exposing the occluded *hok* TIR. This structural remodeling enables coupled reinitiation on the same mRNA, ensuring that toxic Hok protein is produced only when *mok* translation has occurred (Thisted et al., 1995).

RNA may also respond dynamically to environmental cues, such as temperature shifts. RNA thermosensors, or RNA thermometers, are temperature-sensitive RNA structures, usually located in the 5'UTR of bacterial mRNAs. Although thermosensors can be grouped based on common structural motifs (e.g. fourU, ROSE, prfA-like), there is no universal classification system. These RNA elements regulate gene expression by undergoing temperature-dependent conformational changes. At low temperatures (typically at approximately 30ºC for human pathogens), RNA thermometers fold into stable hairpin structures that occlude the SD sequence or the TIR, preventing ribosome binding and thereby blocking translation initiation. As temperature increases (typically at approximately 37ºC for human pathogens), these inhibitory structures are partially or fully disrupted, exposing the TIR and enabling 30S recruitment (Figure 3D). This mechanism allows bacteria to couple gene expression directly to temperature shifts, enabling rapid response to environmental cues (reviewed in (Kortmann & Narberhaus, 2012; Loh et al., 2018; Sharma et al., 2022)). A well-characterized example is the RNA thermosensor located at the 5'UTR of *prfA*. PrfA is the master TF controlling virulence-gene expression in *Listeria monocytogenes*, thereby its expression is important upon infection conditions (higher temperatures) (Chakraborty et al., 1992; Leimeister-Wächter et al., 1990). Melting of the inhibitory structure follows two steps. The first step occurs at lower temperatures, allowing for an initial structural rearrangement that destabilizes the inhibitory hairpin. This does not yet allow for 30S binding but prepares the structure for further unfolding. Then, at higher temperatures a second, more extensive unfolding event takes place, exposing the RBS, and enabling translation initiation (Johansson et al., 2002; H. Zhang et al., 2020). On the other hand, a fourU thermometer controls the expression of the small heat shock protein AgsA in *Salmonella*. FourU elements are characterized by a conserved stretch of four uridines that base-pair with the SD sequence to repress translation at lower temperatures. Unlike the two-step mechanism of the prfA thermometer, fourU unfolds sequentially, inducing changes in stacking interactions, in a 'zipper-like' manner. Importantly, specific base-pairs within the inhibitory structure have been shown to have an inherent regulatory role in its stability (Gaetani & Zubarev, 2023; Leonarski et al., 2019; Waldminghaus et al., 2007). Together, these examples show the mechanistic diversity of RNA thermosensors, highlighting how differences in the RNA structure can influence the response in temperature. Despite their mechanistic differences, these structures enable for rapid

response to temperature shifts, controlling gene expression in different ecological niches.

Another type of structure-dependent post-transcriptional regulation is mediated by translational riboswitches. These *cis*-acting regulatory elements typically function *via* the binding of a small molecule to their aptamer, or ligand-binding domain. Ligand binding induces structural rearrangements in the expression platform (downstream region that includes the RBS) that can either result in exposing the previously sequestered TIR (ON switch) or occlusion of the TIR (OFF switch) (Figure 3E) (reviewed in (Breaker, 2018)). Riboswitches are generally named after their ligand, and numerous distinct classes have been identified so far. Some are widespread across many bacterial species, while others appear more rare (McCown et al., 2017). The Flavin Mononucleotide (FMN) riboswitch is a well-characterized class of riboswitches, that was first described in *Bacillus subtilis*. Upon binding of FMN, a riboflavin (vitamin B_2) derivative, the riboswitch modulates the expression of genes involved in vitamin B_2 biosynthesis (Winkler et al., 2002). In *E. coli*, the FMN riboswitch is located upstream of the *ribB* gene that encodes a vitamin B_2 biosynthetic enzyme. FMN binding leads to sequestration of the SD motif, resulting in translation attenuation. Hence, in high concentrations of the vitamin, its *de novo* production is inhibited. Interestingly this riboswitch can act both transcriptionally and post-transcriptionally (Pedrolli et al., 2015; Vitreschak, 2002). Further work shows that the FMN riboswitch adopts intermediate structures co-transcriptionally, which may influence ligand binding (Eschbach et al., 2024). A similar principle applies to M-box riboswitches. Unlike most riboswitches that respond to a small metabolite, M-boxes bind at least six magnesium ions, important for proper RNA folding (Dann et al., 2007; Wakeman et al., 2009). Notably, Mg^{2+} binding to the M-box located within the 5'UTR of *mgtA*, a component of the Mg^{2+} transport system in *Salmonella*, positively regulates translation of the upstream leader peptide *mgtL* by 'opening' its RBS, which in turn modulates *mgtA* transcription elongation (Cromie et al., 2006; Zhao et al., 2011). In contrast to these small-molecule responsive elements, T-box leaders are a special category of riboswitches commonly found in Gram-positive bacteria. Although they also contain an aptamer domain and an expression platform, their ligand is a tRNA rather than a small molecule. Binding of uncharged tRNA, indicative of amino-acid starvation, promotes translation initiation, whereas binding of charged tRNA signals amino-acid abundance and inhibits translation of nutrient starvation-related genes (reviewed in (Green et al., 2010; J. Zhang, 2020)). It was previously assumed that ligand binding is solely responsible for translation initiation control. However, it was recently shown that the adenine-

sensing riboswitch from *Vibrio vulnificus* requires both ligand binding and 30S ribosomal subunit binding for the stabilization of the ON switch (De Jesus et al., 2021). Nevertheless, riboswitches can affect bacterial translation in response to various environmental cues, ensuring tight regulation under non-permissive conditions.

Effect on RNA degradation

Historically considered short and functionally minimal, bacterial 3'UTRs are now known to carry important regulatory information. Many transcripts terminate with an intrinsic terminator located in their 3'UTR, which includes a hairpin structure followed by a U-stretch. This structural motif promotes release of the nascent transcript from the RNAP, and protects it from exonucleolytic degradation (reviewed in (Hui et al., 2014; Ray-Soni et al., 2016)). In Gram-negative bacteria, exonucleolytic degradation typically proceeds in 3'→5' direction. Notably, the function of the major 3'→5' exoribonucleases RNase II and PNPase is strongly inhibited by stable stem-loop structures (Spickler & Mackie, 2000). As a result, stronger terminators tend to increase mRNA stability, indirectly affecting the protein input (Ren et al., 2025). Similarly, the activity of RNase E, a major endoribonuclease in Gram-negative bacteria, can be inhibited by secondary structures (reviewed in (Mackie, 2013)). RNA structures also modulate interactions with RBPs such as Hfq, ProQ, and CsrA, which serve as global regulators of post-transcriptional control, and RNA degradation (see 'RNA-binding proteins').

Regulatory RNAs

RNA molecules, except for encoding proteins, also have central regulatory roles. For instance, tRNAs and rRNAs are essential components of the translational machinery. The tRNAs deliver amino-acids to the ribosome and decode the ORF of mRNA (reviewed in (Kirchner & Ignatova, 2015; Rodnina, 2018)). Additionally, during amino-acid starvation, uncharged tRNAs bind onto the ribosome-RelA complex, inducing a conformational change in RelA that activates (p)ppGpp synthesis, which in turn reprograms bacterial transcription, a key step in stringent response (Loveland et al., 2016). As discussed earlier, tRNAs can also serve as ligands for T-box riboswitches, where either charged or uncharged tRNA determines the regulatory outcomes (reviewed in (Green et al., 2010; J. Zhang, 2020)). On the other hand, the rRNAs constitute the core functional elements of the ribosome (Noller et al., 1992). As described earlier, rRNAs also contribute to translation initiation, as the initial recognition between the 30S ribosomal subunit and the mRNA involves pairing between the SD sequence and the 3'-end of the 16S rRNA (reviewed in (Gualerzi & Pon, 2015)).

A growing class of regulatory RNAs in bacteria is the sRNAs. Recent technological advancements have allowed for the identification and functional characterization of a plethora of sRNAs. Most sRNAs are approximately 50-300 nucleotides long, and function by base-pairing with their mRNA targets. However, their regulatory capacity is expanding in interactions with other sRNAs, RBPs, and RNases (reviewed in (Adams & Storz, 2020; Barquist & Vogel, 2015; Hör et al., 2020; Storz et al., 2011)). Many are crucial for bacterial stress responses, as integral components of regulatory networks involved in iron homeostasis, membrane stress, DNA damage, biofilm formation, and other processes (reviewed in (Georg et al., 2020; Holmqvist & Wagner, 2017)). Additionally, sRNAs are broadly classified into two categories, *cis*- and *trans*-encoded. *Cis*-encoded sRNAs are transcribed from the DNA strand opposite their target gene, resulting in perfect complementarity, and typically have a single target. In contrast, the *trans*-encoded sRNAs are found at loci separate from their targets and regulate gene expression through short, imperfect base-pairing, often influencing multiple mRNAs. In this case, the interaction is governed by a conserved sub-region of the sRNA, often referred as the seed region. Of note, some sRNAs contain only one seed region, while others carry more, each capable of base-pairing with distinct targets (reviewed in (Gottesman & Storz, 2011; Hör et al., 2020; Updegrove et al., 2015; E. G. H. Wagner & Romby, 2015)). The interaction between sRNAs and their targets may lead to a plethora of regulatory outcomes, as will be discussed in the following section.

Regulatory mechanisms of sRNAs

sRNAs can exhibit their function in various ways both transcriptionally and post-transcriptionally and typically control gene flow by perfect or imperfect base-pairing with mRNAs. They generally engage with their targets by first forming a fast, strong contact *via* a short nucleotide sequence exposed in stem-loop or single-stranded structures. Following this initial interaction, additional base pairing can develop, often accompanied by rearrangements in the RNA secondary structure (reviewed in (Storz et al., 2011)). sRNA implications in transcription are not well-studied, though a number of sRNAs have been shown to mediate Rho-dependent transcription termination, as in the case of the *rpoS* gene (Sedlyarova et al., 2016). On the other hand, sRNAs can act as sponges by sequestering other regulatory sRNAs or RBPs, preventing their regulatory role. As an example, a *Caulobacter crescentus* sRNA can remodel the gene expression during nutrient starvation, acting as a sponge to neutralize other regulatory RNAs (Vogt et al., 2025). Similarly, the sRNA OppX sequesters the sRNA MicF, known repressor of the outer membrane porin regulation mRNA *ompF* in *Salmonella*, resulting in alterations in outer membrane permeability (Matera et al., 2022). In both examples, the global RBP Hfq (see

'RNA-binding proteins') acts as a mediator. Regulatory sRNAs can also associate with other RBPs, such as ProQ, and CsrA, either because these proteins are required to facilitate proper RNA-RNA interactions (see 'RNA-binding proteins') or to alter the proteins' activity. In the latter, the sRNAs can mimic the RBP's target, titrating the protein, as a sponge, and counteracting its activity. As an example, the sRNAs CrsB and CsrC modulate the activity of CsrA (see 'RNA-binding proteins'). CsrB and CsrC accumulate upon entry into stationary phase in response to multiple nutritional stresses, thereby preventing the function of CsrA (reviewed in (Pourciau et al., 2020; Romeo & Babitzke, 2018)).

sRNAs are however most well-known for their effect on translation and mRNA stability by direct binding to an mRNA target. When sRNAs control translation, they typically base-pair to, or in close vicinity of, the TIR. This interaction prevents direct recruitment of the 30S ribosomal subunit on the RBS, thereby inhibiting translation initiation (Figure 4A) (reviewed in (Adams & Storz, 2020; Gottesman & Storz, 2011; E. G. H. Wagner & Romby, 2015). The first characterized example of this mechanism exploited by a *trans*-encoded sRNA is the pair of MicF-*ompF.* Here, the MicF sRNA sequesters the TIR of *ompF* mRNA, blocking its translation, and ultimately controlling the permeability of the outer membrane of *E. coli* (Andersen & Delihas, 1990; Schmidt et al., 1995). Notably, MicF has multiple targets responding to various environmental stresses (Carrier et al., 2023; Holmqvist et al., 2012). Similarly, the sRNA OxyS, with approximately 40 targets (Altuvia et al., 1997), binds to *fhlA* RBS blocking ribosome binding, and thereby affecting *E. coli* metabolism (Altuvia et al., 1998).

sRNA-mediated translation inhibition can also occur through interference with elements located upstream or downstream of the TIR that are essential for translation initiation. When translation of the downstream gene depends on the prior translation of a uORF (see 'Regulatory structures'), repression of uORF translation will consequently reduce expression of the downstream gene (Figure 4B). A classic example of this regulatory mechanism is the *cis*-encoded sRNA CopA of the R1 plasmid, one of the earliest characterized bacterial sRNAs. As described above, expression of the replication initiator RepA depends on translation of the uORF *tap.* When plasmid copy number increases, CopA binds to *tap*, blocking 30S ribosome access to its RBS. In the absence of *tap* translation, *repA* expression decreases rapidly, thereby preventing plasmid replication (Blomberg et al., 1992; E. G. Wagner et al., 1987). As described earlier, translation initiation may also rely on ribosomal standby sites (see 'Regulatory structures') and sRNA-mediated sequestration of such sites can inhibit 30S loading (Figure 4C). A well-studied example is the type 1 TA system *tisB/ istR-1* in *E. coli* (see 'Toxin-antitoxin systems'). Here base-

pairing of the sRNA IstR-1 with the standby site located at the 5'UTR of *tisB* mRNA inhibits toxin translation from a distance, enabling bacterial growth (Darfeuille et al., 2007; Romilly et al., 2019). In contrast, sRNAs can repress translation by destabilizing structural elements located in the ORF of their target mRNAs (Figure 4D). For instance, binding of OmrA and OmrB to a translation-activating RNA structure positioned downstream of the TIR is thought to disrupt this structure, thereby downregulating FepA synthesis (see 'Regulatory structures') (Jagodnik et al., 2017).

Despite their role in directly repressing translation, sRNAs can also activate translation by base-pairing with their target mRNAs. When the TIR is sequestered in a stable stem-loop structure, direct loading of 30S is kinetically unfavorable (see 'Regulatory structures'). In this case, base-pairing of the sRNA can also function as competitor of the RNA structure. Hence, sRNA binding to their mRNA targets in a way that prevents the formation of inhibitory secondary structures, enhances ribosome accessibility to the TIR and thereby promotes efficient translation initiation (Figure 4E) (reviewed in (Hör et al., 2020; Papenfort & Vanderpool, 2015)). This so-called anti-antisense mechanism is known to activate translation of the *rpoS* mRNA in *E. coli.* Here, binding of the sRNA DsrA leads to the liberation of the previous occluded RBS, and translation of the major stress-induced σ factor (Lease et al., 1998; Majdalani et al., 1998; Soper & Woodson, 2008). Similarly, the sRNA RprA, which is induced under environmental cues distinct from those of DsrA, also binds to the *rpoS* 5'UTR at a site overlapping the DsrA binding region and stimulates *rpoS* translation (Majdalani et al., 2001, 2002). In a similar manner, the sRNA ArcZ base-pairs with *rpoS* mRNA at the same region as RprA and DsrA, activating translation (Mandin & Gottesman, 2010). In all three cases, sRNA regulation is mediated by the RBP Hfq (see 'RNA-binding proteins').

A major component of post-transcriptional regulation is mRNA stability, which determines their abundance. Degradation of mRNAs may be regulated either positively or negatively by sRNAs (reviewed in (Adams & Storz, 2020; Storz et al., 2011; E. G. H. Wagner & Romby, 2015)). In Gram-negative bacteria, most mRNAs are degraded *via* endoribonucleolytic cleavage, mainly mediated by RNase E. RNase E recognizes single-stranded cleavage sites within mRNAs, initiating degradation. On the contrary, RNase III can cleave RNA-RNA duplexes. Following endonucleolytic cleavage, the resulting RNA fragments are typically degraded by 3′→5′ exoribonucleases such as RNase II, RNase R, and PNPase (reviewed in (Hui et al., 2014; Mohanty & Kushner, 2018)). Hence, sRNA binding to a cleavage site could restrict RNase E access, thereby stabilizing the target mRNA (Figure 4F). For example, the binding site of the sRNA RydC overlaps with the RNase E cleavage site of the *cfa*

mRNA. Therefore, binding of RydC restricts RNase access, stabilizing the target, promoting protein synthesis, and increasing membrane stability in *Salmonella* (Fröhlich et al., 2013). Similarly, SgrS sequesters the cleavage site of the RNase E-processed *pldb-yigL* transcript, inhibiting further RNase E-mediated degradation. Thus, YigL is expressed, leading to degradation of accumulated phosphorylated sugars and preventing toxicity in *Salmonella* (Papenfort et al., 2013). On the other hand, formation of an sRNA-mRNA duplex can serve as a recognition site for RNase-mediated cleavage (Figure 4G). For example, in the type I TA system *bsrG*/ *sr4* of *Bacillus subtilis*, binding of SR4 induces *bsrG*-SR4 duplex cleavage by RNase III, preventing toxicity (Jahn et al., 2012). Similarly, RatA binding on *txpA* recruits RNase III for toxin mRNA degradation (Meißner et al., 2016). Interestingly, sRNAs can also enhance the function of RNase E. In a recent study, it was demonstrated that the *E. coli* GcvB can accelerate RNase E-mediated degradation by increasing the accessibility of RNase E recognition sites (Vigoda et al., 2024). Similarly, the *E. coli* RyhB can induce rapid degradation of its target genes, that are involved in the iron metabolism, potentially by recruiting RNase E to the mRNA (Massé et al., 2003).

Nevertheless, sRNA-mediated regulation is not always clear. For example, base-pairing of the *E. coli* sRNAs OmrA and OmrB with the *csgD* mRNA (involved in biofilm formation), far upstream of the start codon, prevents ribosome access and thereby inhibits translation in a mechanism not well elucidated yet (Holmqvist et al., 2010). Similarly, RseX and ArrS inhibit the translation of *fepA via* direct interaction with structural elements located in the 5'UTR. Even though the binding seems to be base-pairing independent, the exact mechanism is still unclear (Solchaga Flores et al., 2024). On the other hand, RybB base-pairs with *ompN* coding region, downregulating its expression, and subsequently outer membrane permeability in *Salmonella* (Bouvier et al., 2008). Although, the exact mechanisms of sRNA-mediated downregulation in these cases may not be fully understood yet, the interaction is, or speculated to be, mediated by the global RBP Hfq.

Altogether, the growing body of work on sRNA-mediated regulation demonstrates that post-transcriptional control in bacteria is far more sophisticated than previously appreciated. sRNAs can act as molecular sponges, prevent or enable translation, shield transcripts from nucleases, or alternatively mark them for targeted degradation. Their actions, often coordinated with RBPs like Hfq, form complex networks that allow bacteria to rapidly sense, respond to, and survive environmental challenges. As new sRNAs, co-factors, and regulatory principles continue to be discovered, it is increasingly evident that sRNAs represent one of the central components of bacterial gene regulation.

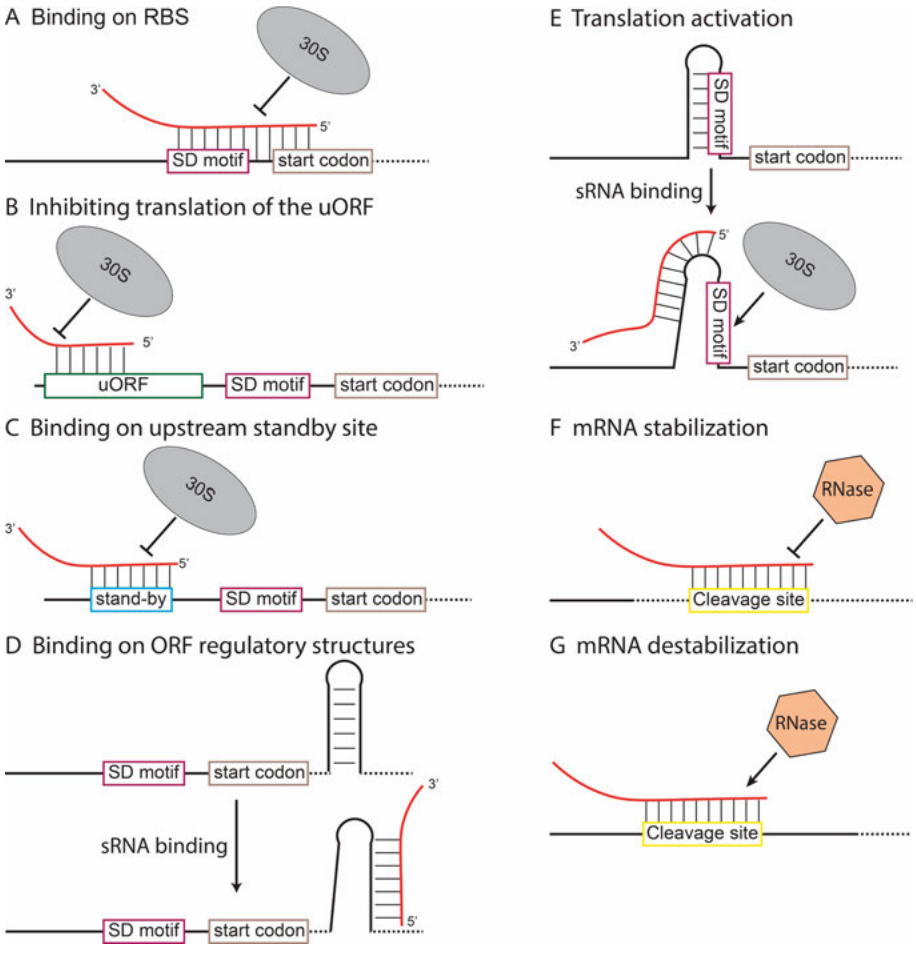

Figure 4: The interaction of sRNAs with their mRNA targets can determine the fate of the mRNA. A) An sRNA can bind to the TIR of an mRNA competing with the 30S ribosomal subunit for access to the RBS. B) When translation of the downstream gene is dependent on a uORF, sRNA binding to uORF results in translation inhibition. C) Binding of an sRNA to a standby site prevents the 30S subunit from accessing that site, thereby inhibiting translation. D) Binding of an sRNA to regulatory structures within the ORF can inhibit translation by unfolding structural elements required for translation initiation. E) sRNA binding to an inhibitory structure that sequesters the SD sequence results in disruption of the structure, allowing for direct 30S recruitment. F) RNase cleavage site sequestration mediated by sRNA binding results in mRNA stabilisation. G) Formation of an sRNA-mRNA duplex can result in recruitment of RNase, leading to targeted degradation of the mRNA.

RNA-binding proteins

RBPs are ubiquitous across all domains of life and participate in most cellular processes. They have a wide and diverse range of RNA targets, including rRNAs, tRNAs, sRNAs, and mRNAs. Some of the most evolutionary

conserved and first studied RBPs are the ribosomal proteins that play essential roles in ribosome architecture. Functionally, they maintain the correct positioning of mRNA and tRNA, and recognize structural elements within mRNAs (reviewed in (Davis & Williamson, 2017)). However, recent technological advances have allowed for global identification of novel RBPs in bacteria, expanding our understanding of their regulatory capacities (Chu et al., 2022; Holmqvist et al., 2016, 2018; Said et al., 2009; Shchepachev et al., 2019; Smirnov et al., 2016; Stenum et al., 2023). Ultimately, RBPs have emerged as key players in gene regulation, particularly in mRNA processing and stability (reviewed in (Christopoulou & Granneman, 2022; Holmqvist & Vogel, 2018; Jørgensen et al., 2020)).

RBPs are divided into two major categories: specialized RBPs, which recognize only a narrow set of RNA targets, and global RBPs, which interact with hundreds of different mRNAs. These proteins associate with RNA *via* dedicated RNA-binding domains (RBDs). Of note, classic RBDs, such as the S1 domain, cold-shock domain, and double-stranded RBD, are widespread among bacterial RBPs, highlighting their importance in gene regulation. Individual RBPs may contain one or multiple RBDs, either identical or different from one another, enabling them to bind one or several RNA targets with varying degrees of affinity and specificity. Many bacterial RBDs are relatively small, and often only a few amino acid residues make direct contact with the RNA. These domains typically recognize short RNA motifs, although some, like the ribosomal protein S1, instead preferentially bind distinct RNA secondary structures (see 'Regulatory structures') (reviewed in (Christopoulou & Granneman, 2022; Holmqvist & Vogel, 2018)).

RBPs can act either alone or in concert with regulatory RNAs to exert their function on transcription, translation or RNA degradation. At the transcriptional level, RBPs can either inhibit or promote transcription termination. In bacteria, termination occurs through two main mechanisms: Rho-dependent and Rho-independent (see 'Effect on RNA degradation'). In Rho-dependent termination, the Rho protein binds an unstructured, C-rich region of the nascent RNA, known as the Rho utilization (*rut*) site, and then translocates along the transcript until it reaches the elongating RNAP, ultimately causing its release (reviewed in (Banerjee et al., 2006; Holmqvist & Vogel, 2018)).

In addition to sRNAs and regulatory structures, post-transcriptional regulation may also occur in *trans* by regulatory proteins. A well-characterized regulator of translation in *Salmonella* and *E. coli* is the global RBP CsrA that regulates carbon usage and motility in *E. coli* as well as virulence in *Salmonella*. CsrA adopts a homodimeric structure, with the two monomers interlocking, forming a barrel-like structure that presents two RBDs. Therefore, CsrA may bind two sites on one RNA (Gutiérrez et al., 2005; Mercante et al.,

2006, 2009). CsrA regulates more than 400 mRNA targets, most often by preventing their translation, and thereby plays a central role in controlling envelope homeostasis and overall cellular growth (Holmqvist et al., 2016; Potts et al., 2017; Pourciau et al., 2023). In addition to its effects on translation, evidence also indicates that CsrA can influence transcription termination by exposing *rut* sites that promote Rho-dependent termination (Figueroa-Bossi et al., 2014). CsrA typically recognizes 5'-GGA-3' motifs located within the 5'UTRs of its mRNA targets, exposed at loops of stem-loop structures (Figure 5) (Dubey et al., 2005; Holmqvist et al., 2016). These same motifs also appear in several sRNAs, most notably CsrB and CsrC, which act as molecular sponges that sequester CsrA away from lower-affinity mRNA targets, as described earlier (see 'Regulatory mechanisms of sRNAs'). Recent work further revealed that CsrA can be compartmentalized into dynamic, membraneless intracellular assemblies in *E. coli*. These structures contain degradosome components, CsrB, CsrC, and specific mRNAs, and their formation occurs alongside a functional shift in CsrA, modulating its role between activating and repressing virulence-associated genes (Aroeti et al., 2025).

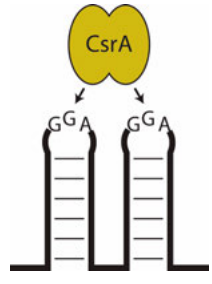

Figure 5: The CsrA-binding motif (5'-GGA-3') is shown located within the loop regions of two separate stem-loop structures. These motifs represent the primary recognition sites through which CsrA interacts with its RNA targets to regulate translation.

In *Salmonella* and *E. coli*, the global RBP Hfq assembles into a hexameric ring structure (Beich-Frandsen et al., 2011; Møller et al., 2002), and binds approximately 100 sRNAs and over 500 mRNAs. Its binding sites are found predominantly in the 5' and 3'UTRs of mRNAs and at the 3'-ends of sRNAs, though Hfq can also associate with additional regions of its sRNA targets (Chao et al., 2012; Holmqvist et al., 2016; Sittka et al., 2008; Tree et al., 2014). Hfq was originally identified as a host factor required for RNA phage Qβ replication (De Fernandez et al., 1972). Since then, it has been shown to affect phage infection more broadly by binding phage-encoded sRNAs, thereby contributing to their maturation and stability (Silverman et al., 2025), and to modulate phage spread (Pershing et al., 2026). Hfq is best known for its role as an RNA chaperone, facilitating sRNA-mRNA base pairing by engaging RNA

targets through distinct binding surfaces. Its proximal face binds U-rich sequences typically found at sRNA 3'-ends, while its distal face preferentially recognizes A-rich motifs commonly located in mRNA 5'UTRs. Additional contacts between the sRNA, mRNA targets, and basic residues on the rim help facilitate RNA-RNA pairing (reviewed in (Santiago-Frangos & Woodson, 2018; Updegrove et al., 2016)). RNA binding by Hfq and its stimulation of sRNA-mRNA pairing leads to several downstream effects. For example, Hfq facilitates the binding between an sRNA and its RNA sponge, thereby influencing the availability of regulatory sRNAs during nutrient depletion (Vogt et al., 2025). Additionally, it can repress mRNA translation, by recruiting sRNAs at mRNA 5'UTRs. This mechanism is exemplified by McaS, OmrA, and OmrB, which all rely on Hfq to bind *csgD* mRNA, prevent its translation, and thereby suppress biofilm formation in *E. coli* (Holmqvist et al., 2010; Thomason et al., 2012). Alternatively, Hfq participates in specialized regulatory pathways, such as the anti-antisense mechanism controlling *rpoS* expression (see 'Regulatory mechanisms of sRNAs'). In this process, Hfq first binds the *rpoS* 5'UTR and destabilizes an inhibitory stem-loop (Peng et al., 2014). It then recruits the sRNA DsrA, promoting base pairing that further opens the repressive structure enabling ribosome access, and ultimately activating translation (Majdalani et al., 1998; Soper & Woodson, 2008). Hfq also contributes to RNA stability control, both by protecting certain mRNAs from ribonucleases and by promoting RNA decay (reviewed in (Santiago-Frangos & Woodson, 2018)). As previously mentioned, the sRNA RydC protects *cfa* mRNA from RNase cleavage in an Hfq-dependent manner (Fröhlich et al., 2013). On the other hand, SgrS that in other cases protects from RNase E cleavage (see 'Regulatory mechanisms of sRNAs'), along with Hfq can recruit RNase E to *ptsG* in *E. coli*, enabling targeted degradation in response to phosphosugar stress (Morita et al., 2005). Finally, Hfq can further stimulate RNA turnover by enhancing poly(A) polymerase I-dependent (PAPI) polyadenylation at 3'-ends, thereby triggering subsequent degradation by exonucleases (Mohanty et al., 2004). Collectively, Hfq and its associated RNAs influence a wide array of cellular processes, including motility, virulence, stress responses, and membrane composition, highlighting its central role in bacterial post-transcriptional regulation.

Another global RBP found in *Salmonella* and *E. coli* is ProQ, a member of the ProQ/FinO-domain family (reviewed in (Olejniczak & Storz, 2017)). Initially identified in *E. coli* for its role in proline uptake (Milner & Wood, 1989), ProQ has since emerged as a major post-transcriptional regulator. In *Salmonella*, ProQ interacts with over 400 transcripts under both standard laboratory conditions and infection-relevant environments. These targets include

mRNAs involved in virulence, motility, and antibiotic persistence, showcasing its role in infection conditions (Bergman et al., 2024; Holmqvist et al., 2018; Rizvanovic et al., 2021, 2022; Smirnov et al., 2016). Structurally, ProQ consists of two globular domains, an N-terminal and a C-terminal domain, connected by an unstructured linker (Gonzalez et al., 2017). The N-terminal domain contributes to both protein stability and RNA binding, while the C-terminal domain appears to modulate regulatory activity, likely through additional RNA contacts (Rizvanovic et al., 2021; Stein et al., 2020; Zhou et al., 2025). Like Hfq, ProQ binds to terminator structures, but with distinct RNA preferences. ProQ typically recognizes stem-loop elements 5' adjacent to A-rich sequences, followed by 3' 4-9 nucleotide U-stretches, a feature that differentiates its targets from those of Hfq (Figure 6), and FinO (Holmqvist et al., 2018; Mamońska et al., 2025; Stein et al., 2020). As for other RBPs ProQ has also been shown to facilitate RNA-RNA interactions (Melamed et al., 2020) and protect from RNA degradation. For example, ProQ facilitates sRNA-dependent repression of *hupA* mRNA, which encodes a protein crucial for chromosome organization in *Salmonella*. Under standard laboratory conditions, *hupA* is expressed, and contributes to the silencing of infection-related genes through nucleoid structuring. During infection, however, ProQ-mediated sRNA repression of *hupA* translation allows for the expression of genes required for successful host colonization (Smirnov et al., 2017). On the other hand, ProQ also promotes RNA stabilization of numerous transcripts (Smirnov et al., 2016; Westermann et al., 2019). It was recently shown that ProQ can shield RNAs from degradation by protecting nascent 3'-ends from PAPI-dependent polyadenylation and subsequent exonuclease attack (Bergman et al., 2025; Holmqvist et al., 2018), a function contrasting with that of Hfq, which enhances polyadenylation-linked decay. Notably, ProQ binds predominantly to mRNAs, whereas Hfq interacts with sRNAs and mRNAs to a more equal extent (Melamed et al., 2020). This distinction suggests that ProQ governs a unique and still only partially understood regulatory repertoire, distinguishing it functionally from other global bacterial RBPs.

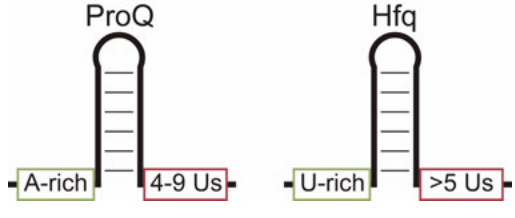

Figure 6: Comparison of ProQ- and Hfq-binding motifs at intrinsic terminators. The ProQ-recognized motif features a stem-loop structure preceded by an A-rich sequence and followed by a U-stretch. In contrast, the Hfq-binding motif includes a U-rich sequence upstream of the stem-loop and a downstream U-stretch of more than 5 Us.

Toxin-antitoxin systems

TA systems are widespread and evolutionary conserved genetic loci found on both prokaryotic chromosomes and plasmids. Notably, it has been proposed that the distribution patterns of TA systems mirror and potentially shape the structure of bacterial communities connected through horizontal gene transfer (Bethke et al., 2024). Classical TA systems consist of two components: a toxin that inhibits self-growth by disrupting essential cellular processes, and typically a labile cognate antitoxin that neutralizes the toxin or blocks its translation to protect the cell. Beyond these bipartite modules, tripartite TA systems, composed of three functional elements rather than the traditional two, have also been described (reviewed in (Harms et al., 2018; Jurėnas et al., 2022)). These tripartite systems are typically encoded by tricistronic operons within mobile genetic elements or prophages and are frequently associated with phage defense mechanisms (Bobonis et al., 2022; Mets et al., 2024). Recent work identified two such tripartite systems that protect bacterial populations upon phage infection. Under normal laboratory conditions, a dedicated chaperone stabilizes the inherently unstable antitoxin and promotes its interaction with the toxin. Upon phage entry, however, the chaperone senses the invading phage. The phage tail protein outcompetes the antitoxin for binding, leading to antitoxin displacement and rapid degradation. The liberated toxin then halts bacterial translation, thereby restricting phage propagation at the population level (Mets et al., 2024). However, the focus of this thesis will be on bipartite TA systems, which are discussed in detail in the following sections.

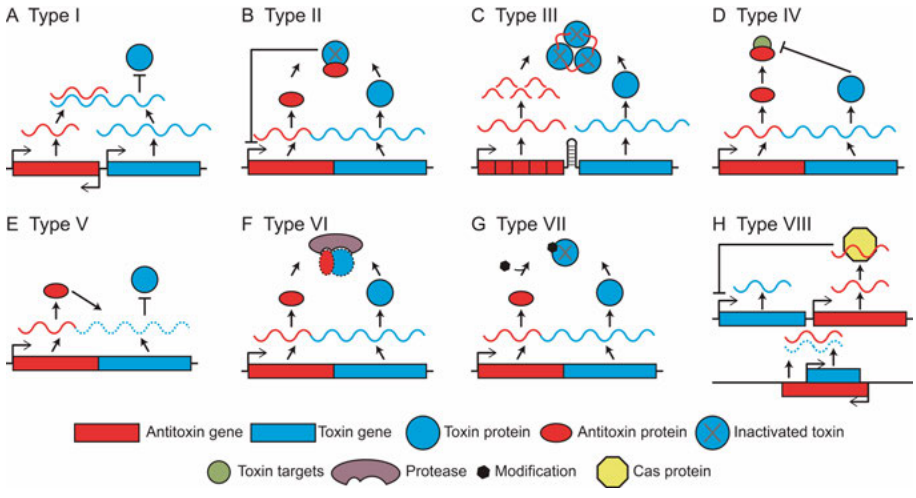

Figure 7: Main regulatory mechanisms of bipartite TA systems reported up to date. Figure adjusted from (Jurėnas et al., 2022). A) Type I TA systems use RNA antitoxins that inhibit toxin mRNA translation. B) Type II systems produce protein antitoxins that bind directly to their cognate toxins, preventing the toxins from interacting with their cellular targets, and suppressing the operon's transcription. C) Type III systems rely on RNA antitoxins, which bind to and sequester the toxin proteins, blocking their activity. D) Type IV TA systems encode protein antitoxins that do not bind the toxins themselves but instead protect the toxin targets, counteracting toxin activity. E) Type V systems include endoribonuclease antitoxins that specifically cleave the toxin-encoding mRNA, thereby preventing toxin synthesis. F) Type VI TA systems include protein antitoxins that direct the toxin proteins towards proteolytic degradation. G) Type VII systems encode protein antitoxins that neutralize their toxins through post-translational modifications. H) Type VIII systems use RNA antitoxins that contribute to the Cas-mediated transcriptional repression of RNA toxin genes, or targeted degradation of the toxin transcript, thereby reducing toxin expression.

The genes encoding TA components are usually genetically linked and arranged in operons. Advances in bioinformatic tools, such as T1TAdb (Tourasse & Darfeuille, 2021), FlaGs, (Saha et al., 2021), TASmania (Akarsu et al., 2019), and TADB 3.0 (Guan et al., 2024) have greatly facilitated the discovery of novel TA loci. In most, up to date, characterized TA systems, the toxin is a protein, whereas antitoxins may be either RNAs or proteins that counteract toxicity at multiple regulatory levels, including inhibition of toxin transcription or translation, direct interference with toxin activity, or destabilization of the toxin protein (Figure 7). TA systems are classified according to the nature and mode of action of the antitoxin. To date, nine types of TA systems (Types I-IX) have been proposed (reviewed in (Harms et al., 2018; Jurėnas et al., 2022; Seo et al., 2026)). However, this thesis will mainly focus on types I-VIII (Figure 7) (reviewed in (Harms et al., 2018; Jurėnas et al., 2022)). In type I TA systems, the antitoxin is a small unstable RNA that represses

toxin synthesis by inhibiting toxin mRNA translation (Figure 7A). These systems will be discussed in more detail later (see 'Type I toxin-antitoxin systems'). Type II TA systems encode protein antitoxins that directly bind and neutralize their cognate toxins, while they also regulate transcription of the TA operon (Figure 7B). In type III systems, structured antitoxin RNAs sequester toxin proteins, preventing their activity (Figure 7C). Type IV toxins function differently, targeting and interfering with the toxin's cellular target rather than neutralizing the toxin directly (Figure 7D). Type V antitoxins are endoribonucleases that degrade toxin-encoding mRNAs (Figure 7E), whereas type VI antitoxins direct toxin proteins to ATP-dependent proteases for degradation (Figure 7F). On the other hand, type VII antitoxins neutralize toxins through post-translational modification (Figure 7G). In type VIII systems, both the toxin and antitoxin are sRNAs. Here, the antitoxin functions either as an antisense RNA or as a Clustered regularly interspaced short palindromic repeats (CRISPR)-RNA mimic that guides a CRISPR-associated (Cas) protein to repress toxin transcription (Figure 7H) (reviewed in (Jurėnas et al., 2022; Qiu et al., 2022)). Finally, a new class of TA systems was proposed recently, type IX, where the protein antitoxin inhibits the protein toxin *via* nucleotide-based signaling (reviewed in (Seo et al., 2026)).

Type I toxin-antitoxin systems

Type I TA systems are a major class of bipartite genetic modules in which toxin expression is regulated by an antisense sRNA. These systems are widely distributed across bacteria and are tightly regulated at both the transcriptional and post-transcriptional levels, to ensure inhibition of toxin translation. The first type I TA system identified is the *hok/ sok* module, initially discovered on the R1 plasmid of *E. coli* (Gerdes, Bech, et al., 1986). Later, studies on plasmid maintenance in the Gram-positive bacterium *Enterococcus faecalis* uncovered the *fst/ RNAII* system located on pAD1 (Weaver et al., 1996; Weaver & Tritle, 1994). Soon after, multiple chromosomal homologues of *hok/ sok* were identified in *E. coli* (Pedersen & Gerdes, 1999), while several type I TA systems are found to be located exclusively on bacterial chromosomes, including within prophages (reviewed in (Shore et al., 2024)).

Although no universal transcriptional regulatory pattern exists for type I TA systems, several cases of tight regulation have been described. For example, one study demonstrated transcriptional activation of the chromosomal *hokB* toxin *via* interaction of (p)ppGpp and the GTP-binding protein Obg, although the precise mechanism remains unresolved (Verstraeten et al., 2015). Additionally, transcription of *txpA* toxin mRNA in *Bacillus subtilis* is also activated by the biofilm master regulator Spo0A (Bloom-Ackermann et al.,

2016). Other well-characterized examples include the chromosomally encoded *tisB/ istR-1*, and *dinQ/ agrB*, whose toxin promoters are controlled by LexA, the master regulator of the SOS response (Fernández De Henestrosa et al., 2000; Vogel et al., 2004; Weel-Sneve et al., 2013). In both cases, the antitoxins IstR-1 and AgrB are constitutively transcribed, while LexA represses the toxin promoters. Upon DNA damage, LexA is degraded, allowing toxin expression to override antitoxin levels and trigger toxicity (Dörr et al., 2010; Vogel et al., 2004; Weel-Sneve et al., 2013). However, leaky toxin expression persists in these systems, making post-transcriptional control essential for maintaining cellular homeostasis (Berghoff et al., 2017; Weel-Sneve et al., 2013). As a result, type I toxins are typically transcribed in inactive forms to prevent co-transcriptional translation, often through sequestration of the SD motif within inhibitory RNA structures (reviewed in (Berghoff & Wagner, 2017; Masachis & Darfeuille, 2018; Shore et al., 2024)).

As mentioned above, all type I antitoxins repress toxin production through antisense base-pairing with their cognate mRNAs. The extent of complementarity varies across systems and, in the examples studied to date, generally occurs independently of RBPs. Despite using a shared mode of recognition, type I antitoxins differ in their repression efficiency. In some cases, antisense inhibition alone is insufficient, so additional inhibitory RNA structures or RNase-mediated degradation act as additional regulatory layers (reviewed in (Berghoff & Wagner, 2017; Masachis & Darfeuille, 2018; Shore et al., 2024)). To date, five distinct modes of antitoxin regulation have been described, with RNA structure and RNA processing emerging as central determinants of toxin inhibition. Increasing evidence shows that type I TA modules rely on more complicated modes than previously assumed, affecting both toxin inhibition and toxin activation (reviewed in (Masachis & Darfeuille, 2018; Shore et al., 2024)).

The *hok/ sok* TA system of *E. coli* exemplifies such multilayered RNA-based regulation. In this system, translation of *hok* depends on translation of the uORF *mok*, whose SD sequence is initially sequestered within the nascent highly structured, translationally inactive *mok-hok* transcript. Over time, 3'-end processing of the primary *mok-hok* transcript triggers structural rearrangements that convert it into a translation-competent mRNA. In cells carrying the plasmid encoding the TA system, the antitoxin sRNA, Sok, binds a U-turn motif exposed in the processed transcript, preventing translation of *mok* and consequently *hok*. The resulting RNA duplex is then cleaved by RNase III and degraded. Thus, *hok* regulation relies on two layers of control: an inhibitory secondary structure that renders the full-length transcript inert and the antitoxin Sok that neutralizes the processed form (reviewed in (Gerdes & Wagner, 2007)). Together, these structural transitions and antisense

interactions maintain precise control over *hok* expression and prevent accidental toxin activation.

Beyond transcriptional control, the chromosomal *tisB* toxin of *E. coli* also undergoes tight post-transcriptional regulation. The full-length *tisB* transcript is inactive and requires 5'-end enzymatic processing to become translationally competent. Even then, the SD sequence remains occluded, making translation dependent on an upstream ribosome standby site. The ribosomal protein S1 recognizes a pseudoknot adjacent to this single-stranded site, and *via* its helicase-like activity, promotes opening of the SD sequence (Darfeuille et al., 2007; Romilly et al., 2019, 2020). However, the antitoxin, IstR-1, targets the ribosome standby site, preventing *tisB* translation. The resulting duplex is subsequently cleaved by RNase III, ensuring efficient toxin repression (Vogel et al., 2004). Thus, similar to *hok*/ *sok*, the *tisB*/ *istR-1* locus employs multilayered post-transcriptional control to allow toxin activation only under defined stress conditions.

The chromosomally encoded *aapA1*/ *isoA1* system of *Helicobacter pylori* follows a distinct but related regulatory strategy. Initially, *aapA1* transcription produces a full-length mRNA whose SD sequence is sequestered within a stable stem-loop structure. Using the FASTBAC-Seq (Functional analysis of toxin-antitoxin systems in bacteria) genetic selection method, single-nucleotide mutations were identified that suppress toxin-induced lethality by stabilizing SD-sequestering hairpins. These transient structures form co-transcriptionally and prevent premature translation (Masachis et al., 2019). Subsequent 3'-end processing generates a shorter RNA that undergoes structural rearrangements to become translation-competent. However, this new conformation also exposes two stem-loops that mediate kissing-loop interactions with the antitoxin, IsoA1, forming a duplex that sequesters the RBS and triggers RNase III-mediated degradation (Arnion et al., 2017). The *fst*/ *RNAII* system of *Enterococcus faecalis,* though non-processed follows a similar type of regulation, with RNAII sequestering *fst* RBS, and thereby inhibiting translation, while protecting the duplex from degradation (reviewed in (Weaver, 2012)).

For the systems presented above, the antitoxin inhibits translation by physically blocking recruitment of the 30S ribosomal subunit. However, some antitoxins induce structural rearrangements that sequester the RBS, thereby inhibiting translation indirectly. For example, the *dinQ* primary transcript is translationally inert due to SD motif sequestration. Upon 5'-end enzymatic processing, the resulting transcript adopts a conformation that exposes the SD for 30S binding. This structural switch is governed by alternative interactions among four sequence elements in *dinQ* mRNA. The processed form is also competent for AgrB binding, which restores SD sequestration and leads to

RNase III-mediated degradation of the *dinQ*-AgrB duplex (Kristiansen et al., 2016; Weel-Sneve et al., 2013). Similarly, in the *Bacillus subtilis* prophage-encoded *bsrG/ sr4* system, binding of Sr4 strengthens sequestration of the already-occluded SD sequence, again promoting RNase III-dependent decay (Jahn et al., 2012; Jahn & Brantl, 2013).

For all the systems presented above, antitoxin binding results in inhibition of 30S recruitment, either directly or indirectly. In contrast, in the *txpA/ ratA,* the first chromosomally encoded type I TA system identified in *Bacillus subtilis* (Silvaggi et al., 2005), antitoxin binding does not structurally alter the toxin's TIR. Instead, binding of RatA to the 3'-end of *txpA* mRNA forms a duplex that is rapidly cleaved by RNase III, an event essential for toxin suppression. Notably, RatA binding does not induce structural rearrangements in the TIR of *txpA* mRNA. However, it has been proposed that *txpA*'s unusually strong SD sequence may itself limit translation efficiency, since the ribosome may struggle to move beyond the SD into elongation (Durand et al., 2012).

As described above, many type I toxin mRNAs become translationally active only after a 5'- or 3'-end processing step, triggering structural rearrangements that expose the RBS and allow 30S subunit recruitment, or antitoxin binding. Yet, for several type I toxins, such as the chromosomally encoded *timP* (Andresen et al., 2020), and the pAD1 encoded *fst* (Weaver, 2012)*,* no such processing event has been detected, suggesting that alternative mechanisms enabling the transition to a translation-competent mRNA remain to be discovered. Interestingly, in the case of *timP*/ *timR*, the antitoxin, TimR, binds approximately fifty nucleotides upstream of the RBS, inhibiting translation probably *via* an alternative mechanism (Andresen et al., 2020). Post-transcriptional regulation of the *Salmonella* chromosomally encoded *timP*/ *timR* is the focus of Papers I, and II.

Type II-VIII toxin-antitoxin systems

Type II TA systems are considered the most prevalent and diverse TA family, found in the genome of the majority of bacteria and in several archaeal species (Leplae et al., 2011; Makarova et al., 2009; Pandey & Gerdes, 2005; Xie et al., 2018). Notably, free-living bacteria typically encode more type II systems than obligate intracellular organisms, a trend that may reflect the greater chromosomal stability of intracellular species (Pandey & Gerdes, 2005). This is consistent with the observation that roughly one-third of all type II loci are associated with mobile genetic elements (Guan et al., 2024). Type II modules are usually arranged as operons in which the toxin gene precedes the antitoxin gene (reviewed in (Chan et al., 2023)). Their transcription is tightly autoregulated, typically *via* a negative feedback-loop. Antitoxins are generally

translated more efficiently than toxins, ensuring a molar excess that fully neutralizes the available toxin pool. Most type II antitoxins contain a DNA-binding domain that allows them to also repress transcription at the operon's promoter. Although these domains bind DNA with relatively low affinity, the formation of TA complexes enhances promoter binding and strengthens transcriptional repression. Once toxin levels rise and saturate the complex, the regulatory assembly dissociates from the promoter, lifting repression and restoring antitoxin synthesis (Cataudella et al., 2012; Chan et al., 2023; Fraikin et al., 2020). In other cases, where the toxin gene is encoded upstream of the antitoxin, the toxin can displace the antitoxin from the promoter, creating a stable complex that inactivates toxin activity. For example, in the case of *msqR*/ *msqA*, the antitoxin MsqA acts as a transcriptional repressor. In the presence of MsqR, MsqA is displaced from the promoter, forming a strong complex with the toxin. This way, it is ensured that under certain conditions transcription is enabled, and toxin may exhibit its function (B. L. Brown et al., 2013). Post-transcriptional regulation also contributes to the control of type II systems. For example, at the *kid*/ *kis* system (*parD* stability system of the R1 plasmid in *E. coli*), limited degradation of the polycistronic *parD* favors antitoxin accumulation, and coupled translation ensures that Kis levels exceed those of the toxin Kid during steady-state growth (Ruiz-Echevarría et al., 1995). The signals that trigger toxin activation remain incompletely understood. While some studies suggest that stress-induced Lon-mediated antitoxin degradation liberates active toxin, conflicting findings across systems indicate that activation cues vary and likely must be assessed individually (reviewed in (Chan et al., 2023; Fraikin et al., 2020)).

Type III TA systems have a similar operon organization as type II loci, with the antitoxin gene located upstream of the toxin. A distinguishing feature of these systems is the presence of Rho-independent terminators separating the antitoxin gene from the toxin ORF. This arrangement may restrict toxin expression while promoting continuous production of the labile antitoxin (reviewed in (Goeders et al., 2016; Jurėnas et al., 2022)). A second regulatory safeguard arises from the processing of the antitoxin sRNA into multiple repeated units, each capable of neutralizing two or three toxin molecules (Figure 7C). This processing maintains a favorable antitoxin-to-toxin ratio and ensures tight control of system stoichiometry (reviewed in (Goeders et al., 2016; Jurėnas et al., 2022)). For instance, two CptI antitoxin monomers neutralize the CptN toxin by forming a heterotetramer (F. Rao et al., 2015). On the other hand, at the *toxN*/ *toxI* system, ToxI monomers associate with three ToxN monomers to form a heterohexamer inhibitory complex (Blower et al., 2011; Short et al., 2013). The ToxI-ToxN complex has also been proposed to repress

transcription from its operon promoter, providing autoregulation similar to that observed in type II TA systems (Blower et al., 2009). Expression of type III toxins has been suggested to be the result of degradation of the labile antitoxin (Guegler & Laub, 2021; Short et al., 2018). Type IV TA systems are likewise encoded in operons, but both components are proteins. The first such system identified, *E. coli cbtA*/ *cbeA*, revealed a toxin, CbtA, that inhibits polymerization of the cytoskeletal proteins FtsZ and MreB, disrupting cell morphology and growth. Unlike type II antitoxins, CbeA does not bind the toxin directly. Instead, it neutralizes toxicity indirectly by enhancing the bundling and stabilization of FtsZ and MreB filaments, thereby shielding these cytoskeletal targets from CbtA activity (Masuda et al., 2012).

Types V, VI, VII, and VIII are comparatively rare within the broader TA system landscape. To date, only a single type V system has been experimentally validated, although additional candidates have been predicted through bioinformatic analyses (Guan et al., 2024). The *ghoS*/ *ghoT* operon encodes two small proteins: the toxin GhoT and its antitoxin GhoS. Unlike antitoxins in types II or III, GhoS does not bind its toxin. Instead, it neutralizes GhoT by specifically cleaving the *ghoT* mRNA, thereby preventing toxin synthesis (X. Wang et al., 2012). Similarly, only one type VI TA module has been characterized so far, *socB/ socA* of *Caulobactercres centus*. This operon follows the typical TA architecture but differs in that the toxin SocB is less stable than its antitoxin. The antitoxin SocA binds SocB and targets it for degradation by the ClpXP protease complex, ensuring that toxin levels remain suppressed under normal conditions (Aakre et al., 2013). Type VII systems are slightly more diverse, with representatives like, *tgiT-menT3*/ *takA-menA3*, *hha*/ *tomB*, and *hepN*/ *mnt*. In all cases, toxin neutralization is achieved through post-translational modification catalyzed by the antitoxin (reviewed in (Sonika et al., 2023)). For example, the antitoxin TakA, a protein kinase, specifically phosphorylates its cognate toxin, thereby neutralizing its activity (Yu et al., 2020). Finally, type VIII TA systems are unique because both the toxin and antitoxin are RNAs. Only two examples have been described so far, and they operate through different mechanisms. In *E. coli*, the *sdsR*/ *ryeA* locus encodes the toxin sRNA SdsR and its antisense antitoxin RyeA, which are transcribed from opposite DNA strands. RyeA binds along the full length of SdsR, forming a duplex that is subsequently cleaved by RNase III, resulting in the simultaneous degradation of both RNAs and preventing toxicity (Figure 7H) (Choi et al., 2018). In contrast, the *creT*/ *creA* system of *Haloarcula hispanica* is embedded within a CRISPR/ Cas locus (see 'The CRISPR/ Cas system'). Here, the antitoxin CreA mimics a CRISPR RNA and recruits the Cascade

complex to repress transcription of the CreT toxin gene by binding to a partially complementary promoter sequence (M. Li et al., 2021).

Recently, TA systems with unique characteristics have been identified, highlighting that TA biology is more diverse and mechanistically flexible than previously appreciated (reviewed in (Sonika et al., 2023)). An interesting example of the TAs diversity is the recent discovery of the Panacea antitoxin domain that can pair with different toxins, overturning the assumption that one antitoxin neutralizes only one toxin (Ernits et al., 2023; Kurata et al., 2022). Together, the mechanistic diversity of the TA systems highlights the importance of tight toxin regulation to avoid accidental production of a toxin that will result in self-bacterium cell death. While the canonical bipartite systems remain the best understood, the expanding catalogue of less common TA types illustrates how bacteria have repeatedly repurposed these modules for finely tuned regulation, defense, and survival. Continued characterization of these systems, especially the rare and unconventional types, will not only deepen our understanding of microbial regulatory networks but may also uncover new molecular strategies for controlling bacterial growth, stability, and interactions within microbial communities.

Biological functions of toxin-antitoxin systems

TA-encoded toxins are typically small proteins that exert their function either in the cytoplasm or at the inner membrane of the host bacterium. Type I TA systems usually encode small membrane-acting toxins that impair cell envelope integrity, as exemplified by Hok, IbsC, ShoB, TisB, and TimP (Andresen et al., 2020; Fozo et al., 2008; Gerdes, Bech, et al., 1986; Gerdes, Rasmussen, et al., 1986; Unoson & Wagner, 2008; Weel-Sneve et al., 2013). Under standard laboratory conditions, deletion of a type I antitoxin often has no obvious phenotype, and toxicity is typically revealed only upon toxin overexpression, indicating their role as stress response elements. Type II toxins are categorized into nine superfamilies based on their structural characteristics. Toxins belonging to the same superfamily may have different modes of action, though most of them directly target core cellular functions, like DNA replication, and translation. Type III toxins, in contrast, are generally endoribonucleases (reviewed in (Qiu et al., 2022; Sonika et al., 2023)). To date, only a few type IV TA systems have been characterized. As described earlier, the toxin CbtA inhibits the polymerization of the cytoskeletal proteins FtsZ and MreB, ultimately disrupting cell shape, halting DNA replication and cell growth (Masuda et al., 2012). On the contrary, the only known type V toxin is a small hydrophobic protein that lyses cells by disrupting the cell membrane, forming ‘ghost’ cells, like Hok (X. Wang et al., 2012). The type VI toxin SocB blocks

replication elongation and triggers an SOS response in *Caulobacter crescentus* (Aakre et al., 2013). Type VII TA systems exhibit slightly different and sometimes conflicting modes of action. For example, the toxin encoded by *Rv1045* (TgIT or MenT3) has been reported to inhibit protein synthesis by preventing tRNA charging through addition of nucleotides to the 3'-CCA acceptor stem in a GTP-independent manner (Cai et al., 2020). However, a study published the same year, suggests that TgIT requires GTP to most likely target genes involved in translation (Yu et al., 2020). Expression of the type VIII toxin CreT sequesters the rare $tRNA^{Arg}$, impairing translation of essential genes and ultimately leading to growth arrest (M. Li et al., 2021). As described above, toxins encoded within TA loci are typically inducing cell death or growth arrest. Notably, the type II toxin MazF challenges this notion, since recent work shows that physiological MazF levels contribute to genome stability and stress recovery (Fleurier & Matic, 2025).

In many of these systems, toxicity is observed upon toxin overexpression. However, even in cases where toxicity is observed upon antitoxin deletion, the environmental signals that activate toxin expression remain poorly understood.

Toxin-antitoxin systems as addiction elements

The discovery of the TA systems originated from studies on plasmids, where these modules were identified as mediators of plasmid inheritance. Characterization of the first two systems, *hok/ sok* (Type I), and *ccdB/ ccdA* (Type II) led to the establishment of the post-segregational killing model, describing how daughter cells that fail to inherit the plasmid are eliminated (Gerdes, Rasmussen, et al., 1986; Ogura & Hiraga, 1983). Because cell survival depends on retaining the plasmid carrying the TA genes, this mechanism became widely referred to as 'plasmid addiction'. Chromosomally encoded TA systems may though counteract plasmid addiction by neutralizing plasmid-encoded toxins. For instance, the chromosomally encoded antitoxin of the *ccd* system in *Dickeya dadantii* protected the bacteria from post-segregational killing caused by a plasmid-encoded TA system (Saavedra De Bast et al., 2008). Nevertheless, a growing body of studies highlight the roles of TA systems in an extensive repertoire of bacterial processes, including defense against phages, persistence and virulence. Although they are widely described as stress response elements, their biological roles remain controversial.

Effects of toxin-antitoxin systems in virulence

Bacterial TA systems contribute to intracellular survival and virulence by helping pathogens withstand stress and adapt to the host environment. For example, in *Salmonella*, deletion of the type II toxin *hha* results in reduced colonization of the infected mice (P. Paul et al., 2022). Similarly, deletion of three

mazF homologues from *Mycobacterium bovis* attenuates infection in guinea pigs (Tiwari et al., 2015). In contrast, deletion of *mazF* in *Staphylococcus aureus* conferred the opposite effect, promoting bacterial virulence (Ma et al., 2019). In another example, the plasmid-encoded type II toxin PumA from *Pseudomonas* aeruginosa affects virulence in a quorum-sensing dependent manner. In the absence of autoinducers, *pumA* expression is low and no toxin-dependent phenotype is observed. Addition of exogenous autoinducers restores *pumA* mRNA levels, toxin activity, and virulence demonstrating how quorum sensing can directly regulate a TA module to time virulence based on population density (Hernández-Ramírez et al., 2020).

TA systems also influence virulence indirectly by modulating biofilm formation and persister cell development. For example, MazF inhibits biofilm formation in *Staphylococcus aureus* (Ma et al., 2019). Additionally, deletion of the *yoeB*/ *yefM* and *relE*/ *relB Streptococcus pneumonia* type II TA systems severely impairs biofilm formation (Chan et al., 2018), a phenotype observed also for the *prrT /prrA* system in *Pseudomonas aeruginosa* (Shmidov et al., 2022). Interestingly, deletion of *yoeB*/ *yefM* resulted in increased biofilm formation in *Edwardsiella piscicida* (Ma et al., 2021), a contradictory phenotype that has also been observed for MazF. Type I TA systems can similarly influence these processes. For instance, *timP/ timR* has been suggested to affect biofilm formation and virulence in pathogenic *E. coli* strains (Bessaiah et al., 2021; Ranjith et al., 2019). Additionally, overexpression of the type I toxin TxpA hinders biofilm formation and lyses pre-established biofilms, suggesting a role in removing defective cells no longer needed in the biofilm community (Bloom-Ackermann et al., 2016).

The plasmid encoded Hok was initially shown to create ‘ghost’ cells, resulting in post-segregational killing. However, its chromosomal counterpart *hokB* contributes to persister cell formation by inducing membrane depolarization during stationary phase (Verstraeten et al., 2015). Chromosomal deletion of *tisB* similarly reduces persister levels following ciprofloxacin treatment, highlighting the role of type I toxins in persistence (Berghoff et al., 2017; Dörr et al., 2010). Type II TA systems have been particularly well studied in this context. In *Salmonella*, multiple type II modules are activated within the acidic, nutrient-limited *Salmonella*-containing vacuole, rapidly generating non-replicating persisters (Helaine et al., 2014). For example, type II acetyltransferases may halt bacterial translation, and ultimately drive *Salmonella* into a persistent state (Cheverton et al., 2016; Rycroft et al., 2018). Finally, low levels of the type V toxin GhoT also promote persister formation through membrane damage (X. Wang et al., 2012).

Effects of toxin-antitoxin systems in phage defense

Early studies suggested that TA systems may act as phage-defense elements. Activation of certain toxins triggers killing of the infected bacterium, ultimately protecting the population (see 'Abortive infection') (Guegler & Laub, 2021) (Pecota & Wood, 1996). However, not all toxins necessarily lead to bacterial cell death. For example, the DarT toxin ADP-ribosylates the phage DNA, inhibiting phage DNA replication in *E. coli* (Jankevicius et al., 2016). Antitoxins can also affect phage infection directly. For example, the type II antitoxin CrlA in *Pseudomonas aeruginosa* can inhibit phage infection, potentially by binding phage DNA through its DNA-binding domain (Ni et al., 2022). Phages can also exploit TA systems to modulate their own infection cycle. A recent study revealed that the type II *mazE/ mazF* system suppresses lytic propagation of arbitrium-encoding phages, which use quorum-sensing-like signals to choose between lytic and lysogenic cycles (see 'Bacteriophages'). The phage-encoded protein YopM engages MazE, allowing MazF to exert its function, and ultimately promote lysogeny over lysis (Cui et al., 2022). The effect of type I TA systems in phage infection is the focus of Paper III.

Bacteriophages

Bacteriophages (phages) are viruses that specifically infect bacteria, hijacking their cellular machineries. They are commonly classified based on their infection cycle, morphology, taxonomy, supergroup, or 'Baltimore classes' (Baltimore, 1971; Koonin et al., 2021; Valencia-Toxqui & Ramsey, 2024). Phages can follow either a lytic or a lysogenic lifestyle (described as temperate phages as well). Lytic phages are strictly virulent, taking control of the host machineries to produce all the viral components important for their survival. The resulting progeny phages are released from the bacterial cells upon lysis (Figure 8A). In contrast, the temperate phages can choose between the lytic cycle and lysogeny. During lysogeny, the phage genome is integrated in the bacterial chromosome as a prophage. This prophage is replicated together with the host genome under conditions that promote bacterial growth and chromosome maintenance. Upon stress conditions that threaten host viability, prophages can be induced, switching to lytic cycle, ensuring viral propagation and survival (Figure 8B) (Brady et al., 2021). Prophages can become cryptic, losing their ability to enter the lytic cycle and produce new viral particles. Cryptic prophages can be selectively maintained in the bacterial chromosome, providing evolutionary advantage to the host (Howard-Varona et al., 2017; X. Wang et al., 2010). Phages, like all viruses, can be categorized by morphology. The two major groups are tailed, and non-tailed phages. Tailed phages can be

further classified into short-, contractile-, or flexible-tailed. On the other hand, non-tailed phages can form pleomorphic, filamentous, or polyhedral (occasionally enveloped) virion particles (Valencia-Toxqui & Ramsey, 2024). Sequence-based comparisons offer yet another classification, grouping phages phylogenetically according to whole-genome nucleotide similarity (Valencia-Toxqui & Ramsey, 2024). Finally, the 'Baltimore' classification, originating about 50 years ago, categorizes viruses into six classes, based on nucleic acid type and replication strategy. These classes are: double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA), positive-sense single-stranded RNA (ssRNA), negative-sense ssRNA and the reverse transcribing RNA viruses (Baltimore, 1971). Additional classes have since been described, including dsDNA viruses that rely on an RNA intermediate and reverse transcription for efficient replication (Koonin et al., 2021).

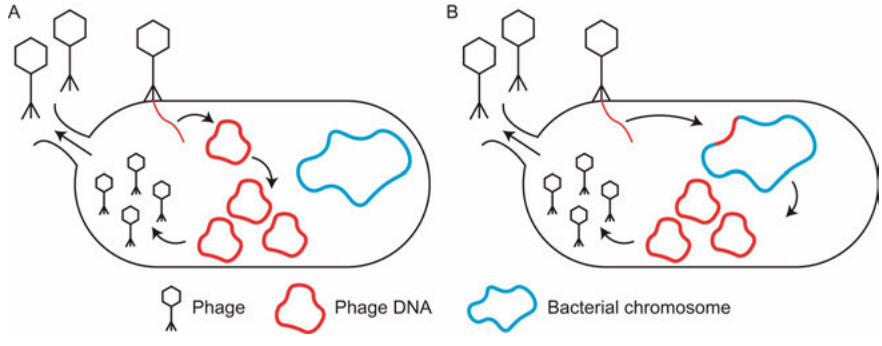

Figure 8: The life cycle of bacteriophages. A) The lytic phages infect the bacterial cell, where the phage DNA circularizes separately from the host DNA. The phage DNA replicates, and new phage particles are assembled. Subsequently, the bacterium lyses, releasing the progeny virions. B) Temperate phages infect the bacterial cell and integrate their genome into the bacterial chromosome, where they remain in a dormant, prophage state. When conditions become favorable or the host experiences stress, the prophage DNA is excised from the chromosome. It is then replicated and new virions are produced. Finally, the bacterium undergoes lysis, releasing the new phage particles.

The phage genes are typically organized into early, middle, and late, allowing the infection cycle to be fine-tuned for maximum efficiency and evasion of host defenses. Gene expression in DNA phages is mainly regulated at the level of transcription (Chang et al., 1995; Pavlova et al., 2012; Yang et al., 2014), whereas RNA phages regulate gene expression at the level of translation (Duin, 1999; Poot et al., 1997). The DNA phages can either reprogram the host RNAP or encode their own RNAP to ensure efficient gene transcription. Even when phages encode their own polymerase, they can still rely on the host RNAP for transcription of early genes. Alternatively, phages that depend

entirely on host machinery can modify its function *via* early-expressed RNAP-altering factors. In both cases, expression of middle and late genes is orchestrated by the regulatory events of the early gene expression (Yang et al., 2014). In contrast, RNA phages typically rely on RNA inhibitory structures located within the middle and late genes, inhibiting ribosome access to the translation initiation site. Hence, expression of these genes is strictly dependent on translation of the early genes, which relieves these secondary structures, exposing downstream ribosome binding sites (Duin, 1999). Gene expression can be further regulated *via* phage-encoded sRNAs. Some lytic genes can be repressed by phage-encoded sRNAs, contributing to maintenance of the prophage within the bacterial population (Blasdel et al., 2017; Bloch et al., 2023; Nejman-Faleńczyk et al., 2015). On the other hand, phage-encoded sRNAs can also facilitate the entrance to the lytic cycle by hijacking the host regulatory machineries (Silverman et al., 2025; Sprenger et al., 2024).

The interplay between bacteria and phages

With an estimated number of 10^{31} phages on Earth, they could vastly outnumber the bacterial population across different environmental niches (Hendrix et al., 1999; Mushegian, 2020). Given their high abundance and narrow host-range specificity, phage-host interactions are often described as an evolutionary arms race, where bacteria and phages constantly adapt to outcompete one another for survival and replication (Hampton et al., 2020; Stern & Sorek, 2011; S. Zhang et al., 2025). This constant battle imposes strong evolutionary pressure on both organisms, driving rapid genomic changes. Recent metagenomic studies have provided deeper insight into this co-evolutionary landscape. Bacteria must constantly protect themselves against phage infection, implementing a repertoire of defense systems (Georjon & Bernheim, 2023). Likewise, the phages encode an extensive anti-defense arsenal to overcome bacterial immunity, while phage-encoded sRNAs can influence regulatory processes on both the phage and bacterial sides (Lewandowska et al., 2025; Murtazalieva et al., 2024).

Defense systems

As in mammals, the bacteria have evolved immunity to defend themselves against parasitic organisms like phages. Their innate immunity provides rapid, non-specific, sequence-independent protection. Contrary, adaptive immunity offers slower, highly-specific and heritable defense against previously encountered phages (Ledvina & Whiteley, 2024). The anti-phage arsenal, though previously considered to be limited, is now recognized as extensive and

remarkably diverse. Notably, approximately 78% of the sequenced bacterial genomes encode for more than one phage-defense system, indicating a functional interplay between the antiviral strategies (Georjon & Bernheim, 2023). Several of these defense mechanisms are also integral to normal bacterial physiology. For example, systems such as homologous recombination, restriction-modification, and CRISPR/ Cas not only protect against phage attack but also contribute to maintaining chromosome integrity and regulating DNA replication (reviewed in (Rocha & Bikard, 2022)).

The RecBCD complex

RecBCD, a helicase-nuclease complex, has a key role in dsDNA break repair through homologous recombination in *E. coli*. These breaks can arise naturally during DNA replication and lead to 'replication fork collapse' (Dillingham & Kowalczykowski, 2008). Upon encountering a blunt dsDNA end, RecBCD initiates repair by unwinding and processing the DNA to generate a 3′ ssDNA overhang suitable for RecA loading (Taylor & Smith, 1985). A key regulatory step occurs when RecC recognizes Chi (crossover hotspot instigator) octamer sequences. Binding to a Chi site triggers conformational changes within the complex, halting the unwinding activity of RecD and potentially inducing a nick at the Chi locus (Smith, 2012; Zheng et al., 2025). This conformational change further facilitates RecA loading onto the newly generated 3' single-stranded end. RecA then drives strand invasion and homologous DNA exchange, allowing replication to resume (Anderson & Kowalczykowski, 1997; Bianco & Kowalczykowski, 1997; Cheng et al., 2020; Handa et al., 2005; Taylor et al., 2014). Of note, Chi site recognition is not as efficient as previously assumed. However, avoidance of self-destruction is achieved *via* high density of Chi loci within the bacterial chromosome (Cheng et al., 2020; Levy et al., 2015).

Phages lacking the Chi sites are typically vulnerable to RecBCD-mediated degradation. Many phages, such as λ phage, completely lack Chi sites, rendering their genomes highly susceptible to RecBCD processing (Cheng et al., 2020; Levy et al., 2015). However, recent work suggests that the destructive function of the complex is dependent on the ability of the DNA fragment to undergo homologous recombination, rather than the presence of Chi sites (Zheng et al., 2025). Nevertheless, degraded foreign DNA fragments may subsequently be recovered by the CRISPR/ Cas (see 'The CRISPR/ Cas system')), or the Argonaut system further targeting the incoming phage DNA (Kuzmenko et al., 2020; Levy et al., 2015). To protect themselves against the RecBCD activity, phages have evolved dedicated anti-RecBCD mechanisms. Lambdoid phages, that generally lack Chi sites, often encode for inhibitors,

such as the Gam protein (Enquist & Skalka, 1973; Friedman & Hays, 1986; Murphy, 1991). The λ-encoded Gam protein binds directly to the RecBCD complex inhibiting its activity (Karu et al., 1975; Murphy, 1991). On the other hand, the Mu-encoded Gam is localized directly on DNA ends rather than RecBCD itself, slowing the progress of the complex, and promoting non-homologous DNA end joining repair instead (Bhattacharyya et al., 2018).

Restriction-modification systems

Restriction-modification (RM) systems are by far the most abundant antiviral defense mechanisms in prokaryotic genomes (Tesson et al., 2022). These bifunctional systems protect the host by methylating its own DNA at specific recognition sites while cleaving unmethylated foreign DNA, such as incoming viral genomes. The RM systems are typically categorized in three major types, type I, II, and III, based on their mechanism of action, recognition sequence patterns and enzyme structure (Anton et al., 2025; Patel, 2016). More subtypes are described in literature, but this thesis will only focus on these three classes. Type I RM systems consist of three genes, *hsdS* encoding for the DNA specificity domain*, hsdM* encoding for the methyltransferase subunit*,* and *hsdR* encoding for the restriction endonuclease (reviewed in (Loenen et al., 2014)). The active holoenzymes typically assemble in R_2:M_2:S_1 stoichiometry, while M_2:S_1 has also been observed. Type I RM systems recognize bipartite DNA motifs, typically converting an adenine to *N6*-methyladenine (m6A), with strong preference for semi-methylated DNA substrates (Figure 9). When both strands of the recognition sequence are unmethylated the restriction subunit cleaves the DNA (see 'The EcoKI RM system') (reviewed in (Loenen et al., 2014)). Notably, it has been shown that the RecBCD complex can then engage the resulting DNA ends, displacing the type I RM machinery and further degrading the restricted DNA through its potent helicase-nuclease activity (Bianco & Hurley, 2005). Type II RM systems are the most abundant across prokaryotes and are widely used in molecular biology. However, they are more heterogeneous than other types and further categorized into multiple different subtypes. Type II RM systems typically function as homodimers that recognize short palindromic sequences, producing blunt or 'sticky' DNA ends (Pingoud et al., 2005). Type III RM systems, consist of two genes, *mod* that encodes the methyltransferase, and *res* which encodes the restriction endonuclease. The functional complex typically forms in Res_2:Mod_2 stoichiometry. Unlike type I systems, the sequence specificity domain is located within Mod. Mod recognizes asymmetric motifs and methylates only one strand. Restriction by Res occurs only when two unmethylated recognition sites are present in an inverted orientation relative to one another. Here, the restriction

activity is dependent on the relative arrangement of the recognition sites (Anton et al., 2025; D. N. Rao et al., 2014).

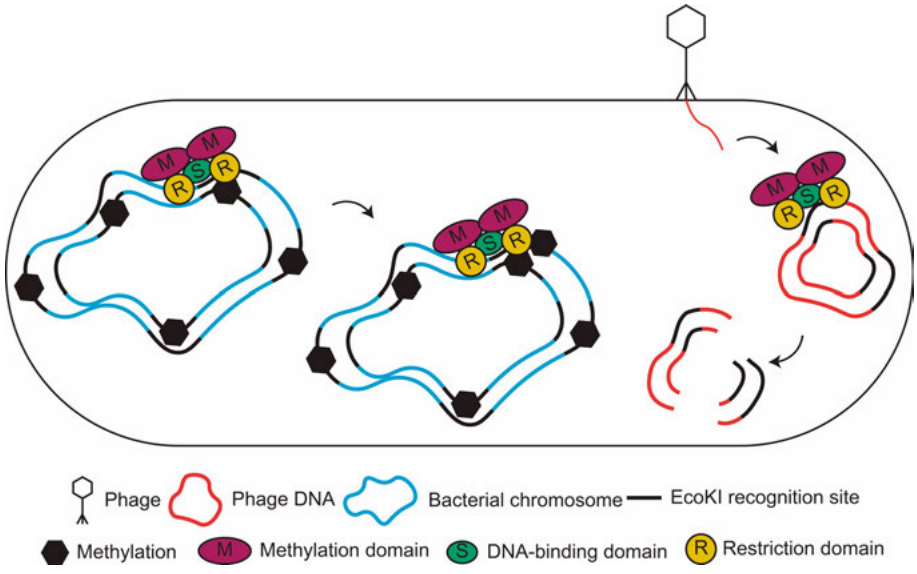

Figure 9: Schematics of the type I RM mechanism. The recognition sites are highlighted in black. Under normal conditions (left) the R_2:M_2:S_1 complex binds semi-methylated host DNA and adds methyl groups to unmethylated adenine. Upon phage infection the R_2:M_2:S_1 complex binds on the recognition sequence, where the restriction domain induces DNA translocation and subsequent DNA cleavage (right).

The EcoKI RM system

EcoKI is the canonical type I restriction-modification (RM) system of *E. coli* K-12, encoded by the chromosomal *hsdRMS* locus (reviewed in (Loenen et al., 2014)). The system forms a pentameric holoenzyme with R_2:M_2:S_1 stoichiometry that recognizes the bipartite DNA sequence 5′-AAC(N_6)GTGC-3′ (Kan et al., 1979). Within this complex, the HsdS subunit confers sequence specificity, the two HsdM subunits methylate adenines within the recognition motif to protect host DNA, and the two HsdR subunits use ATP to translocate bidirectionally along foreign, unmethylated DNA. When two EcoKI complexes converge on an invading DNA molecule, this collision triggers double-strand breaks that often occur many kilobases away from the original recognition site (Figure 9). Efficient cleavage requires both ATP and S-adenosylmethionine (Bickle et al., 1978; Davies et al., 1999; Eskin & Linn, 1972; García & Molineux, 1999). The methylation subunit shows a strong preference for semi-methylated recognition sequences. However, unmethylated motifs can still be methylated, with slower kinetics, to avoid self-destruction. Unmodified recognition sequences may appear transiently during replication,

recombination-mediated repair, or after DNA damage such as ultraviolet irradiation. In this case, the bacterial genome is theoretically susceptible to degradation by the restriction subunit. This paradox is resolved by ClpXP-mediated restriction alleviation (Makovets et al., 1999). Here, the ClpXP protease targets HsdR, inducing its proteolysis, during dsDNA translocation on circular DNA but not on linear DNA, due to the longer residence time of the enzyme on circular substrates (Doronina & Murray, 2001; Simons et al., 2014). This way, ClpXP-mediated restriction alleviation is mainly effective on the host's chromosome, rather than on incoming linear foreign DNA. A second putative restriction-alleviation pathway in *E. coli* involves the *lar* or *ralR* gene of the Rac prophage (described as type I TA system (Y. Guo et al., 2014)), whose gene product may enhance methylation at unmodified sites (Blakely & Murray, 2006). The function of RalR in phage infection is the subject of Paper III.

Beyond antiviral defense, EcoKI plays a regulatory role in horizontal gene transfer. Although it restricts incoming DNA, it does not completely block conjugation (Roer et al., 2015), allowing for acquisition of beneficial genes. Gene transfer is more likely between closely related bacterial strains that share similar RM systems, occupy overlapping ecological niches, and possess compatible sequences that recombine more readily. The advantages conferred by new genes might be higher when transfer takes place between more similar genetic backgrounds. In this way, EcoKI acts not only as a defense system but also as an evolutionary filter that shapes the landscape of horizontal gene flow, promoting exchange within related species while restricting acquisition from more divergent sources. Additionally, phase variation of the *hsdS* gene can result in variable genome-wide methylation patterns within a bacterial population. This variable epigenetic landscape drives changes in gene expression (De Ste Croix et al., 2017; Huang et al., 2020), allowing for more rapid bacterial adaptation. Studies also indicate that alterations in the HsdS specificity domain can shift target recognition patterns, linked to changes in virulence phenotypes in several bacterial species (Elsener et al., 2026; Manso et al., 2014), with similar effects likely applicable to EcoKI.

EcoKI also functions as a major innate immune defense system against phage infection. Phage genomes often enter the cell unmethylated, a pattern recognizable by EcoKI, leading to HsdR-mediated restriction (Figure 9) (Bickle & Krüger, 1993). In response, viruses and other mobile genetic elements have evolved a wide range of anti-restriction strategies, including acquiring host-derived methylation, encoding their own methyltransferases, hyper-modifying their DNA with noncanonical bases, producing anti-restriction proteins that mimic DNA, or eliminating host recognition sequences (reviewed in (Loenen et al., 2014)). For example, the λ-encoded Ral protein

enhances methylation over restriction, resulting in restriction alleviation (Loenen & Murray, 1986; Zabeau et al., 1980). On the other hand, the Ocr protein encoded within the early T7 phage genes, mimics the structure of DNA and strongly binds to the RM complex, completely inhibiting its activity and enabling phage propagation (Atanasiu et al., 2002; Moffatt & Studier, 1988; Walkinshaw et al., 2002). P1 phage uses a different anti-restriction strategy. Here, P1 packages and delivers its own methyltransferase into the host cell along with its genome, allowing immediate protection against EcoKI cleavage (Iida et al., 1987; Łobocka et al., 2004). Base hypermodification provides yet another escape mechanism, exemplified by T4 phage, whose use of 5-hydroxymethyl-dCTP impairs EcoKI recognition and subsequent cleavage (H. Kang et al., 2025; Luria & Human, 1952).

The CRISPR/ Cas system

CRISPR/ Cas systems provide bacteria with adaptive immunity by acquiring short spacer sequences from past phage infections, and storing them in CRISPR arrays, enabling precise targeting of the previously encountered phages (Barrangou et al., 2007; Bolotin et al., 2005; Pourcel et al., 2005). CRISPR loci are characterized by short, direct repeat sequences. Upon transcription, these loci produce a precursor CRISPR RNA (pre-crRNA), which is subsequently processed into mature crRNAs that guide Cas effector complexes to complementary invading nucleic acids for silencing. Cas proteins participate in all three stages of CRISPR immunity: spacer acquisition (adaptation), crRNA biogenesis, and interference with foreign DNA. During adaptation, a short copy of foreign nucleic acid, the protospacer, is integrated into the CRISPR array. New spacers are typically inserted at one end of the locus, maintaining a relative chronological record of past infections. In the crRNA biogenesis phase, the pre-crRNA is processed with the assistance of Cas proteins to generate mature crRNAs. Subsequently, the crRNAs are incorporated into effector complexes, guiding the complex to the invading, complementary nucleic acid, inducing its degradation (reviewed in (Terns & Terns, 2011)). CRISPR/ Cas systems distinguish between host and foreign DNA. Short sequences, adjacent to the protospacer, termed PAMs, affect this recognition (reviewed in (Terns & Terns, 2011)). Another layer of discrimination may arise from host genome architecture. Acquisition of new spacers depends largely on RecBCD-mediated processing of dsDNA. The higher density of Chi sites on the bacterial chromosome, combined with the greater number of replication forks on invading phage DNA, biases RecBCD activity toward foreign DNA and promotes selective acquisition of phage-derived spacers (Levy et al., 2015). Phages, in turn, encode a wide variety of anti-CRISPR

(Acr) proteins that inhibit different steps of CRISPR-Cas activity, allowing them to evade nucleic acid degradation (reviewed in (Y. Li & Bondy-Denomy, 2021)). Despite this well-documented role in bacterial defense, these systems also influence normal bacterial physiology. They have been shown to modulate bacterial virulence by affecting host-cell invasion (Louwen et al., 2013). Additionally, the CRISPR/ Cas systems can regulate gene expression (Medina-Aparicio et al., 2021; Viswanathan et al., 2007), and contribute to broader cellular functions such as stress responses and overall bacterial physiology (Devi et al., 2022).

Abortive infection

Abortive infection (Abi) was initially used as a broad term describing any situation in which phages fail to complete the infection process (Gross, 1954). The definition later narrowed to describe a specific defense mechanism in which an infected bacterial cell undergoes premature cell death before phage replication is completed, thereby preventing the spread of the phage to neighboring cells. Therefore, Abi is usually considered an altruistic phenomenon, protecting the bacterial population at the cost of individual cells. Multiple phage defense systems can trigger Abi, and it is generally activated only as a last resort when other antiviral mechanisms, such as RM systems, have failed (reviewed in (Aframian & Eldar, 2023; Lopatina et al., 2020)). For example, activation of the type III TA system *toxIN* can result in bacterial 'suicide'. During T4 infection, host transcription shuts off, leading to rapid degradation of the sRNA antitoxin ToxI and release of the ToxN toxin. Free ToxN then cleaves both host and phage mRNAs extensively, collapsing cellular metabolism and causing abortive infection (Guegler & Laub, 2021). The *E. coli* type I TA *hok*/ *sok* has also been suggested to induce abortive infection upon T4 infection (Pecota & Wood, 1996), though this phenotype could not be reproduced (Messineo et al., 2026). Anti-anti-restriction systems can also induce Abi. One example is the phage anti-restriction-induced system (PARIS), found in 5.2% of prokaryotic genomes, and activated in the presence of the T7-encoded Ocr protein (Deep et al., 2024; Rousset et al., 2022).

Current investigation

The work included in this thesis has focused on the post-transcriptional regulation and biological functions of type I TA systems. Although genetic, structural, and biochemical studies have significantly advanced our understanding of how these systems are regulated, many mechanistic details remain unresolved. The primary focus of this thesis has been to characterize the regulatory mechanisms underlying the recently identified *timP*/ *timR* TA system in *Salmonella enterica* serovar Typhimurium. As outlined in the introduction, type I TA encoded toxins do not share a common regulatory strategy, and their translation initiation mechanisms are highly diverse. The work in this thesis highlights the molecular determinants governing *timP* post-transcriptional regulation and expands our understanding of the role of type I TA systems during phage infection. In Paper I, we examined the contribution of individual regions of *timP* 5'UTR to translation initiation and elucidated how the antitoxin TimR inhibits TimP synthesis. In Paper II, we investigated how a long-range intramolecular interaction within the *timP* mRNA forms a structural switch that controls toxin expression. In paper III, we explored the effect of *E. coli* chromosomally encoded type I TA systems in phage infection.

Paper I

The type I TA system *timP*/ *timR*, recently identified and, widely conserved among enterobacterial species, encodes the toxin TimP, a small-hydrophobic inner-membrane protein (Andresen et al., 2020), previously annotated as the non-coding RNA *ryfA* (Wassarman et al., 2001). Overexpression of TimP leads to membrane leakage and growth inhibition. A puzzling feature of the system is that *timP* mRNA, unlike many other type I toxin transcripts, does not undergo ribonucleolytic processing (Andresen et al., 2020). This suggests that transcription-translation decoupling must occur *via* an alternative mechanism. TimR is an sRNA that represses TimP synthesis by base-pairing with a complementary sequence within the 145 nucleotide-long *timP* 5'UTR (Andresen et al., 2020). Interestingly, its predicted binding site lies approximately fifty

nucleotides upstream of the TIR. This implies that TimR does not prevent translation by sterically blocking 30S ribosomal access to the RBS.

Paper I focused on uncovering how translation initiates on *timP* mRNA and how TimR mediates repression. Using structural, biochemical and genetic approaches, we determined how *timP* escapes co-transcriptional translation, identified the 5'UTR elements that are essential for its expression, and uncovered how TimR inhibits translation initiation. Our results showed that full-length *timP* folds into four stable stem-loops, one of which sequesters the RBS. Furthermore, our data strongly indicated that *timP* adopts at least two mutually exclusive conformations: one that is translation competent, and one that is translationally inactive. The translationally active conformation features an evolutionary conserved pseudoknot structure within the *timP* 5'UTR that is essential for efficient translation. Moreover, our data indicated that, in analogy with other type I TA systems, TimR preferentially binds to this active conformation. Upon TimR binding, the pseudoknot gets destabilized, thereby preventing translation initiation. Based on this, we suggested an alternative two-step regulatory mechanism for controlling expression of a type I toxin. Rather than relying on enzymatic processing, the primary *timP* transcript undergoes 'structural processing' from an inactive to an active conformation. TimR then specifically targets the translationally competent mRNA, preventing toxin synthesis. Taken together, the findings from Paper I expand our knowledge on the tight regulatory mechanisms that protect the cell from leaky toxin expression.

Paper II

The observations from Paper I provided a plausible explanation for how the 30S ribosomal subunit might initially engage *timP* mRNA despite the absence of an exposed RBS. As in the case of *tisB*, ribosomal protein S1 could potentially recognize the pseudoknot structure, facilitating 30S binding (Romilly et al., 2019, 2020). However, this model failed to answer how the 30S subunit subsequently gains access to the downstream RBS, which remains structurally occluded in all predicted *timP* mRNA conformations. Consequently, the molecular events that follow pseudoknot formation were only partially understood. Saturation mutagenesis combined with phenotypic sorting and deep sequencing has proven to be a powerful approach for dissecting bacterial post-transcriptional regulation (Holmqvist et al., 2013; Rizvanovic et al., 2021; Rutherford et al., 2015). Among these methods, FASTBAC-Seq has been successfully implemented in the characterization of type I toxin mRNAs. In this method, the toxin mRNA is initially subjected to mutagenesis, in the

absence of the antitoxin. FASTBAC-Seq takes advantage of the growth-inhibitory effect of type I toxins to select for mutations that abolish toxicity (Le Rhun et al., 2023; Masachis et al., 2018, 2019).

In Paper II we used an integrated experimental strategy, combining FASTBAC-Seq, compensatory mutation analysis, and RNA structural probing, to show that *timP* mRNA is controlled by a binary RNA conformational switch. Our results uncovered a previously unrecognized, evolutionarily conserved long-range interaction that competes directly with formation of the SD-sequestering stem-loop structure. Based on these findings, we proposed a new mechanistic model in which the active conformation contains both a pseudoknot and an additional long-range interaction that fully exposes the RBS, whereas the inactive conformation is stabilized by a third competing long-range interaction that prevents pseudoknot formation and traps the RBS within a stable stem-loop. Although in Paper I we speculated that the 30S subunit could move along the *timP* mRNA and access the RBS during transient unfolding of the inhibitory structure, our current findings indicate that such access is enabled only when the 5'UTR undergoes a structural reorganization. This arrangement likely provides a safety-key to prevent inadvertent toxin production while allowing rapid activation upon permissive conditions. Together, these findings uncover an unexpected level of RNA-mediated regulation and expand the repertoire of translation initiation strategies used by bacteria.

Paper III

Bacteria live in ever-changing environments, facing a plethora of challenges, including infection by bacteriophages. Bacteria and phages are constantly in an arms race, adapting to outcompete each other for survival (Hampton et al., 2020; Stern & Sorek, 2011; S. Zhang et al., 2025). To withstand phage attack, bacteria deploy an extensive repertoire of defense systems that target nearly every stage of the phage life cycle (Georjon & Bernheim, 2023). In response to bacterial defense systems, phages have evolved a variety of countermeasures. For instance, the T7 Ocr protein mimics bent DNA and binds EcoKI with high affinity, thereby blocking its activity and allowing the phage to successfully infect the host (Atanasiu et al., 2002; Moffatt & Studier, 1988; Walkinshaw et al., 2002). TA systems have been successfully deployed by bacteria in phage defense (Dy et al., 2014; Fineran et al., 2009; Guegler & Laub, 2021; T. Zhang et al., 2022). Type I TA systems are characterized as stress response elements, that encode a stable toxin mRNA and an unstable antitoxin sRNA. Hence, a mechanism in which rapid degradation of the labile antitoxin, and rapid translation of the already existing toxin mRNA is

an intriguing hypothesis that would position type I TA systems as key players in phage infection. Indeed, the plasmid-encoded type I TA system *hok*/*sok* has been suggested to induce abortive infection upon T4 infection (Pecota & Wood, 1996).

In Paper III, we investigated the role of *E. coli* chromosomally encoded type I TA systems in phage infection using a combination of genetic, biochemical, and infection assays. To this end, we cloned eight chromosomal type I TA modules into a multi-copy plasmid and challenged these strains with a panel of fifteen distinct phages. Our results revealed that one prophage-encoded module unexpectedly enhanced phage infection, leading to increased phage propagation. We hypothesized that the type I toxin, RalR, encoded within this system was responsible for the phenotype. Using a series of genetic constructs, we demonstrated that RalR indeed modulates phage infection, and importantly, does so independent of its previously reported toxicity. We next confirmed that RalR expression is specifically induced upon phage infection, supporting the idea that its regulatory function is activated under these conditions. Finally, we examined whether the major *E. coli* innate immune system, EcoKI, contributes to the observed phenotype. Consistent with this possibility, infection enhancement was found to be EcoKI-dependent, suggesting that the RalR-mediated effect may selectively target phages entering the cell with an unmethylated genome. Overall, our results uncover an unexpected contribution of type I TA systems to phage-host interactions, expanding their functional repertoire.

Future perspectives

The findings of this thesis have provided new insights into the role of RNA-mediated control in post-transcriptional regulation and have expanded the functional repertoire of TA systems. These findings add to our understanding of the molecular mechanisms underlying toxin control in bacteria. More importantly, the work presented here has led to new scientific questions about post-transcriptional gene regulation, RNA folding, and the biological functions of type I TA systems.

In Paper I, we reveal that translation of *timP* toxin is dependent on the formation of a pseudoknot structure within its 5'UTR. Removal of the pseudoknot results in impaired toxin translation, as well as antitoxin binding. Since *timP* SD is sequestered in a stable stem-loop structure, we have speculated that the pseudoknot may act as a binding platform for the ribosomal protein S1, engaging 30S when the SD sequence is occluded within a stable stem-loop structure, in a manner similar to *tisB* (Romilly et al., 2019, 2020). This way, 30S ribosomal subunit would be able to initiate translation when the SD-sequestering stem-loop transiently unfolds (De Smit & Van Duin, 1993, 2003). Whether this pseudoknot structure is actively implicated in 30S recruitment remains to be elucidated both *in vitro* and *in vivo*. Further, we have identified that TimR destabilizes the pseudoknot, using a mutant where this tertiary interaction was favored. In wild-type mRNA, only a small fraction carries the pseudoknot structure, making it difficult to detect such changes in experiments that rely on bulk measurements. Future *in vitro* single-molecule experiments would enable the characterization of TimR-mediated pseudoknot destabilization on wild-type mRNA molecules. Furthermore, more type I toxins such as the *fst* in *Enterococcus faecalis* (Greenfield et al., 2001), and the *txpA* in *Bacillus subtilis* (Silvaggi et al., 2005) seem to not rely on enzymatic processing of the primary transcript. Hence, it would be interesting to see if 'structural processing' is implicated in regulation of these toxins as well. Finally, the pseudoknot formed within the *timP* mRNA represents one of the few known examples in which such a tertiary RNA structure is essential for activating translation initiation. It is plausible, however, that such elements are far more widespread, particularly in highly structured toxin mRNAs. Future studies will be required to uncover the full extent of these regulatory structures.

In Paper II, we elucidate the mechanism by which the pseudoknot mediates translation initiation of *timP* mRNA. Using FASTBAC-Seq, we identified a previously uncharacterized region of the 5'UTR that plays a central role in *timP* translation initiation. Through multiple complementary experimental approaches, we found that this region competes with pseudoknot formation in the inactive conformation, and with the SD-sequestering stem-loop in the active conformation. This dual competition explains how the RBS becomes accessible for 30S binding and, subsequently, initiation of translation. Nevertheless, our findings raised a number of questions for future studies to be addressed. It is still unknown whether the transition from the inactive to the active conformation is stochastic or induced by specific environmental cues. In several aspects, the identified RNA switch resembles an 'ON' translational riboswitch. Magnesium concentration is an environmental signal that promotes virulence in *Salmonella* (Fàbrega & Vila, 2013; Jennings et al., 2017). Thus, Mg^{2+}-dependent activation of *timP*, similar to what is observed for *mgtL* (Zhao et al., 2011), represents a plausible hypothesis that remains to be experimentally tested. Although the switch can form *in vitro* in the absence of additional factors, it is unclear whether a stable active conformation also requires *trans*-acting elements *in vivo.* Another important issue is the timing of the formation of the active conformation. Since the sites that form the inactive structure are transcribed first, this conformation is likely to arise co-transcriptionally. This resembles the early formation of metastable structures reported for the *hok* and *aapA3* toxin mRNAs, which transiently fold in a manner that sequesters the SD sequence within a stable stem-loop (Gultyaev et al., 1997; Masachis et al., 2019). It also remains unresolved whether the pseudoknot, rather than the second long-range interaction, is formed co-transcriptionally. To address these, single-molecule approaches will be necessary to map the co-transcriptional folding events of *timP*. Additional open questions include why TimR shows a preference for the pseudoknot-containing conformation of *timP* and how its binding leads to pseudoknot destabilization. Addressing these issues will require high-resolution structural characterization of the different *timP* molecules, for example through Cryo-Electron Microscopy, and single-molecule approaches.

In Paper III, we showed that a chromosomally encoded type I TA system in *E. coli* can unexpectedly enhance phage infection, acting in coordination with the EcoKI RM system. Although this reveals a new regulatory role for type I toxins in phage-host interactions, many questions remain. It is still unknown whether the toxin interacts directly with EcoKI or influences it indirectly. Co-immunoprecipitation of the candidate proteins could shed more light into this question. Our data further indicate that infection enhancement

depends on the DNA-methylation state of the incoming phage. Whether the phages we tested differ in their methylation profiles, and whether this mechanism is more broadly applicable across phages, needs to be elucidated. Additionally, it was previously shown that both the toxin and the antitoxin are expressed under normal laboratory conditions (Y. Guo et al., 2014). However, a different study shows that *ralR* is expressed at background levels in LB medium (Thomason et al., 2015). Hence, further investigation needs to be conducted to determine the RNA levels of both the toxin and the antitoxin in our conditions. Nevertheless, the fate of the antitoxin during infection remains unclear. Whether the antitoxin is rapidly degraded, as seen for ToxI (Guegler & Laub, 2021), or if the toxin is transcriptionally activated, overriding the antitoxin levels, as in the case of *tisB* (Vogel et al., 2004), remains to be elucidated. Moreover, while our data indicate that toxin expression is selectively induced upon phage entry, the trigger for this activation, whether a cellular factor or a phage-encoded protein is still unclear. Finally, although the presence of RalR appears disadvantageous for the host under the tested conditions, whether it provides benefits under alternative stresses and is thus maintained in bacterial populations is an important question for future work.

Taken together, the findings presented in this thesis advance our understanding of both the diverse mechanisms underlying translation initiation and antitoxin regulation of type I toxins, as well as RNA-mediated regulation in bacteria. We also expand the functional repertoire of type I toxins by uncovering previously unrecognized roles that should be further investigated. Future research could elucidate the full landscape of RNA-based regulatory mechanisms in bacteria, define the molecular factors that modulate toxin expression under various stress conditions, and determine how widespread these newly identified mechanisms are across species. Further work combining high-resolution structural approaches, single-molecule analyses, and comparative genomics will be essential to characterize conformational switches, identify additional regulatory RNAs, and understand how TA modules integrate into broader cellular networks. Ultimately, such efforts will deepen our understanding on how bacteria sense, respond to, and survive environmental challenges through RNA-mediated control.

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