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Insight into the environmental cues modulating the expression of bacterial toxin–antitoxin systems

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Abstract

Bacteria require sophisticated sensing mechanisms to adjust their metabolism in response to stressful conditions and survive in hostile environments. Among them, toxin–antitoxin (TA) systems play a crucial role in bacterial adaptation to environmental challenges. TA systems are considered as stress-responsive elements, consisting of both toxin and antitoxin genes, typically organized in operons or encoded on complementary DNA strands. A decrease in the antitoxin–toxin ratio, often triggered by specific stress conditions, leads to toxin excess, disrupting essential cellular processes and inhibiting bacterial growth. These systems are categorized into eight types based on the nature of the antitoxin (RNA or protein) and the mode of action of toxin inhibition. While the well-established biological roles of TA systems include phage inhibition and the maintenance of genetic elements, the environmental cues regulating their expression remain insufficiently documented. In this review, we highlight the diversity and complexity of the environmental cues influencing TA systems expression. A comprehensive understanding of how these genetic modules are regulated could provide deeper insights into their functions and support the development of innovative antimicrobial strategies.

Keywords: toxin–antitoxin systems; regulation; environmental cues; microbial community; host immunity; antibiotics

Introduction

Forty years ago, the first toxin–antitoxin (TA) system, a type II TA system named CcdA/CcdB, was discovered on the *Escherichia coli* mini-F plasmid (Ogura and Hiraga 1983). Since then, numerous TA systems were identified in bacterial and archaeal genomes. TA systems are genetic modules composed of two genes: one encoding a stable toxin, whose overexpression leads to growth arrest or cell death, and the other one encoding a labile antitoxin that counteracts toxin's activity. Under certain conditions, the antitoxin no longer inhibits the toxin, allowing it to affect essential cellular processes such as DNA replication, translation, ATP synthesis, and cell division (Jurénas et al. 2022). TA systems distribution is not homogeneous within bacterial genomes. They are abundant in free-living prokaryotes but seem to be absent in obligate host-associated organisms such as *Chlamydia trachomatis* (Pandey and Gerdes 2005). Moreover, the TA systems repertoire varies from one species to another. For instance, at least 93 TA systems have been identified in *Mycobacterium tuberculosis* (Sundaram et al. 2023), whereas 35 have been described in *E. coli* K-12 MG1655 (Harms et al. 2018). As interest in TA systems increased, bioinformatics tools were developed to facilitate their identification and annotation. The first of these, RASTA-Bacteria, appeared in 2007 to identify TA loci in prokaryotes (Sevin and Barloy-Hubler 2007), followed by TADB 3.0 (Guan et al. 2023), TASmania (Akarsu et al. 2019), and T1TAdB (Tourasse and Darfeuille 2021). Ongoing efforts to characterize TA systems revealed that they are classified into eight types, according to the antitoxin's mode of action and nature (RNA in types I, III, and VIII and protein in types II, IV, V, VI, and VII) (Song and Wood 2020). Toxins are proteins in all types of TA systems, except in the recently discovered type VIII TA system,

where it is an RNA (Choi et al. 2018, Li et al. 2021). As mentioned above, TA systems were initially discovered on plasmids, where they contribute to plasmid maintenance (Ogura and Hiraga 1983, Gerdes et al. 1986). For TA systems located on bacterial chromosomes, various novel biological functions have been identified, such as mobile genetic element maintenance, defense against phages, biofilm formation, antibiotic resistance, and persistence (Pecota and Wood 1996a, Ren et al. 2004, Kim et al. 2009, Kim and Wood 2010). Under favorable growth conditions, antitoxins are sufficient to inactivate their cognate toxin and counteract toxicity. This general mechanism of regulation mediated by antitoxins is essential for maintaining bacterial homeostasis and has been extensively described (Jurénas et al. 2022, Bonabal and Darfeuille 2023). However, under environmental cues, the antitoxin–toxin ratio decreased leading to toxin excess, disrupting essential cellular processes and inhibiting bacterial growth. It is noteworthy that the environmental cues influencing TA systems expression are still inadequately documented. Therefore, in this review, we provide insights into the regulation of TA systems expression, both at the RNA and protein levels, when bacteria are (i) present within a microbial community, (ii) targeted by the host's immune response, and (iii) exposed to xenobiotics. These environments generally expose bacteria to multiple stresses that may induce toxin expression and/or activity.

Influence of the microbial environment on TA systems expression

In natural environments, bacteria reside within microbial communities, sharing a common living space in which they can

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be considered as predators of some microorganisms, as preys of phages or other microorganisms, or can form symbionts in biofilms. In this section, we will focus and summarize the current state of art on the regulation of TA systems expression during natural competence, phage infection, and biofilm formation. All these events are mainly under the control of the quorum-sensing (QS) and are summarized in Table 1.

TA systems expression during natural competence

Natural competence is the ability of bacteria to acquire foreign DNA from their environment (Dubnau and Blokesch 2019). DNA uptake is a source of nucleotides, chemicals elements, and energy. This mechanism also participates to horizontal gene transfer and, therefore, to bacterial evolution, antibiotic resistance, and virulence gene acquisition (Cooper et al. 2017, Cordero et al. 2022). In *Haemophilus influenzae*, the expression of competence genes is controlled by two transcription factors: CRP (cAMP receptor protein) and the competence activator Sxy (Jaskólska and Gerdes 2015). Under sugar starvation, an increase in intracellular cAMP level leads to CRP activation. CRP binds to DNA promoters at a CRP canonical site to activate the transcription of *sxy* and sugar utilization genes. Then, CRP and Sxy act together to bind CRP-S sites, which differ from standard CRP sites as they require both CRP and Sxy for activation. These CRP-S sites are located on promoters of competence genes, leading to DNA uptake and natural transformation. The *toxA* gene, encoding the type II antitoxin of the ToxTA TA system, contains a CRP-S site on its promoter (Findlay Black et al. 2020). RNA-sequencing analysis showed that *toxT* and *toxA* transcript levels are upregulated in a competence-inducing starvation medium, while deletion of either the *crp* or *sxy* genes prevents this upregulation. These results demonstrate that *toxTA* operon is controlled by the CRP–Sxy complex, which is involved in the regulation of competence genes (Findlay Black et al. 2020). In the same study, the authors showed that the deletion of *toxA* results in an increase of *toxT* transcript levels and a decrease in transformation efficiency. However, deletion of *toxT* has no impact on bacterial competence. Thus, the exact role of ToxT in natural competence in *H. influenzae* has yet to be elucidated. ToxTA is not the only example of a TA system controlled by CRP–Sxy. Even though *E. coli* is not considered naturally competent, overexpression of *sxy* causes upregulation of the *chpSB* and *higBA* type II TA systems and the *hokD* type I toxin genes (Sinha et al. 2009). Moreover, *hicAB* operon is controlled by two promoters, one of them, the P1 promoter, contains a CRP-S site and is also regulated by the CRP–Sxy complex in *E. coli* (Turnbull and Gerdes 2017).

TA systems expression during phage infection

Phages are categorized based on their life cycles as lytic or temperate. Upon infecting a bacterium, lytic phages initiate a cycle that leads to the production and release of viral particles during host lysis. Temperate phages, on the other hand, can enter either a lytic or a lysogenic cycle. During the lysogenic cycle, the phage genome integrates into the bacterial chromosome as a prophage. The prophage replicates along with the bacterial genome until environmental triggers, such as nutrient deprivation, induce phage activation into the lytic cycle. In response to phage infections, bacteria have evolved phage defense mechanisms, including restriction/modification (RM) systems, CRISPR-Cas, and abortive infection (Abi) systems (Kelly et al. 2023).

Interestingly, an emerging function of TA systems is their role in phage defense (Kelly et al. 2023). During phage infection, TA sys-

tems can be activated to prevent phage replication, thereby protecting the bacterial population. The first TA system involved in the defense against T4 phage infection is the *E. coli* type I *hok/sok* system (Pecota and Wood 1996b). Since then, numerous studies have demonstrated the involvement of various TA systems in anti-phage mechanisms (Kelly et al. 2023, Saunier et al. 2024). Among them, the AbiEi/AbiEii TA system was initially identified as an Abi system conferring phage defence in *Lactococcus lactis* (Garvey et al. 1995), and was later characterized as a type IV TA system, consisting of the AbiEi antitoxin and the AbiEii toxin (Dy et al. 2014). Unlike classical Abi systems that promote cell death in infected hosts, recent findings suggest that toxins may provide phage defense by interfering with or blocking virion production without necessarily killing the cell. Instead, they may exert bacteriostatic effects upon activation. Homologues of AbiEii found in *Serratia* sp. and *M. tuberculosis* have been shown to disrupt tRNA loading, leading to a global reduction of translation and consequent growth arrest, which coincides with reduced phage replication (Cai et al. 2020, Hampton et al. 2020). In *E. coli*, the bacterial phage antirestriction-induced system functions as a type II TA system (Deep et al. 2024). In uninfected cells, the antitoxin AriA, a hexamer related to SMC-family ATPases, binds to up to three monomers of the toxin AriB, keeping them in an inactive state. Upon infection by the T7 phage, the phage antirestriction protein Ocr binds to the AriA hexamer, inducing a structural rearrangement. This interaction triggers the release of AriB, allowing it to dimerize and become active. As a result, AriB, a toprim/OLD-family nuclease, cleaves lysine tRNA, thereby inhibiting protein translation, which leads to cell growth arrest and prevents phage propagation (Deep et al. 2024). Additionally, the DarTG system, a type II/IV TA hybrid, prevents bacterial death and blocks phage replication by inhibiting DNA synthesis. This occurs through ADP-ribosylation of phage DNA by the DarT toxin, which is activated upon DarG inhibition (LeRoux et al. 2022). The authors hypothesized that a phage-derived factor could inhibit, sequester, or degrade DarG, ultimately leading to the release of DarT. In *Pseudomonas aeruginosa*, overexpression of the CrIA type II antitoxin protects against phage infection independently of its cognate CrIT toxin, which cleaves bacterial mRNAs and induces growth arrest (Ni et al. 2022). The authors suggested that CrIA's anti-phage activity results from direct binding to phage DNA via its DNA-binding domain, thereby reducing phage replication (Ni et al. 2022).

Another well-documented example of TA systems activation in response to phage infection is the ToxIN type III TA system (Fineran et al. 2009, Blower et al. 2012, Guegler and Laub 2021). This system, composed of the *toxI* RNA antitoxin (encoded by 5.5 repeats of 36 bp) and the endoribonuclease ToxN toxin, protects *E. coli* against phages. T4 phage infection induces a transcriptional shutoff in *E. coli*, leading to the rapid degradation of ToxI RNA (half-life: 2.5 min), which allows the release of the ToxN toxin. ToxN then cleaves viral transcripts at a GAAAU motif, thereby preventing phage protein synthesis and the production of new phages (Fig. 1). In this case, T4 phage infection is counteracted by ToxIN due to the inherent instability of ToxI RNA rather than increased expression of ToxN (Guegler and Laub 2021). AvcID is a type III TA system composed of the AvcD deoxycytidine deaminase toxin and the AvcI RNA antitoxin, expressed by *Vibrio cholerae* (Hsueh et al. 2022). This system protects bacteria against phage infection by disrupting cytosine availability. T5 phage infection induces transcriptional arrest in the bacterial host, leading to rapid degradation of AvcI RNA. Consequently, AvcD deaminates dCTP to dUTP, increasing uracil incorporation in phage DNA and resulting in defective T5 virions. Subsequent studies demonstrated that heterol-

Table 1. Role of the microbial community on TA systems expression.

Environmental event	TA system	Type	Bacteria	Mechanism of regulation	References
Natural competence	ToxTA	II	<i>Haemophilus influenzae</i>	Transcriptional upregulation	Findlay Black et al. (2020)
	ChpSB, HigBA, HicAB	II	<i>Escherichia coli</i>	Transcriptional upregulation	Sinha et al. (2009), Turnbull and Gerdes (2017)
Phage infection	Hok-Sok	I	<i>Escherichia coli</i>	Antitoxin degradation induced by transcriptional repression	Pecota and Wood (1996b)
	RlnAB	II	<i>Escherichia coli</i>	Antitoxin degradation induced by transcriptional repression	Koga et al. (2014)
	ToxIN	III	<i>Escherichia coli</i>	Antitoxin degradation induced by transcriptional repression	Guegler and Laub (2021)
Biofilms	AvcID	III	<i>Vibrio cholerae</i>	Antitoxin degradation induced by transcriptional repression	Hsueh et al. (2022)
	RelBE	II	<i>Pseudomonas aeruginosa</i>	Upregulation of mRNA level	Mahmoudi et al. (2022)
	SprG1/SprF1	I	<i>Staphylococcus aureus</i>	Upregulation of <i>sprG1</i> mRNA level	Karimaei et al. (2021)
	MazEF, RelBE	II	<i>Staphylococcus aureus</i>	Upregulation of <i>mazF</i> and <i>relE</i> mRNA level	Karimaei et al. (2021)
	ParDE ₄	II	<i>Caulobacter crescentus</i>	Transcriptional downregulation	Berne et al. (2023)
Quorum sensing	SmuATR	II	<i>Streptococcus mutans</i>	Upregulation of mRNA level	Perry et al. (2009), Dufour et al. (2018)
	PumAB	II	<i>Pseudomonas aeruginosa</i>	Upregulation of mRNA level	Hernández-Ramírez et al. (2020)

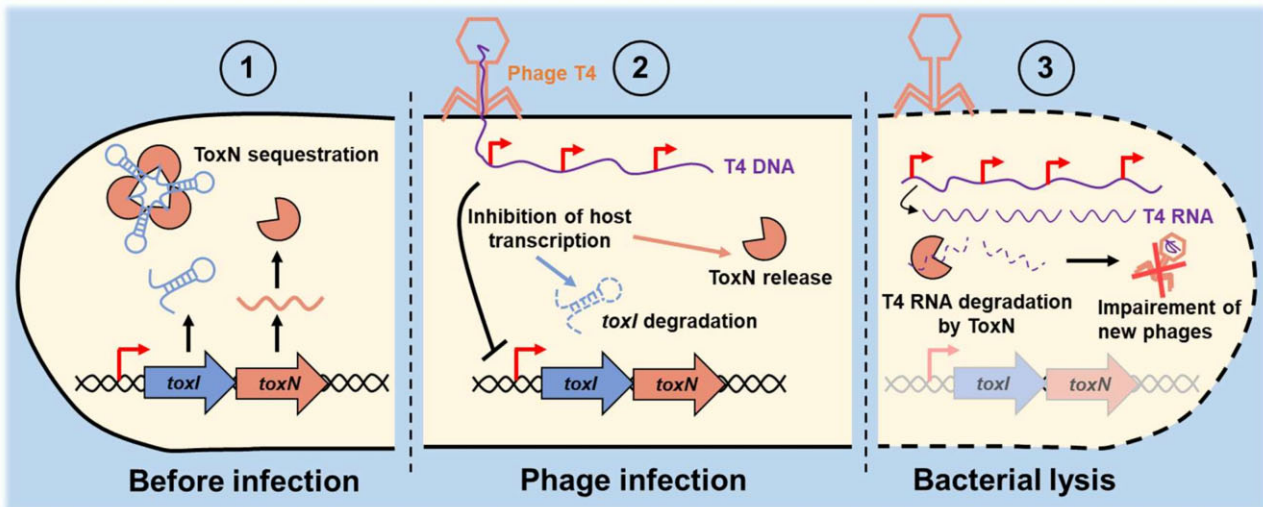


Figure 1. T4 phage infection leads to host shut-off transcription, ToxI antitoxin degradation and ToxN release.

ogous expression of the *avcID* locus from *Vibrio parahaemolyticus* in *E. coli* protects against T5 but not T7 phages (Hsueh et al. 2023). AvcID effectively defends against phages with longer replication cycles, such as T5, but is ineffective against rapidly replicating phages like T7. The authors proposed that the effectiveness of AvcID in phage defense is contingent on the length of the phage replication cycle. Similar mechanisms of antitoxin–toxin imbalance due to transcriptional shutdown were proposed for the RnLAB and Hok-Sok TA systems, where degradation of labile antitoxins releases free toxins (Pecota and Wood 1996a, Koga et al. 2011).

Interestingly, a recent study reported that the Retron-Sen2 system in *Salmonella* provides phage defense and can be considered as a tripartite TA system comprising the effector toxin RcaT, a non-coding RNA, and a reverse transcriptase responsible for generating multicopy single-stranded DNA (msDNA) (Bobonis et al. 2022). Activation of RcaT is coupled with disruption of the msDNA–RT antitoxin complex, likely through degradation or methylation of msDNA by phage components. RcaT is hypothesized to hydrolyze nucleosides and nucleotides, thereby interfering with phage replication (Bobonis et al. 2022). The authors demonstrated that the Dam protein methylates msDNA to activate the retron TA system, while the RacC protein directly counteracts RcaT toxicity (Bobonis et al. 2022). Recently, the MqsR/MqsA/MqsC tripartite TA system was shown to enhance *E. coli* survival following T2 phage infection by inducing the formation of persister cells, a subpopulation of bacteria initially described by Joseph Bigger in 1944 (Bigger 1944) that can transiently survive to antibiotics and resume growth after antibiotic removal (Balaban et al. 2019). Additionally, this TA system cooperates with the RM system to effectively inactivate phages (Fernández-García et al. 2024).

Despite the growing number of TA systems identified as phage defense mechanisms, further research is needed to elucidate the precise triggers that activate the transcription of TA locus during on TA mediated-phage defense.

TA systems expression in biofilms

Biofilms are structured, surface-attached microbial communities embedded in an extracellular matrix composed of polysaccharides, extracellular DNA (eDNA), and other components. This lifestyle protects bacteria from hostile environmental conditions, such as the host immune response and antibiotics, and is con-

sequently associated with therapeutic treatment failures (Vuotto and Donelli 2019).

TA systems can participate in biofilm formation or disruption. For instance, the deletion of five type II TA systems (MazEF, RelBE, ChpB, YoeB/YefM, and YafQ/DinJ) reduces biofilm formation after 8 hours but enhances it after 24 hours due to decreased biofilm dispersal in *E. coli* (Kim et al. 2009). This study highlights the role of the uncharacterized YjgK (TabA) protein, which is induced upon TA system deletion and negatively regulates type 1 fimbriae, a key factor in biofilm attachment (Kim et al. 2009). In *P. aeruginosa*, deletion of the HigA antitoxin leads to a 28-fold induction of *higB* mRNA transcription, which in turn reduces biofilm formation (Wood and Wood 2016). Moreover, the *relBE* locus is upregulated in stronger biofilm-producing *P. aeruginosa* isolates (Mahmoudi et al. 2022). Similarly, RelBE promotes biofilm formation in *V. cholerae* (Wang et al. 2015). In *Staphylococcus aureus*, the relative expression of *mazF*, *relE1*, and *relE2* type II toxin mRNAs, as well as *sprG1* type I toxin mRNA, is upregulated in biofilms compared to planktonic bacteria (Karimaei et al. 2021). However, the functional significance of these upregulations in biofilm formation has not yet been elucidated. Another example of a TA system regulated under biofilm conditions is the ParDE₄ type II TA system, consisting of the ParD antitoxin and the ParE gyrase inhibitor in *Caulobacter crescentus* (Berne et al. 2023), a Gram-negative bacterium living in lakes and streams. In this bacterium, when living conditions in the biofilm deteriorate, eDNA serves as a signal to mediate biofilm dispersal. Interestingly, deletion of the ParDE₄ TA system results in an increase in biofilm formation and a decrease in eDNA release (Berne et al. 2023). Further investigations, using a transcriptional *lacZ* reporter system, demonstrated that the *parDE*₄ promoter activity decreases in areas where oxygen availability is reduced within the biofilm. Taken together, these results suggest that, when oxygen availability is reduced, the ParD₄ antitoxin is rapidly degraded by proteases, allowing the ParE₄ toxin to induce programmed cell death, promoting eDNA release and biofilm disruption.

TA systems expression and quorum sensing

Quorum sensing (QS) is a cell-to-cell communication system used by bacteria to coordinate social behaviors in a cell density-dependent manner. QS involves the production of diffusible or se-

creted signaling molecules. As the bacterial population density increases, these signaling molecules accumulate in the extracellular medium and interact with their cognate receptors. This induces the expression of target genes that contribute to bacterial adaptation, survival, and successful interactions with other organisms.

The *Streptococcus mutans* ComCDE QS system is composed of the ComC signal peptide precursor, which, after modifications, generates the competence-stimulating peptide (CSP) alarmone (Perry et al. 2009). Extracellular CSP is detected by the ComD membrane receptor, that leads to the activation of the ComE response regulator. Then, ComE binds the promoters of CSP-responsive genes. Transcriptomic study showed that the SmuATR chromosomal type II TA system is a member of the CSP regulon (Perry et al. 2009). This tripartite system, composed of the SmuA antitoxin, SmuT toxin, and SmuR transcriptional repressor, is upregulated by the CSP alarmone (Perry et al. 2009, Dufour et al. 2018). Noteworthy, the CSP alarmone is involved in the formation of persister cells. The involvement of the SmuATR TA system in the CSP-inducible persister phenotype was illustrated using deletion mutants lacking *smuAT* and *smuATR*, both preincubated with or without the CSP alarmone prior an ofloxacin treatment (Dufour et al. 2018). However, further studies are needed to demonstrate that ComE can bind the promoter of *smuATR* locus.

The plasmid-encoded PumAB type II TA system of *P. aeruginosa* is another TA system regulated by QS (Hernández-Ramírez et al. 2020). This system is involved in plasmid stability, and the PumA toxin is associated with bacterial virulence (Hernández-Ramírez et al. 2017). In *P. aeruginosa*, LasI–LasR and RhlI–RhlR are two QS pathways leading to the expression of QS responsive genes (Lee and Zhang 2015). LasI and RhlI are acyl-homoserine lactone synthases that produce QS signal molecules, 3-oxo-C12-AHL or C4-AHL, respectively. RT-qPCR (Reverse-Transcription coupled to quantitative Polymerase Chain Reaction) analysis showed that the plasmid-derived *pumA* mRNA level decreases in the *P. aeruginosa* *lasI/rhlI* double mutant strain compared to the parental strain (Hernández-Ramírez et al. 2020). In the same study, the authors demonstrated that in the *lasI/rhlI* double mutant, virulence conferred by *pumA* overexpression decreases in a lettuce leaf or in *Caenorhabditis elegans* infection models. Interestingly, extracellular addition of 3-oxo-C12-AHL or C4-AHL restored *pumA* mRNA relative expression levels, and *pumA* overexpression conferred virulence in *C. elegans*. The authors showed that PumA-mediated virulence is exacerbated when 3-oxo-C12-AHL is added in the culture medium compared to the addition of C4-AHL. This suggests that the LasIR QS system is more effective to induce *pumA* mRNA expression than the RhlIR QS system. These results indicate that *pumA* gene expression and virulence mediated by PumA are QS dependent.

To summarize, in this section, we reported examples demonstrating that TA systems expression can be influenced by transcriptional regulators associated with natural competence or QS, destabilization of antitoxin–toxin ratio following phage infection, and biofilm conditions.

Influence of the host's immune response and their related stresses on TA systems expression

During infection, some bacteria survive within their host's immune cells (macrophages and neutrophils) while being confronted to various stresses such as acid stress, nutrient starvation,

or oxidative stress (Weiss and Schaible 2015). Here, we present the effects of these stresses on TA systems expression in ESKAPEE or non ESKAPEE pathogens (Table 2). ESKAPEE refers to seven pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, *Enterobacter* spp., and *E. coli*) that are often responsible for healthcare-associated infections and have developed resistance to many antibiotics (Pendleton et al. 2013).

Influence of the host's immune system on TA systems expression in ESKAPEE

Staphylococcus aureus

S. aureus, a Gram-positive bacterium, is a major public health threat causing a wide range of infections (Tong et al. 2015). Several studies have investigated the effect of different stresses encountered by *S. aureus* during host infection on the RNA levels of type I TA systems. In this bacterium, two major type I TA systems have been extensively characterized: the SprA/SprA_{AS} and the SprG/SprF systems, which are present in two or four copies in the *S. aureus* genome, respectively (Sayed et al. 2012, Germain-Amiot et al. 2019, Riffaud et al. 2019). The expression of the SprA1_{AS} antitoxin RNA, which belongs to the SprA1/SprA1_{AS} TA system, decreases by 25% after acid stress and by 50% under oxidative stress (Sayed et al. 2012). Consequently, SprA1 toxin level is upregulated under acid and oxidative conditions, even though no variation in *sprA1* RNA level is observed (Sayed et al. 2012). Since SprA1 exhibits cytolytic effects on human cells, the authors suggested that under the acid and oxidative conditions predominant in the phagolysosomes of host immune cells, the decreased SprA1_{AS} level promotes SprA1 expression. This could lead to *S. aureus* lysis, release of SprA1 toxins in the phagolysosome, and the destruction of the host cell membrane. For the SprA2/SprA2_{AS} TA system, SprA2_{AS} RNA levels decrease during nutritive starvation or after osmotic stress (Germain-Amiot et al. 2019). Since SprA1_{AS} RNA level are not impacted by osmotic stress (Sayed et al. 2012), this indicates that these homologous TA systems do not respond to the same triggers. Moreover, SprA2_{AS} RNA levels decrease at low temperature (18°C) but are upregulated under acid stress. Unlike SprA2_{AS}, there is no difference in *sprA2* mRNA levels under nutritive starvation. However, *sprA2* mRNA levels significantly decrease after oxidative stress, which is not the case for the antitoxin (Germain-Amiot et al. 2019). Thus, *sprA2* and SprA2_{AS} RNA expression are driven by different stresses. The effect of stresses on the expression of the four SprG/SprF homologous TA systems (SprG1/SprF1, SprG2/SprF2, SprG3/SprF3, and SprG4/SprF4) was also investigated in the *S. aureus* HG003 strain (Riffaud et al. 2019). RNA expression levels of the SprF antitoxins decrease during osmotic and oxidative stresses. Additionally, SprF1 and *sprG1* RNA levels decrease after *S. aureus* internalization in THP-1 macrophages. However, as *sprG1* mRNA levels are not influenced by oxidative stress, this suggests that the decrease of *sprG1* mRNA levels in macrophages is due to another trigger that remains to be uncovered. Future studies are needed to investigate the effect of these stresses, encountered by *S. aureus* during host infection, on the levels of the cytolytic toxins SprA2, SprG1₃₁, and SprG1₄₄. It can be speculated that the release of these toxins may represent an “altruistic behavior” in which the toxin-producing cells sacrifice themselves to provide nutrients for the remaining population, thereby promoting the spread of the host infection. Furthermore, a separate study performed in *S. aureus* N315 strain demonstrated that during hyperosmotic stress, the level of SprF1 RNA increases due to enhanced stability. Subsequently, SprF1 ac-

Table 2. Impact of host stresses on TA systems expression.

Bacteria	TA system	Type	Stress	Mechanism of regulation	References
ESKAPEE <i>S. aureus</i>	SprA1/SprA1 _{AS}	I	Acid and oxidative stresses	Downregulation of SprA1 _{AS} RNA level and upregulation of SprA1 mRNA level	Sayed et al. (2012)
	SprA2/SprA2 _{AS}	I	Starvation and osmotic stresses	Downregulation of SprA2 _{AS} RNA level	Germain-Armiot et al. (2019)
<i>P. aeruginosa</i>	SprC1/SprF1, SprG2/SprF2, SprG3/SprF3	I	Osmotic and oxidative stresses	Downregulation of SprF antitoxin RNA level (except SprF4)	Riffaud et al. (2019)
	SprC1/SprF1	I	Hyperosmotic stress	Upregulation of SprF1 RNA level	Pinel-Marie et al. (2021)
	PacTA	II	Iron starvation	Upregulation of pacT mRNA level and PacT protein level	Song et al. (2022)
	HigBA HokB/SokB	II	Antimicrobial peptide (LL-37) Stringent response	Transcriptional upregulation Upregulation of hokB mRNA level	Song et al. (2024) Verstraeten et al. (2015)
<i>E. coli</i>	MazEF RelBE	II	Amino acid starvation	Upregulation of mRNA level	Christensen et al. (2001, 2003)
	HicAB	II	Amino acid starvation	Upregulation of mRNA level	Jørgensen et al. (2009)
	MazEF, CptAB	II	Bile salt, acid stress, and macrophages	Upregulation of toxin mRNA level	Bustamante and Vidal (2020)
Non-ESKAPEE <i>Salmonella</i>	14 putative TAS	II	Nutrient starvation, macrophages	Upregulation of mRNA level	Helaine et al. (2014)
	Hok/Sok, LdrA/RdlA, TisB/IstR-1 T2 _{ST} /T4 _{ST} /T5 _{ST} , VapBC2	I	Fibroblasts epithelial cells	Upregulation of toxin mRNA and protein levels	Lobato-Márquez et al. (2015)
<i>M. tuberculosis</i>	RelBE	II	Fibroblasts epithelial cells	Upregulation of toxin mRNA and protein levels	Lobato-Márquez et al. (2015)
	HigBA1/BA2, VapBC31/BC46, MazEF1/EF5, UcAT10 MbcTA	II	Nitrogen-limiting and oxidative stresses Chemical and/or nutritional stresses	Upregulation of mRNA level	Korch et al. (2015) Gupta et al. (2017)
<i>Helicobacter pylori</i>	AapA1/IsoA1	II	Nutrient starvation, oxidative or nitric stresses, macrophages	Transcriptional upregulation of MbcA	Aniyachaokum et al. (2020)
<i>Xenorhabdus nematophila</i>		I	Oxidative stress	Transcriptional downregulation and degradation of IsoA1 antitoxin	El Mortaji et al. (2020)
	HipBA ^{Xn2}	II	Nutrient starvation, heat shock	Transcriptional upregulation	Yadav and Rathore (2022)

cumulates on polysomes to attenuate protein synthesis (Pinel-Marie et al. 2021).

Interestingly, it was revealed that the transcriptional regulator SarA positively regulates the *mazEF* type II TA system (Donegan and Cheung 2009). Additionally, a study expanded the SarA regulon using transcriptomic and chromatin immunoprecipitation approaches (Oriol et al. 2021). Among the newly identified SarA targets, two genes belonging to type I TA systems were identified: *sprG2*, encoding the SprG2 toxin and belonging to the SprG2/SprF2 TA system, and *sprA2_{AS}*, encoding the SprA2_{AS} antitoxin from the SprA2/SprA2_{AS} TA system. In a *sarA* deleted strain, northern blot experiments demonstrated that *sprG2* and SprA2_{AS} RNA levels increase. Also, EMSA (Electrophoretic Mobility Shift Assay) showed that SarA can directly and specifically bind *sprG2* and *sprA2_{AS}* promoters. These results demonstrate that SarA represses *sprG2* and *sprA2_{AS}* expression by binding to their promoters. Interestingly, under the conditions used in this study, the homologous TA systems, SprG1/SprF1, SprG3/SprF3, SprG4/SprF4, and SprA1/SprA1_{AS}, were not regulated by SarA. This indicates that homologous systems do not necessarily belong to the same regulon, complexifying our understanding of the TA systems regulatory network. Therefore, exploring the link between SarA, host immune stresses, and TA systems regulation could be of great interest.

Pseudomonas aeruginosa

The Gram-negative bacterium, *P. aeruginosa*, is a common environmental organism and a significant opportunistic pathogen, particularly in patients with cystic fibrosis, due to its ability to form biofilms (Laborda et al. 2022). So far, 10 type II TA systems have been experimentally characterized in this species (Li et al. 2023a). Type II TA systems are generally organized in operons, with two distinct genetic organization (Jurénas et al. 2022). In one organization, the antitoxin gene is encoded before the toxin gene, and the locus is transcribed from a single promoter. In the other, the toxin gene is upstream of the antitoxin gene, with two promoters present: one for the entire locus and the second, located within the toxin gene, responsible for antitoxin gene expression. These genetic organizations are autoregulated thanks to the DNA-binding domain of the antitoxins, which acts as transcriptional repressors. When the toxin:antitoxin ratio is in favor of the antitoxin, the toxin assists the antitoxin to repress the operon; however, when the toxin concentration is high, derepression occurs, a phenomenon known as “conditional cooperativity” (Jurénas et al. 2022). A recent study showed that an excess of toxin leads to a transition from an hexameric TA complex to an octameric complex, resulting in DNA deformation and operon derepression (Grabe et al. 2024). In *P. aeruginosa*, the *pacTA* locus, encoding the PacTA type II TA system, is located on the chromosome and is composed of the toxin gene upstream the antitoxin gene (Li et al. 2023a). This system consists of the PacA antitoxin and the PacT toxin, containing a GCN5-related N-acetyltransferase domain, that can arrest translation via tRNAs acetylation. Under iron starvation, the growth of *pacTA* or *pacT* mutant strains is impaired compared to the parental strain (Song et al. 2022). Moreover, proteome analysis showed that in a *pacTA* deletion mutant strain, iron uptake genes, such as *feoC* and *feoB* (encoding ferrous iron transport proteins) or *pigA* (encoding a heme oxygenase) are downregulated. These genes are under the control of Fur, the major regulator of iron homeostasis, which represses genes involved in iron import and storage. Interestingly, the PacT toxin can bind the Fur repressor, attenuating its DNA-binding ability. These results indicate that the PacTA TA system is involved in iron home-

ostasis. During iron starvation, *pacT* expression is induced, allowing the PacT toxin to associate with Fur. This complex prevents Fur to binding its regulon, thus leading to an activation of genes involved in iron import and storage. However, how the *pacTA* promoter senses iron starvation is not elucidated yet.

To combat invading bacteria, the host innate immune system produces antimicrobial compounds such as the LL-37 peptide. In *P. aeruginosa*, recognition of LL-37 by the CprS sensor, a component of the CprRS two-component system, triggers the phosphorylation of the CprR regulator (Song et al. 2024). Upon activation, this regulator binds a palindromic region within the *higBA* type II TA system locus, increasing the production of the endonuclease HigB toxin. Overexpression of HigB subsequently induces the expression of a type III secretion system, enabling the bacteria to modulate the host immune response.

Escherichia coli

E. coli is a Gram-negative bacterium commonly found in intestinal flora of humans and animals. However, pathogenic strains, such as the enterotoxigenic *E. coli* and enterohemorrhagic *E. coli*, are responsible for human diseases. Under environmental stress conditions, such as nutrient starvation, *E. coli* induces the stringent response. This response is mediated by the (p)ppGpp alarmone (3',5'-bispyrophosphate) and affects vital cellular processes, enabling the bacteria to adapt to these stressful conditions (Irving et al. 2021). (p)ppGpp is synthesized and hydrolyzed by proteins belonging to the RSH superfamily (RelA/SpoT homologues), which includes small alarmone synthetases, and small alarmone hydrolases. (p)ppGpp allows the expression of the type II *mazEF* TA system in *E. coli* (Aizenman et al. 1996). Artificially increasing (p)ppGpp levels by inducing the *relA* gene with IPTG leads to *mazEF* RNA expression and subsequently bacterial death. However, some studies demonstrated that transcription of *mazEF* and *relBE* genes is upregulated upon amino acid starvation in a (p)ppGpp-independent manner (Christensen et al. 2001, 2003). These studies suggest that chromosomally encoded TA systems may act as stress response elements, reducing global translation during nutritional stress independently of (p)ppGpp.

Further research has revealed a link between the stringent response and TA systems regulation. In *E. coli*, transcription of the *hicAB* type II TA system is upregulated under amino acid starvation (Jørgensen et al. 2009), and the expression of the *hokB* type I toxin gene is induced by (p)ppGpp in an ObgE-dependent manner (Verstraeten et al. 2015). Overexpression of *obg* gene, encoding the ObgE GTPase, requires (p)ppGpp to enhance persister cells formation in *E. coli*. Interestingly, *hokB* deletion abolished the ObgE-mediated persistence phenotype, demonstrating that ObgE mediates persistence by activating *hokB* transcription. The HokB peptide, composed of 49 amino acid residues, forms pores in the membrane, leading to membrane depolarization, ATP leakage, and persister cells formation (Fig. 2) (Wilmaerts et al. 2018). Recently, the mechanism involved in HokB pore formation was deciphered (Wilmaerts et al. 2019). The periplasmic C46 residue of HokB is essential for dimerization and pore formation, facilitated by the periplasmic oxidoreductase DsbA, which forms a disulfide bond between two HokB C46 residues. Consequently, membrane depolarization, ATP leakage, and persister cells formation do not occur in a cysteine 46-to-serine substitution mutant, where pore formation is abolished. HokB monomerization by the DsbC oxidoreductase and, then, degradation by the DegQ protease, are responsible for pore disassembly, membrane repolarization, and ATP production contributing to HokB persister cells awakening (Wilmaerts et al. 2019).

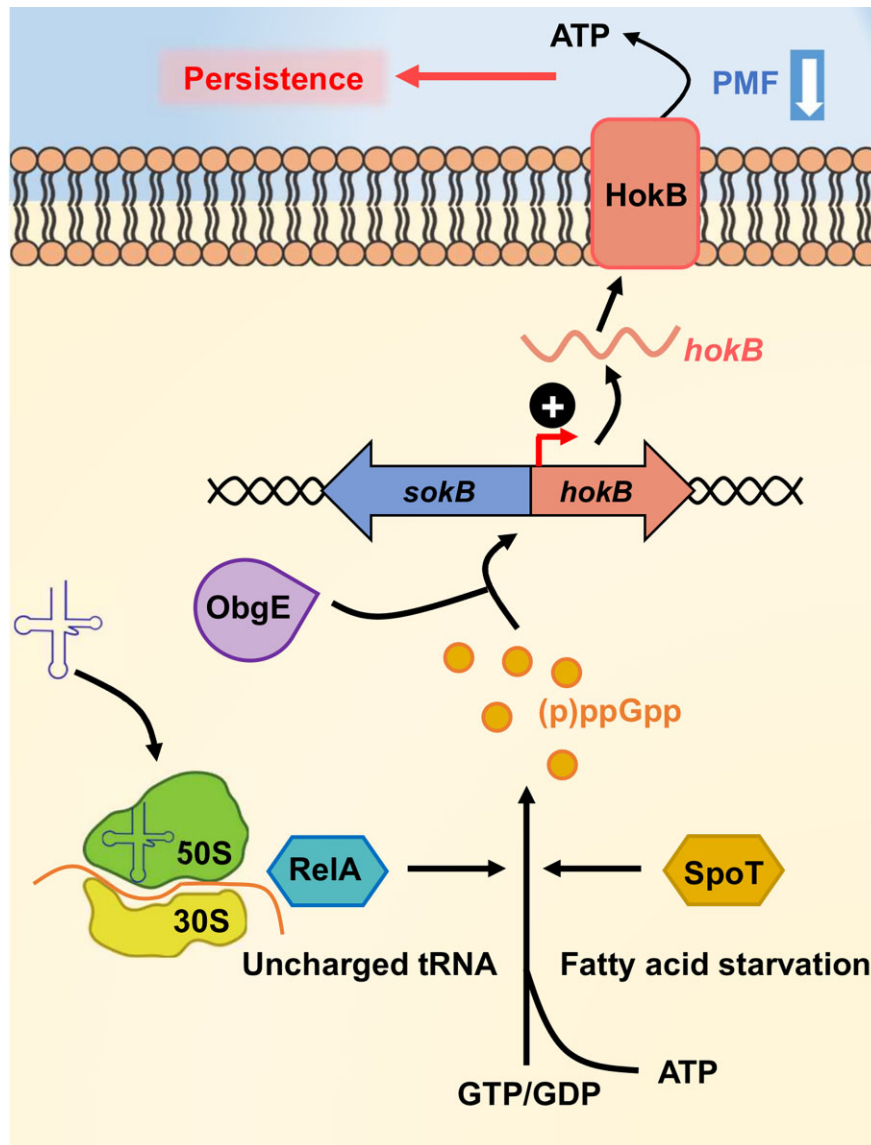


Figure 2. ObgE GTPase and stringent response activate *hokB* transcription in *E. coli*. Interaction between ObgE GTPase and (p)ppGpp alarmone, produced by RelA or SpoT synthetases under nutrient starvation, increases *hokB* transcription. Thus, HokB toxin, by causing pores formation, ATP depletion, and membrane depolarization, is involved in bacterial persistence. PMF: proton motive force.

A recent study investigated the toxin expression levels under *in vitro* stress conditions encountered by adherent-invasive *E. coli* (AIEC) in the intestine and within macrophages (Bustamante and Vidal 2020). AIEC is proposed as a possible agent triggering Crohn's disease and characterized by its ability to adhere and invade epithelial cells and survive and replicate inside macrophages. Under *in vitro* bile salt and acid stress conditions or within macrophages, AIEC strains respond by inducing the expression of various toxin genes, such as *ccdB*, *yafO*, *parE*, *yoeB*, *mazF*, *cptA*, *hipA1*, and *ortT* (Bustamante and Vidal 2020). The authors found that toxin genes upregulated within macrophages, such as *mazF*, *cptA*, and *ortT*, do not necessarily respond to acid stress *in vitro*, suggesting that they may respond to different stresses within the intramacrophage environment. Moreover, in *A. baumannii*, *cptAB* genes are down-regulated under oxidative and antibiotic stress (ElBanna et al. 2021), emphasizing the variability within TA systems and the need to study each system in its natural genetic context to decipher its contribution to bacterial physiology. The study also revealed that the *ghoT* gene was upregulated under acid stress and in-

tramacrophage conditions but downregulated in response to bile salts. These findings suggest that TA genes can respond to different stress conditions and that activation of various toxin genes by diverse intramacrophage stresses collectively contribute to AIEC survival.

Influence of the host's immune system on TA systems expression in non-ESKAPEE

Salmonella

Salmonella, an intracellular pathogen, belongs to the *Enterobacteriaceae* family with some species within the genus responsible for salmonellosis after ingestion of contaminated food. The *Salmonella* genome contains 14 putative type II TA systems, all of them exhibit increased mRNA levels after phagocytic uptake in bone marrow-derived macrophages (Helaine et al. 2014). This upregulation is related to the activation of the stringent response, mediated by the production of (p)ppGpp by the RelA and SpoT synthetases after *Salmonella* internalization by macrophages. In fact, in

a *relA/spoT* double mutant, these 14 TA systems are no longer overexpressed. In parallel, transient acidification of the culture medium or starvation induced by serine hydroxamate also activates the expression of both antitoxin and toxin transcripts for these 14 TA systems. Thus, the activation of these systems by stresses encountered within macrophages can promote persister cells formation. In a typhoid fever mouse model, the authors demonstrated that the ShpAB type II TA system contributes to the formation of persister cells (Helaine et al. 2014).

Another study confirmed that *S. Typhimurium* upregulates functional toxins encoded by type I (Hok, LdrA, and TisB) and type II (T2_{ST}, T4_{ST}, T5_{ST}, and VapC2) TA systems in fibroblasts, using RT-qPCR and western blot analysis (Lobato-Márquez et al. 2015). Deletion mutants of *hok-sok*, *ldrA-rdIA*, *tisB-istR*, *ta4*, and *vapBC2* showed reduced intracellular survival of *Salmonella* inside fibroblasts. Notably, only the *vapBC2* deletion mutant exhibited reduced intracellular survival within HeLa epithelial cells (Lobato-Márquez et al. 2015). Collectively, these results demonstrate that the type I toxins Hok, LdrA, and TisB and the type II toxins T4_{ST} and VapC2, promote *Salmonella* survival inside fibroblasts and epithelial cells.

Mycobacterium tuberculosis

Mycobacterium tuberculosis, a member of the *Mycobacteriaceae* family, is the causative agent of tuberculosis. RT-qPCR analysis has shown that the TA genes of the RelBE family are highly expressed under nitrogen-limiting and oxidative stress conditions, and downregulated under hypoxia (Korch et al. 2015). A genome-wide analysis revealed that the genes *higBA1*, *higBA2*, *vapBC31*, *vapBC46*, *mazEF1*, *mazEF5*, and *ucAT10* are highly expressed under chemical and/or nutritional stress conditions (Gupta et al. 2017). The transcriptional regulation of the type II MbcTA TA system has been studied (Ariyachaokun et al. 2020). The *mbcTA* locus encodes the MbcA antitoxin and the MbcT toxin, which leads to phosphorylation of NAD⁺ to trigger bacterial death (Freire et al. 2019). Using the *mbcA* antitoxin promoter and a fluorescent reporter system, the authors demonstrated that *mbcA* promoter expression increases upon nutritive starvation, oxidative, or nitric stresses (Ariyachaokun et al. 2020). The *mbcA* promoter is also induced after phagocytosis in human or murine macrophages. It is likely that the activation of the *mbcA* promoter in macrophages is due to nutritive starvation, oxidative or nitric stresses encountered by *M. tuberculosis* after its internalization. Although individual deletions of TA genes have not been found to impair bacterial survival in a mouse infection model (Singh et al. 2010), this does not rule out a role for TA loci in *M. tuberculosis* infection and survival in the human host.

Helicobacter pylori

Helicobacter pylori, a Gram-negative bacterium, represents a significant risk for the development of stomach cancer due to its ability to survive in an acidic environment (Camilo et al. 2017). In the gastric mucosa, *H. pylori* is also challenged by reactive oxygen species (ROS) produced by the host. Using northern blot analysis and by cloning the promoter of AapA1/IsoA1 type I TA system genes fused with a β -galactosidase reporter, it was shown that in response to oxidative stress, the promoter activity of the IsoA1 antitoxin decreases while IsoA1 transcript processing increases. Consequently, *H. pylori* depletes the RNA antitoxin IsoA1, leading to increased AapA1 production. AapA1 is a membrane toxin composed of 30 amino acid residues that facilitates the morphological transformation from spiral-shaped to coccoid cells, which are dormant forms of *H. pylori* (El Mortaji et al. 2020). Therefore, AapA1

is part of the *H. pylori*'s survival strategy within the stomach and potentially contributes to persistent infections.

Xenorhabdus nematophila

The study of TA systems expression is not limited to human pathogenic bacteria. In *Xenorhabdus nematophila*, an entomopathogenic bacterium, the transcriptional regulation of the type II HipBA^{Xn2} TA system has been investigated (Yadav and Rathore 2022). The two genes are organized in an operon with the *hipB* antitoxin gene located upstream of *hipA* toxin gene, encoding a serine/threonine kinase. Transcriptional regulation of the HipBA^{Xn2} TA system under stress conditions was analyzed by RT-qPCR and by cloning the promoter of these genes in fusion with a β -galactosidase reporter. Various stresses, such as nutritive starvation or heat shock, were shown to upregulate *hipBA^{Xn2}* promoter activity. Similarly, under these same stressful conditions, RT-qPCR experiments confirmed that *hipA^{Xn2}* and *hipB^{Xn2}* transcript levels were upregulated. These results suggest that this TA system may be involved in *X. nematophila*'s adaptation to stressful conditions (Yadav and Rathore 2022).

In this part, we have listed the stresses encountered by pathogens in different niches inside their hosts, such as nutrient starvation, bile salts, low pH conditions, and oxidative stress, to which TA systems respond. Hence, TA systems could modulate bacterial physiology and consequently play a crucial role in bacterial virulence and pathogenesis.

Influence of xenobiotics exposure on TA systems expression

In this third section of the review, we will discuss the effect of xenobiotics on the regulation of TA systems expression in ESKAPEE and non-ESKAPEE pathogens. The examples presented in this section are summarized in Table 3.

Influence of xenobiotics exposure on TA systems expression in ESKAPEE

Staphylococcus aureus

In *S. aureus*, it was elegantly demonstrated that SprF1 is a dual-function type I antitoxin. With its 3'-end, SprF1 acts as an antitoxin to counteract SprG1 toxicity against competing bacteria and host cells (Pinel-Marie et al. 2014). Additionally, thanks to a purine-rich sequence located at its 5'-end, SprF1 interacts with a subset of polysomes and ribosomes, potentially promoting translation attenuation and antibiotic persister cells formation (Pinel-Marie et al. 2021). In this study, the authors observed that the level of SprF1 RNA increased following vancomycin exposure, while it remained stable when subjected to ciprofloxacin treatment. Conversely, the level of *sprG1* RNA substantially decreased. These findings emphasize the response of *S. aureus* to antibiotic exposure, where the reduction of SprG1-encoded peptide toxicity is essential for improving the formation of antibiotic persister cells, achieved by increasing SprF1 RNA antitoxin level.

Klebsiella pneumoniae

Klebsiella pneumoniae is an opportunistic Gram-negative pathogen known to cause hospital-acquired infections. As TA systems are stress responsive modules, the expression of various type II TA systems after subinhibitory exposure to different antibiotics has been explored (Narimisa et al. 2020). Using RT-qPCR, the authors demonstrated that exposure of *K. pneumoniae* to nalidixic acid or ceftazidime results in a decrease in the expression of all the type II

Table 3. Effect of xenobiotics exposure on TA systems expression.

Bacteria	TA system	Type	Xenobiotic	Mechanism of regulation	References
ESKAPEE <i>S. aureus</i>	SprC1/SprF1	I	Vancomycin	Upregulation of SprF1 RNA level, downregulation of sprG1 mRNA level	Pinel-Marie et al. (2021)
<i>K. pneumoniae</i>	RelEB1, RelEB2, MazEF, VapBC, HipBA, doc/phd	II	Nalidixic acid, ceftazidime	Downregulation of mRNA level	Narimisa et al. (2020)
	RelEB1, MazEF, HipBA, doc/phd	II	Gentamicin	Upregulation of mRNA level	Narimisa et al. (2020)
	RelEB2, VapBC	II	Gentamicin	Downregulation of mRNA level	Narimisa et al. (2020)
<i>A. baumannii</i>	KacAT	II	Meropenem	Upregulation of mRNA level	Li et al. (2023b)
	HicAB	II	Ciprofloxacin	Upregulation of mRNA level	Kashyap et al. (2021)
	HigBA2	II	Rifampicin	Downregulation of mRNA level	Armalyté et al. (2018)
	CptBA	II	Ciprofloxacin, meropenem	Downregulation of mRNA level	ElBanna et al. (2021)
<i>E. coli</i>	TisB/istR-1	I	Ciprofloxacin, ofloxacin (SOS response)	Upregulation of tisB mRNA level	Dörr et al. (2010), Cayron et al. (2024)
	HipBA	II	Nanoalumina	Upregulation of hipB mRNA level	Wang et al. (2022)
Non-ESKAPEE <i>V. cholerae</i>	HipBA, MazEF, YefM-YoeB	II	Antidepressants	Upregulation of mRNA level	Wang et al. (2023b)
<i>Brucella</i>	HigBA	II	Chloramphenicol, kanamycin, spectinomycin	Upregulation of mRNA level	Budde et al. (2007)
	RelE/RHH-Like, Fic/Phd, BrnT/BrnA	II	Gentamicin	Upregulation of mRNA level	Amraei et al. (2020)
<i>H. pylori</i>	HP0315/HP0316, HP0892/HP0893, HP0894/HP0895, HP0967/HP0968	II	Chloramphenicol, kanamycin	Upregulation of mRNA level	Cárdenas-Mondragón et al. (2016)
	Rel family	II	Rifampicin, gentamycin, levofloxacin	Upregulation of rel mRNA level	Provedvi et al. (2009), Singh et al. (2010), Miallau et al. (2013)

TA systems studied (*relE1/relB1*, *relE2/relB2*, *vapC/vapB*, *mazF/mazE*, *hipA/hipB*, and *doc/phd*). Interestingly, when exposed to gentamicin, the type II TA systems studied did not show the same expression profile: *relE1/relB1*, *mazF/mazE*, *hipA/hipB*, and *doc/phd* were upregulated, whereas *relE2/relB2* and *vapC/vapB* expression decreased. This study suggests that homologous TA systems can respond differently to the same antibiotic stress, as *relE1/relB1* expression is upregulated by gentamicin while *relE2/relB2* is downregulated. The KacAT type II TA system provides another example of a TA system induced by antibiotics in *K. pneumoniae*. Specifically, treatment with meropenem elevates the transcript levels of *kacA* and *kacT* (Li et al. 2023b). The authors elucidated that meropenem treatment enhances the production of the Lon protease, leading to the degradation of the KacA antitoxin. Consequently, with the TA molecular ratio favouring the toxin, repression of the *kacAT* promoter is relieved, resulting in increased transcription of *kacA* and *kacT*. These findings provide light on how the TA ratio is controlled under antibiotic exposure to modulate TA systems transcription.

Acinetobacter baumannii

A. baumannii is an emerging threat due to extensive antimicrobial resistance (Ibrahim et al. 2021) and is ranked as a critical priority for the development of new therapeutic strategies by the WHO (Tacconelli et al. 2018). Like other pathogens, *A. baumannii* can form persister cells, which complicates treatments (Kashyap et al. 2021). A transcriptomic study was performed to decipher genes driving survival and persistence of *A. baumannii* after ciprofloxacin exposure (Kashyap et al. 2021). Ciprofloxacin induces DNA damages, leading to the activation of the SOS response for DNA repair. Under normal growth conditions, LexA binds the promoters of SOS response genes to repress their expression. When single-stranded DNA is detected in the bacterium, RecA is activated, leading to LexA cleavage. As expected, genes involved in the SOS response, including *recA*, *umuC*, *umuD*, and *ddrR*, were upregulated after ciprofloxacin treatment in *A. baumannii* (Kashyap et al. 2021). Moreover, the authors demonstrated that the *hicAB* transcript, part of the type II HicAB TA system encoding the HicA endonuclease, is upregulated. These results suggest that ciprofloxacin-induced *hicAB* gene expression is controlled by the SOS response. Another study examined the effect of antibiotics on the RNA levels of the HigBA type II TA system. This system is composed of the HigB endoribonuclease toxin and the HigA antitoxin, which is present in two copies in *A. baumannii*: *higBA1* being located on the chromosome and *higBA2* on the pAB120 plasmid (Armalyt e et al. 2018). The authors observed that treatment with gentamicin or meropenem did not modulate the RNA levels of *higB2* or *higA2*, whereas rifampicin induced a decrease in *higB2* and *higA2* RNA levels. As mentioned earlier, treatment with ciprofloxacin or meropenem downregulates the RNA levels of *cptA* antitoxin and *cptB* toxin (ElBanna et al. 2021). These data suggest that TA systems expression is specifically influenced by different antibiotics.

Escherichia coli

As previously mentioned for the *hicAB* TA system expressed by *A. baumannii*, the first and well-described TA system under the control of the SOS response in *E. coli* is the *tisB/istR-1* type I TA system (Vogel et al. 2004). This system was discovered in 2001 through genome-wide analysis (Argaman et al. 2001, Wassarman et al. 2001). In 2004, it was shown that the TisB toxin is produced from the *tisAB* mRNA, whose expression is controlled by LexA and the SOS response (Vogel et al. 2004). The authors demonstrated that in a *E. coli* K-12 WT strain the deletion of either *istR-1*, *tisAB*, or the entire *istR-1-tisAB* locus has no effect on bacterial

growth. Conversely, in a *E. coli* K-12 *lexA51*-deficient strain with a constitutive SOS response, deletion of *istR-1* was impossible. Thus, while the *istR-1* antitoxin is constitutively expressed from a sigma 70 promoter, transcription of *tisAB* is under the control of the SOS response regulator LexA. Other studies demonstrated that ciprofloxacin SOS-dependent induction of *tisB* is associated with persistence (D orr et al. 2010) (Fig. 3). After ciprofloxacin treatment, DNA damage leads to SOS response activation and *tisB* mRNA translation. TisB, a 29 amino acid pore-forming toxin, provokes membrane damage, proton motive force breakdown, and ATP depletion, contributing to persister cells formation. Furthermore, using an *E. coli* mutant in which *tisB* expression no longer relies on the SOS response, resulting in increased persister formation, the authors observed differential responses to antibiotics within the same family (Edelmann et al. 2021). While *tisB* overexpression confers protection against ciprofloxacin, it paradoxically increases susceptibility to mitomycin C, a DNA-damaging agent. Recent findings also indicate that the TisB toxin orchestrates metabolic disruptions following ofloxacin treatment, a DNA gyrase inhibitor (Cayron et al. 2024). Upon activation of the SOS response by ofloxacin, *tisB* expression is induced. Subsequently, TisB promotes cytoplasmic condensation, followed by proton motive force collapse and intracellular H₂O₂ accumulation. Apart from the *tisB/istR-1* TA system, the SOS response induces other type I TA systems in *E. coli*, such as *symE/symR*, *hokE/sokE*, and *dinQ/agrB* (Fern andez De Henestrosa et al. 2000, Weel-Sneve et al. 2013). In these systems, only the toxin gene is regulated by the SOS response. Conversely, in the *yafN/yafO* type II TA system of *E. coli*, both antitoxin and toxin genes are upregulated by the SOS response (Singletary et al. 2009).

The chromosomally encoded type I TA system ZorO–OrzO consists of the ZorO toxin and OrzO antitoxin. In *E. coli*, *zorO–orzO* overexpression improved bacterial growth in the presence of aminoglycosides and increased the minimum inhibitory concentration against these antibiotics (Bogati et al. 2021). These effects were not observed for other antibiotics tested, suggesting that the system plays a role in the response to aminoglycosides and highlights that this system is to some extent antibiotic-specific. However, endogenous expression of this system under aminoglycoside stress was not studied. Thus, the regulation mechanism of the *zorO–orzO* locus under these conditions remains to be deciphered (Bogati et al. 2021).

Given the growing emergence of multidrug-resistant bacteria, new therapeutic strategies are needed to fight bacterial infections. Among these strategies, nanomaterials with bactericidal effects could be used (Wang et al. 2022). However, as with antibiotics, some bacteria can survive to nanomaterial exposure. It has been shown that ROS production and QS are involved in *E. coli* persister cells formation after nanoalumina treatment (Wang et al. 2022). RT-qPCR analysis demonstrated that *hipB* toxin gene expression was significantly upregulated after nanoalumina treatment. This result suggests that this toxin may participate in persister cells formation, although its deletion has no effect on the persisters fraction, indicating that more than a single player is involved in persister formation following nanoalumina treatment.

Recently, the effect of drugs not used for treating bacterial infections but to which bacteria may be exposed while living in their host's body, was tested on bacterial resistance and persistence. For instance, antidepressants, widely consumed drugs, could contribute to antibiotic resistance and persistence in *E. coli* (Wang et al. 2023b). Using genome-wide RNA sequencing, the authors showed that toxin and antitoxin genes of, among others, HipBA, MazEF, and YefM–YoeB type II TA systems are upregulated when

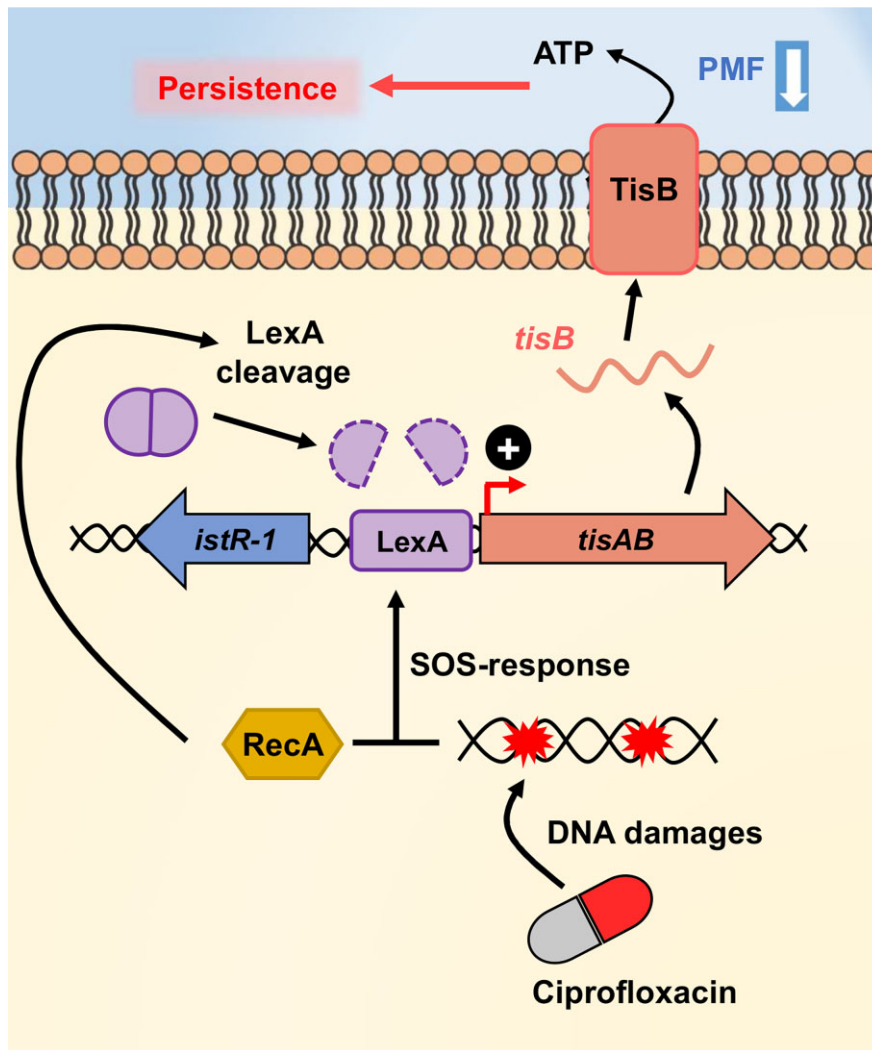


Figure 3. The SOS response activates *hokB* transcription in *E. coli*. Ciprofloxacin treatment induces DNA damages and SOS response activation. Following LexA cleavage by RecA, *tisB* mRNA is transcribed and the TisB toxin is produced. TisB acts as a pore-forming toxin causing membrane damages, ATP depletion, proton motive force (PMF) breakdown and, subsequently, persister cells formation.

bacteria are exposed to Sertraline or Duloxetine, two antidepressant drugs.

Influence of xenobiotics exposure on TA systems expression in non-ESKAPEE

Vibrio cholerae

The Gram-negative bacterium *V. cholerae* is responsible for the diarrheal disease cholera. In *V. cholerae*, several putative TA systems were found in a superintegron on chromosome II. Among them, the expression of the *higBA* type II TA system was studied (Budde et al. 2007). Like other type II TA systems, *higBA* genes are transcribed from the same promoter that is repressed by HigA. The transcription of *higBA* is induced after exposure to chloramphenicol, kanamycin, and spectinomycin (Budde et al. 2007). However, endogenous activation of *higB* gene under these conditions is not bactericidal, raising questions about the role of HigA activation after antibiotic exposure in *V. cholerae*. Interestingly, in *A. baumannii*, gentamicin, an aminoglycoside belonging to the same family as kanamycin, has no effect on *higBA2* expression (Kashyap et al. 2021). Thus, in different bacterial species, an homologous TA system can have different regulation patterns. Moreover, since *higBA*

is upregulated by ciprofloxacin in *C. crescentus* (Qi et al. 2021), it would be relevant to study the effect of ciprofloxacin on *higBA* expression in *V. cholerae*.

Brucella spp

Brucella, an intracellular pathogen, is the causative agent of brucellosis. The effect of gentamicin on TA systems expression was studied by RT-qPCR on fifty *Brucella* spp isolates (Amraei et al. 2020). The authors showed that the RNA levels of three type II TA systems (RelE/RHH-Like, Fic/Phd, and BrnT/BrnA) were upregulated after gentamicin exposure. Surprisingly, upregulation occurs for both toxin and antitoxin transcripts. Since it has been shown that Lon protease is induced by aminoglycosides in *P. aeruginosa* (Marr et al. 2007), it would be interesting to analyse the antitoxin level after gentamicin challenge in a Lon-deficient mutant to determine if Lon protease degrades antitoxins and promotes toxin action in *Brucella*.

Helicobacter pylori

Using RT-qPCR, expression of four type II TA systems (HP0315/HP0316, HP0892/HP0893, HP0894/HP0895, and HP0967/HP0968) belonging to the Vap family was investigated

following antibiotic treatments (ampicillin, chloramphenicol, tetracycline, and kanamycin) (Cárdenas-Mondragón et al. 2016). Chloramphenicol and kanamycin, which interfere with protein synthesis, induce an upregulation in the expression of genes encoding the toxin or the antitoxin of the four TA systems studied. In contrast, ampicillin, which inhibits cell wall synthesis, has no effect on *hp0894/hp0895* genes and decreases *hp0967/hp0968* expression. Moreover, for the two other TA systems, ampicillin upregulates the toxin gene but has no effect on the antitoxin gene expression.

Mycobacterium tuberculosis

In *M. tuberculosis*, some studies revealed that *rel* toxin genes (*relE*, *relG*, and *relK*) are upregulated in response to rifampin, gentamycin (*relG* and *relK*), and levofloxacin (*relG* and *relK*), contributing to antibiotic persistence and increased survival in a drug- and toxin-specific manner (Provvedi et al. 2009, Singh et al. 2010, Millallau et al. 2013).

Collectively, these results demonstrate the complexity of TA systems regulation, as homologous systems exhibit divergent responses to identical antibiotics. Moreover, the expression of TA systems can also be influenced by other drugs, such as antidepressants.

General discussion, concluding remarks, and therapeutic opportunities

In this review, we emphasize the diversity and complexity of the mechanisms regulating TA systems expression, which leads to their activation. Bacteria encounter various environmental stresses that require rapid and precise adaptation by modulating the expression of genes and proteins. TA systems are extensively studied as general stress response modules that enable bacteria to adapt to these stresses. Environmental stresses, including antibiotics, nutrient starvation, and temperature fluctuations, can either upregulate or downregulate TA systems expression. Conceivably, due to the difference in half-life between the toxin and antitoxin components, conditions that impair antitoxin synthesis favor toxin accumulation, thereby activating the system. For example, during critical situations such as antibiotic exposure, nitrogen limitation, or oxidative stress, toxin-induced growth repression enables bacterial survival and persistence beyond the stress period. Environmental factors also regulate toxin activity through their antitoxins. For instance, the activity of the type VII TomB antitoxin is oxygen-dependent (Marimon et al. 2016). When oxygen is available, TomB enhances oxidation of its cognate toxin, HhA, introducing a negative charge and destabilizing its structure, thereby reducing its toxicity. While antibiotics generally increase TA systems expression, current studies primarily focus on type I and II TA systems, with less information available on other TA systems families. Moreover, these studies often lack a detailed description of the underlying molecular mechanisms. TA systems expression is typically measured using RT-qPCR, which cannot differentiate between transcriptional regulation and RNA stability modifications. Some studies have explored in more details the effect of antibiotics on TA systems expression. For instance, ciprofloxacin induces DNA damage, activating the SOS response, which in turn promotes the expression of *tisB* toxin mRNA (Vogel et al. 2004). Despite evidences that environmental stresses can affect TA systems expression, the role of TA systems during stress response remains controversial. Even if *mazEF* and *relBE* TA systems expression is triggered by vari-

ous stress conditions (Hazan et al. 2004), deletion of five type II TA systems in *E. coli*, including *mazEF* and *relBE*, has no impact on stress responses under amino acid starvation, acid stress or rifampicin treatment (Tsilibaris et al. 2007). These results raise questions regarding the potential role of these systems in stress adaptation. However, TA systems are present in multiple copies in bacterial genomes, and this redundancy can compensate for the deletion of certain systems. Moreover, little work has investigated the impact of TA systems regulation at the transcriptional or posttranscriptional levels in a single cell level. It is important to note that TA systems are often found in genomic islands like transposons or prophages, suggesting that they play roles in genomic conflicts that promote replicon maintenance (Jurėnas et al. 2022).

To gain deeper insight into these systems, exploring the impact of environmental stresses on TA systems expression at both transcriptional and translational levels would be insightful. For this purpose, the nFCM-TC-FlAsH strategy has been developed to monitor quantitative MqsA type II antitoxin production at the single cell level under various stresses (Wu et al. 2019). Using this method, the authors observed that bile acid stress causes MqsA degradation, whereas heat shock induces its production. This methodology could also be expanded to investigate toxin production. Another ingenious strategy, FASTBAC-Seq (Functional Analysis of Toxin-Antitoxin Systems in Bacteria by Deep Sequencing), has been employed to study TA systems chromosomal expression (Masachis et al. 2018, Le Rhun et al. 2023). This method, developed in *H. pylori* and *E. coli*, allows the identification of single-nucleotide substitutions that influence toxin expression or toxin activity inhibition. Recently, a high-throughput genetic screening method called toxin activation-inhibition conjugation (TAC-TIC) was developed to identify genes that trigger or block TA systems toxicity (Bobonis et al. 2024). This technique involves transferring genome-wide *E. coli* single-gene overexpression libraries into strains carrying the full TA system or only the toxin on inducible vectors. Colony fitness assessment of double-plasmid transconjugants reveals genes that promote a TA to inhibit growth (TAC) or prevent its action (TIC). Using this sophisticated method, researchers identified multiple triggers (*dam*, *rdgC*, *recE*, *tfaP*, *ymfH*, *RT-Eco1*, and *B21_03469*) and blockers (*racC*, *ydaW*, *yjFH*, *yjHC*, and *dicC*) derived from phage for the RcaT toxin (Bobonis et al. 2022). In contrast to type II TA systems genes, which are typically transcribed as part of operons, type I TA systems genes have their own promoters, allowing for transcription independence between toxin and antitoxin. It is worth noting that competition for transcription may occur among many type I TA systems due to their divergent promoter organization. Adjacent type I TA genes may be susceptible to RNA polymerase collisions and potential promoter interference, as RNA polymerase interacts with both DNA strands during transcription (Courtney and Chatterjee 2014). Environmental stresses can induce TA systems expression through signaling pathways such as the SOS response, stringent response, or QS. Additionally, transcription factors play a crucial role in regulating TA systems transcription. These multilayered regulatory mechanisms contribute to the complexity of TA systems expression studies.

Antibiotic resistance is a major public health threat. TA systems can contribute to this threat by promoting the formation of biofilms or persister cells, enabling bacteria to evade antibiotics (Kędzierska and Hayes 2016). Consequently, there is an urgent need for novel antimicrobial agents, with TA systems emerging as promising antibacterial targets. One effective strategy in-

volves using antimicrobial peptides derived from toxins. For instance, Pep16 and Pep19, peptides derived from the SprA1 type I toxin of *S. aureus*, have demonstrated efficacy against methicillin-resistant *S. aureus* in a mouse sepsis model (Solecki et al. 2015, Nicolas et al. 2019). ParELC3 is another example of a toxin-derived peptide (Sanchez et al. 2021). This peptide is a bacterial topoisomerase inhibitor and can be transported within the bacteria using rhamnolipid-based liposomes. Another strategy is the direct activation of the toxin to promote cell death. To this end, preventing TA interaction or blocking antitoxin expression are two potential approaches (Lee and Lee 2016, Kang et al. 2018, Równicki et al. 2020). In *M. tuberculosis*, mutation of two amino acids in the VapB2 or VapB21 antitoxins prevents their interaction with the VapC2 and VapC21 toxins, causing bacterial cell death (Chauhan et al. 2022). Thus, preventing VapB–VapC interactions could enable the development of new bactericidal antitubercular agents. Finally, combining TA systems with other therapeutic strategies has also shown promise. For instance, the CreTA type VIII TA system combined with CRISPR antimicrobials effectively eliminates multidrug resistant *A. baumannii* clinical isolates (Wang et al. 2023a). Additionally, a CcdB type II toxin-intein-based tool has been engineered to specifically target pathogenic *V. cholerae* bacteria without harming the host microbiota (López-Igual et al. 2019). TA systems could potentially be applied in anticancer therapies. For example, the Kid toxin from the Kid-Kis type II TA system induces apoptosis in human cancer cells (Turnbull et al. 2019). Beyond medicine, TA systems have potential applications in agriculture. TA systems can combat bacterial phytopathogens. For example, MqsR toxin from the MqsRA type II TA system inhibits *Xanthomonas citri* subsp. *citri* growth, reducing citrus canker symptoms in transgenic citrus plants (de Souza-Neto et al. 2022). MazEF type II TA system could also be used to develop new insecticides (Zhang et al. 2021).

In conclusion, understanding TA systems regulation under physiological conditions rather than solely through overexpression will enhance our knowledge of their biological functions. This understanding is crucial for developing new effective therapeutic strategies against antibiotic-resistant bacteria and recurrent infectious diseases, but also innovations in agriculture and medicine fields.

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