Transcriptional and Post-transcriptional Regulatory Mechanisms Controlling Type III Secretion

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Contents

Abstract Type III secretion systems (T3SSs) are utilized by numerous Gram-negative bacteria to efficiently interact with host cells and manipulate their function. Appropriate expression of type III secretion genes is achieved through the

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integration of multiple control elements and regulatory pathways that ultimately coordinate the activity of a central transcriptional activator usually belonging to the AraC/XylS family. Although several regulatory elements are conserved between different species and families, each pathogen uses a unique set of control factors and mechanisms to adjust and optimize T3SS gene expression to the need and lifestyle of the pathogen. This is reflected by the complex set of sensory systems and diverse transcriptional, post-transcriptional and post-translational control strategies modulating T3SS expression in response to environmental and intrinsic cues. Whereas some pathways regulate solely the T3SS, others coordinately control expression of one or multiple T3SSs together with other virulence factors and fitness traits on a global scale. Over the past years, several common regulatory themes emerged, e.g., environmental control by two-component systems and carbon metabolism regulators or coupling of T3SS induction with host cell contact/translocon-effector secretion. One of the remaining challenges is to resolve the understudied post-transcriptional regulation of T3SS and the dynamics of the control process.

1 Introduction

Type III secretion systems (T3SS) are complex nanomachines of Gram-negative bacterial pathogens of plants, animals and humans that export proteins (called effectors) from the bacterial cytoplasm across the cell envelope and inject them into host cells during infection (Büttner 2012; Portaliou et al. 2016; Galan et al. 2014; Deng et al. 2017; Hueck 1998). Depending on the individual pathogen, the injected effectors act as toxins, adhesins or enzymes and can promote attachment and invasion into host cells, intracellular survival and replication or prevent host defenses. The T3SS is composed of 30–40 proteins that are assembled into a needle-like injectisome structure where they are incorporated as single molecules or in few or multiple copies (Galan et al. 2014; Deng et al. 2017; Diepold and Wagner 2014). Consequently, the synthesis of the T3SS proteins that form or utilize the machinery follows a strict hierarchy (Galan et al. 2014; Diepold and Wagner 2014). Moreover, synthesis, recruitment and unfolding as well as secretion and delivery of the effector proteins into host cells are highly energy consuming processes. This is particular evident by the fact that several pathogens stop growth and replication when the T3SS is induced (Büttner 2012; Portaliou et al. 2016; Deng et al. 2017). Thus, it is not surprising that the production of the T3SS in pathogens is tightly controlled by a plethora of regulatory factors in response to numerous environmental cues and the secretory activity of the injectisome. They act on the transcriptional, post-transcriptional and post-translational level and form a complex feedback-controlled network. In the following overview, we focus on the wealth of T3SS control factors of important human pathogens and discuss current challenges to examine their functions.

2 T3SS Regulation on the Transcriptional Level

2.1 Control by Central Transcriptional Activators

The first central factors identified to regulate the expression of the T3SS components were dimeric, **transcriptional activators of the AraC/XylS family**. This family of activators includes LcrF/VirF in human pathogenic yersiniae, HilD, HilC and RtsA in *Salmonella enterica*, MxiE in *Shigella flexneri* and ExsA in *Pseudomonas aeruginosa* (Francis et al. 2002). A common regulatory principle of these key activators involves the coordinate control of all or of a large subset of components and substrates of the T3SS in the respective pathogen. In several cases, multiple binding sites have been identified upstream the target genes and operons (Schwiesow et al. 2015).

In general, all transcriptional activators of this class are composed of an N-terminal sensing domain for environmental signals and a C-terminal helix-turn-helix (HTH) DNA-binding domain per subunit. DNA binding occurs through the recognition helix in the HTH that binds to specific DNA residues within the major groove (Bustos and Schleif 1993; Schleif 2010). Interestingly, some of these master activators, such as LcrF/VirF of *Yersinia* and ExsA of *P. aeruginosa*, are highly homologous and can complement each other. They were shown to interact with common nucleotide sequence motifs that are often highly variable regarding the distance to the transcriptional start sites, directionality and sequence conservation (Schwiesow et al. 2015; King et al. 2013). Notably, despite the resemblance of the DNA motifs, the oligomeric state of the LcrF/VirF (dimer) and ExsA (monomer) proteins is different when they are recruited to the binding sites. This ultimately results in distinct binding affinities, promoter bending and different kinetics of transcriptional activation (Schwiesow et al. 2015; King et al. 2013). Some of the N-terminal domains were further shown to bind co-factors that induce conformational changes and influence the ability of the protein to regulate transcription. One recent study for example showed that the crucial regulator HilD of *S. enterica* binds long-chain fatty acids such as oleate, which prevents binding of the activator to its target sites (Golubeva et al. 2016) (for more details, see Sect. 4.2).

Additionally, many important bacterial pathogens, including *S. enterica*, possess multiple T3SSs in which the key regulators of the systems are also implicated in the control of the master regulator of the other T3SSs. For instance, HilD coordinates the expression of two other type III secretion machineries of *Salmonella*, the flagellar T3SS and the *Salmonella* pathogenicity island 2 (SPI-2) encoded injectisome required for host defenses (Ellermeier and Slauch 2007) (Fig. 1).
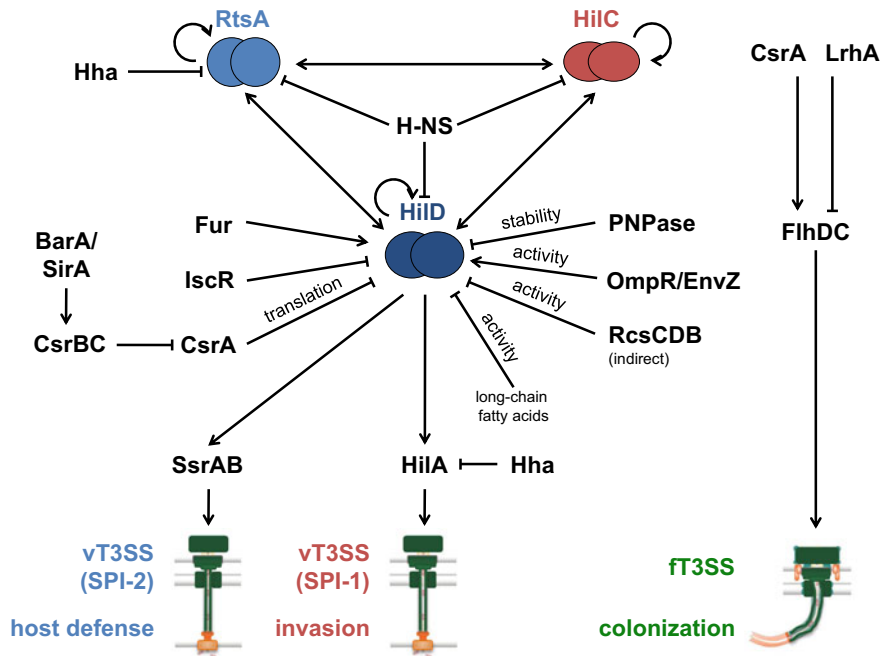
Fig. 1 Regulation of the T3SSs of *Salmonella enteric* serovar Typhimurium. The regulatory network of the flagellar and the two virulence-associated T3SSs (T3SS-1 and T3SS-2) encoded on SPI-1 and SPI-2 is shown. The overview illustrates sensory and regulatory factors, which control the T3SSs on the transcriptional and post-transcriptional level

2.2 Transcriptional Control of the Key T3SS Regulators

Appropriate expression of T3SS components is generally achieved through the integration of several regulatory factors and pathways in the form of a highly structured network that ultimately control the activity of the central AraC/XylS-type transcription activator. This enables the bacteria to tightly control and adjust the production of the T3SS in response to unique environmental cues encountered in the respective host niches. In *S. enterica,* control of the T3SS-1 (encoded on the *Salmonella* pathogenicity island 1 (SPI-1)) is mediated by the AraC/XylS proteins HilC, HilD and RtsA (Erhardt and Dersch 2015) (Fig. 1). They regulate each other and the transcription of their own gene and independently activate the promoter of the OmpR/ToxR family activator HilA. HilA primarily induces transcription of the structural components of the SPI-1 gene cluster, including *prg/org* and *inv/spa* (Ellermeier et al. 2005). Most environmental signals that regulate SPI-1 gene expression are sensed by **two-component systems** and integrated on the level of HilD. The response to (i) osmolarity, desiccation and low temperature occurs through the two-component systems OmpR/EnvZ and the RcsCD/RcsB, and

(ii) presence of certain metabolites is sensed through the BarA/SirA two-component system (Erhardt and Dersch 2015). Hence, HilD constitutes the key activator of *Salmonella* T3SS genes, whereas HilC and RtsA rather act as amplifiers of the activating signal. Control of the key regulator LcrF/VirF of the *Yersinia* T3SS, which is encoded on the *Yersinia* virulence plasmid (pYV), is similarly complex (Fig. 2). Its expression is tightly controlled by nutrients, Mg^{2+} and pH, through the BarA/SirA, PhoP/PhoQ-CsrABC cascade and the cyclic AMP receptor protein (Crp). In addition, diverse cell stresses can be sensed through the two-component systems RcsCD/RcsB and CpxA/CpxR (Schwiesow et al. 2015).

Expression of the T3SS master activators is often further influenced by the action of **specific transcriptional regulators** that promote activator transcription only under certain environmental conditions. Examples of important environmental parameters in this context are the availability of oxygen and ferric/ferrous ions. In this context, Ellermeier and Slauch and Teixido et al. (Ellermeier and Slauch 2008; Teixido et al. 2011) found that the ferric uptake regulator Fur controls HilD expression and more recent studies identified the iron–sulfur cluster coordinating transcriptional regulator IscR as an important transcriptional repressor of the LcrF/VirF and HilD master regulators of *Yersinia* and *Salmonella* (Miller et al. 2014; Vergnes et al. 2017) (Figs. 1 and 2). It is assumed that iron limitation, oxidative
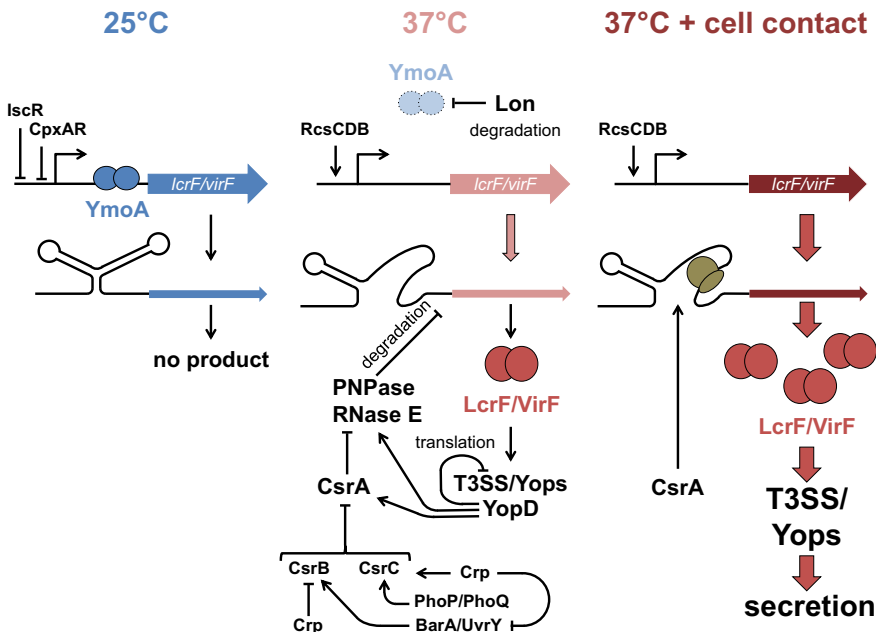

Fig. 2 Regulation of the Ysc/Yop T3SS of *Yersinia*. The regulatory network controlling the virulence plasmid-encoded Ysc/Yop T3SS in *Y. pseudotuberculosis* is illustrated. The most important global and *Yersinia*-specific RNA and protein regulators influencing T3SS gene expression at 25 °C (environment), 37 °C (host entry) and 37 °C upon host cell contact are indicated

stress, as well as oxygen limitation as a result of Fe–S cluster damage, affect the activity of IscR. Another common global regulatory protein is the LysR homologue LrhA. While this regulator represses flagella T3SS expression in *Escherichia coli* and *Salmonella*, it, on the contrary, induces the *espA* gene of the pathogenicity island *locus of enterocyte effacement* (LEE) of enterohemorrhagic *E. coli* (EHEC) and the *rtsB* regulator gene of SPI-1 genes in *Salmonella* (Erhardt and Dersch 2015; Shimizu et al. 2015).

Another common feature is that expression of the master T3SS regulators is also governed by global regulators. They are needed for metabolic adaptation and can additionally coordinate expression of the T3SSs with available carbon sources. Among these regulators is Crp. Crp primarily helps the pathogens to manage and optimize their metabolism by checking and ranking uptake and utilization of available and readily digestible carbon sources (Görke and Stülke 2008; Poncet et al. 2009). In this context, Crp is also important to link the nutrient status and carbon metabolism with the regulation of the T3SS either directly or via the control of the post-transcriptional carbon storage regulator system (Csr), which affects the expression of multiple master regulators in many pathogens (for details see Sect. 3.1.2) (Kusmierek and Dersch 2017; Vakulskas et al. 2015).

2.3 Silencing and Activation of T3SS Master Regulator Expression by Modulator Proteins

Under non-inducing conditions, e.g., outside the host, T3SS genes are often subjected to silencing by ancestral **nucleoid-structuring proteins** of the H-NS and the Hha/YmoA family. These global modulators of gene expression are implicated in the xenogeneic repression of many virulence genes with a low GC-content that were acquired by horizontal gene transfer (Navarre et al. 2006; Dorman 2007). H-NS and HhA/YmoA preferentially bind to AT-rich bent promoter regions, and their binding sites often overlap with binding sites of positive transcriptional activators that are able to alleviate transcriptional repression. This silencing and anti-silencing mechanism was found for H-NS and Hha for all three AraC/XylS-type transcriptional activators RtsA, HilC and HilD which control T3SS genes in *S. enterica* (Olekhnovich and Kadner 2007) (Fig. 1). Similarly, Ler (LEE-encoded regulator), a master regulator of the LEE operons of enteropathogenic *E. coli* (EPEC) and EHEC, is able to relieve H-NS mediated silencing of the LEE5 promoter (Laaberki et al. 2006) (Fig. 3). The LEE-encoded T3SS promotes the establishment of intimate attachment structures (intimin-mediated pedestals) of EPEC/EHEC via translocated effectors leading to attaching and effacing (A/E) lesions and severe damage of the intestinal villi (Katsowich et al. 2017; Bhatt et al. 2009). This is enabled by the significantly higher binding affinity (about 40-fold) of the T3SS master activator Ler to the LEE target DNA sequences, in comparison to H-NS (Choi et al. 2016).
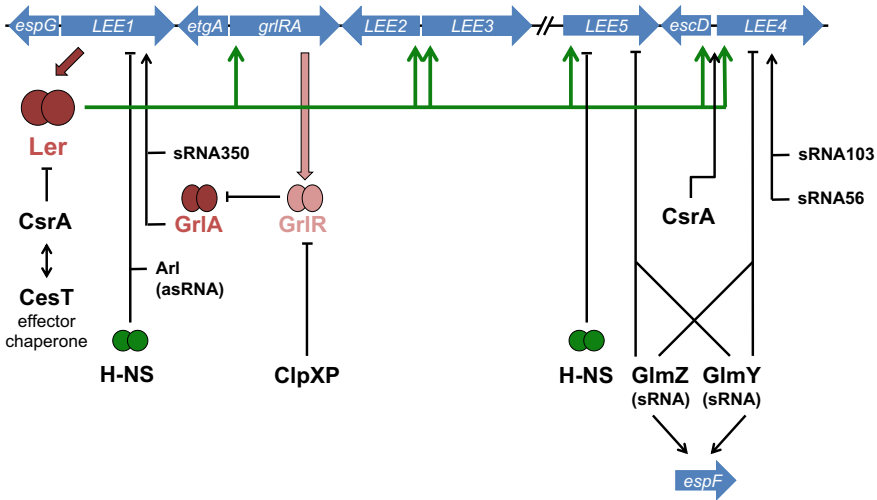
Fig. 3 Regulation of the LEE-encoded T3SS genes of EHEC. The locus of enterocyte effacement (LEE) pathogenicity island of EHEC, including the operons *LEE1-5*, the bicistronic operon *grlRA* and other monocistronic genes, is illustrated. The most important global and EHEC-specific RNA and protein regulators influencing LEE gene expression are indicated

Interestingly, members of the H-NS and Hha/YmoA family are also able to form heterodimers. How this interaction modulates expression of their target genes is not clear yet, as the influence of both family members on gene expression varies significantly between the different pathogens. However, some evidence exists that heterodimer formation influences the binding affinity to their target sites and links control of the affected genes to other environmental control systems (Madrid et al. 2002, 2007). One striking example is the formation of H-NS and YmoA complexes in human pathogenic yersiniae. YmoA is important to block transcription of the T3SS master regulator gene *lcrF/virF* in *Yersinia* to silence sequences downstream of the *lcrF/virF* promoter (Böhme et al. 2012) (Fig. 2). In contrast to its *E. coli* homologue, it is preferentially degraded by the Lon and Clp proteases at body temperature, leading to derepression of *lcrF/virF* transcription upon infection (Jackson et al. 2004).

3 T3SS Regulation on the Post-transcriptional Level

3.1 Control of Translation

3.1.1 Sensory RNAs—RNA Thermometers

One of the first post-transcriptional control mechanisms of T3SS gene expression was discovered in *Yersinia*. A comparison of the amount of the T3SS master

regulator at different growth temperatures revealed that the efficiency of *lcrF/virF* mRNA translation was strongly increased at mammalian body temperature (i.e., 37 °C) (Hoe and Goguen 1993). Initial mRNA secondary structure predictions suggested that the *lcrF* ribosome binding site (RBS) is incorporated into a stem-loop. In a later study, the 5′-untranslated region (5′-UTR) of the *lcrF/virF* mRNA was mapped, and structure probing identified a unique *cis*-acting RNA element which forms a two stem-loop structure at moderate temperatures. The first stem-loop stabilizes the second stem-loop which sequesters the *lcrF/virF* RBS by a stretch of four uracils. Opening of this structure is favored at 37 °C and permits ribosome binding at host body temperature (Böhme et al. 2012; Rhigetti et al. 2016) (Fig. 2). The biological relevance of this **RNA thermometer** was verified in animal models with two different *Yersinia pseudotuberculosis* strains expressing a stabilized and a labile variant of the RNA thermometer. The stabilized variant was strongly reduced in its ability to disseminate into the Peyer's patches, liver and spleen and had fully lost its lethality due to the lack of *lcrF* expression, whereas the destabilized version of the thermosensor was attenuated or exhibited a similar, but not a higher mortality (Böhme et al. 2012). This illustrated that the evolved RNA thermometer provides just the appropriate amount of the T3SS master regulator at the appropriate condition and time for an optimal infection efficiency of *Yersinia*.

### 3.1.2   Translational Control by CsrA/RsmA RNA-Binding Proteins

CsrA/RsmA-type RNA-binding proteins belong to a multicomponent, post-transcriptional regulatory control system that adjusts expression of T3SS genes in several Gram-negative pathogens. A key function of CsrA/RsmA protein family members is the coordinate adaptation of virulence traits, metabolic functions and physiological properties to optimize virulence and fitness of the pathogen during the different stages of the infection (Kusmierek and Dersch 2017; Vakulskas et al. 2015). CsrA/RsmA proteins most commonly prevent the translation of their numerous target mRNAs, including multiple T3SS gene transcripts. They usually bind to GGA-containing sequences that are exposed in single-stranded loop regions of two or more stem-loop structures residing in the 5'-UTRs of the impacted mRNAs, of which one includes the RBS. Simultaneous interaction of the dimeric CsrA/RsmA proteins predominantly hinders ribosome access and translation, which often also reduces the stability of the target RNA (Mercante et al. 2009; Lapouge et al. 2013; Dubey et al. 2005). However, in several cases, CsrA/RsmA protein binding prevents the formation of translation-blocking RNA structures or hinders access of RNases leading to an increase of the total translational efficiency and transcript stability (Ren et al. 2014; Yakhnin et al. 2013). CsrA/RsmA protein activity can be immediately inhibited through sequestration of CsrA by certain non-coding RNAs (e.g., CsrB and CsrC in *Enterobacteriaceae*), which harbor multiple GGA-containing hairpins. Alternatively, CsrA can be inactivated by binding to specific highly abundant GGA-rich mRNAs as well as by interacting proteins (Kusmierek and Dersch 2017).

CsrA/RsmA deficiency impairs synthesis and function of several T3SSs implicated in motility, host colonization and host defense and strongly attenuates virulence. For instance, CsrA induces the expression of the master regulator FlhDC of the flagellar T3SS in *E. coli* (Yakhnin et al. 2013). CsrA activates *flhDC* expression by protecting the *flhDC* mRNA from decay. mRNA degradation studies in *E. coli* support a model in which CsrA binding activates *flhDC* expression by inhibiting the 5′ end-dependent RNase E cleavage pathway (Yakhnin et al. 2013). In addition, the synthesis of the LEE-encoded T3SS for the formation of the intimate attachment structures by EPEC/EHEC is regulated by CsrA on multiple levels (Fig. 3) (Katsowich et al. 2017; Bhatt et al. 2009). First, CsrA directly activates expression of the *escD* and the LEE4 genes and represses expression of several LEE operons through the regulator Ler and GrlA (Bhatt et al. 2009). Moreover, CsrA was found to interact with the T3SS effector chaperone CesT when it is released from its effector under effector-secreting conditions. Sequestration of CsrA by CesT relieves CsrA-mediated repression of *nleA/espI* effector mRNA translation, which enables the bacteria to adjust T3SS gene expression in response to host cell contact (Katsowich et al. 2017).

The CsrA equivalent RsmA protein of *P. aeruginosa* is also implicated in the bacterial response to host cell contact, but the underlying molecular mechanism is quite different. In this pathogen, RsmA controls the expression of the major T3SS transcriptional activator ExsA. The activity of ExsA is controlled by a complex 'partner-switching' cascade involving ExsE, ExsC and ExsD, which is triggered upon host cell contact and secretion of the ExsE effector (Fig. 4, for details see Sect. 4.1) (Intile et al. 2014; Dasgupta et al. 2004; Urbanowski et al. 2005). RsmA activity itself is regulated through the magnesium transporter MgtE and the GacAS two-component system via activation of the Csr/Rsm-type RNAs RsmY and RsmZ (Chakravarty et al. 2017).

CsrA has also a very strong impact on the expression of different virulence-relevant T3SSs in *Yersinia*. Similar to *E. coli*, CsrA activates the FlhDC master regulator of the flagellar T3SS and is thus required for *Yersinia* motility (Heroven et al. 2008). Recent work further demonstrates that CsrA has a major influence on the virulence plasmid-encoded T3SS (Ysc/Yop). Expression of the CsrB and CsrC RNAs in *Y. pseudotuberculosis* is downregulated in the Peyer's patches during acute infection (Nuss et al. 2017), suggesting that more active CsrA protein is required for virulence. In fact, a *csrA*-deficient mutant of *Yersinia enterocolitica* was characterized by reduced secretion of the T3SS effectors YopE and YopH, and a *Y. pseudotuberculosis csrA* mutant was unable to secrete all known Yop effectors, which are usually injected into neutrophils and macrophages to prevent their phagocytic attack (Nuss et al. 2017; Ozturk et al. 2017). In addition to the virulence plasmid-encoded T3SS, *Y. enterocolitica* harbors a second chromosomally encoded T3SS (Ysa). This T3SS is only expressed under very special environmental conditions in vitro and seems to promote intracellular virulence (Bent et al. 2015). This Ysa T3SS system is also influenced by CsrA, but in contrast to the Ysc/Yop T3SS effectors, Ysp effector proteins are over-secreted in the *csrA* mutant (Ozturk et al. 2017).
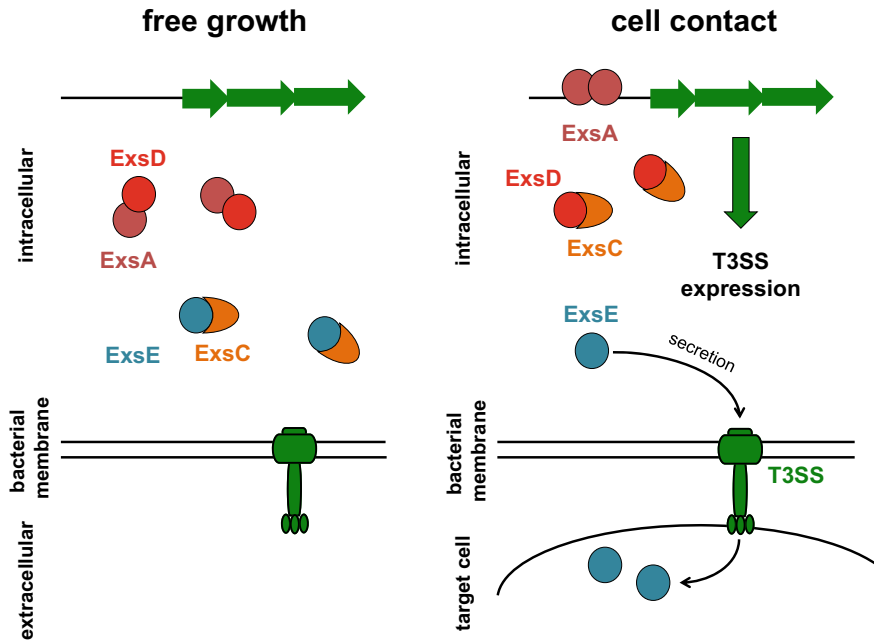
Fig. 4 Control network and partner switching regulating T3SS in *P. aeruginosa*. The regulatory network controlling the T3SS of *Pseudomonas* and the partner switching upon host cell contact is presented. The most important transcriptional and post-transcriptional regulators are illustrated, and the partner-switching mechanisms is presented when the bacteria change from free to the host cell bound stage

Similar to *Yersinia*, CsrA also controls the virulence-associated T3SS of *Salmonella typhimurium*: the cell invasion-promoting T3SS-1, encoded on SPI-1, and the T3SS-2 mediating *Salmonella* replication within macrophages (Fig. 1). CsrA regulates the expression of the common regulator HilD by binding near the ribosome binding site on the *hilD* transcript leading to a decrease of HilD translation and a reduced stability of the *hilD* mRNA (Altier et al. 2000; Martinez et al. 2011).

3.2 Control of T3SS mRNA Structures and Stability— Influence of Helicases and RNases

The overall efficiency of mRNA translation depends on the stability and the structure of the transcript. Bacteria generally have several RNA helicases that unfold intrinsic hairpin and other secondary RNA structures. Of special interest here is a certain class of RNA-binding helicases, the **DEAD-box helicases**. They are named after their conserved DEAD amino acid sequence in their catalytic domain and can hydrolyze ATP to dissolve inhibitory duplex RNA structures. One

of a set of seven DEAD-box helicases, the DeaD helicase, stimulates translation of the T3SS master regulator ExsA in *P. aeruginosa* to promote expression of the injectisome (Intile et al. 2015). DeaD seems to directly stimulate *exsA* mRNA translation, as DeaD-dependent activation is specific to the *exsA* coding region and the native RBS. The purified protein is able to promote ExsA synthesis using in vitro translation assays (Intile et al. 2015). RNA secondary structure predictions of the 5′-UTR of *exsA* and the proximal coding sequence revealed extensive base pairing with the RBS, indicating that DeaD enhances ribosomal access. DeaD does not alter T3SS expression through RsmA (Intile et al. 2015), but whether DeaD and RsmA function independently or are dependent upon each other is currently unknown.

Both T3SS regulators and structural components are also targeted by different **RNases**. One important RNase implicated in T3SS regulation is the polynucleotide phosphorylase (PNPase). PNPase belongs to the group of 3′-exoribonucleases. It can act alone or it functions as part of the multicomponent degradosome complex together with RNase E, the glycolytic enzyme enolase and helicase RhlB (Mohanty and Kushner 2016). In *Yersinia*, optimal functioning of the virulence plasmid-encoded T3SS was shown to require PNPase. While a *pnp* deletion mutant of *Y. pseudotuberculosis* possessed enhanced levels of the T3SS-encoding transcripts and proteins in contrast to wildtype, secretion of the Yop effectors was strongly reduced (Rosenzweig et al. 2007). However, this is in contrast with initial results of our laboratory in which both T3SS gene transcription and Yop secretion was strongly increased in the absence of PNPase in *Y. pseudotuberculosis* (Kusmierek et al., unpublished). The reason for this discrepancy is unclear, and we are currently testing whether a different genetic background or differences in the growth conditions may be responsible for this discrepancy. It was further found by Rosenzweig et al. that normal T3SS activity was restored when a 70 amino acid peptide (S1 domain) containing one of the RNA-binding domains of PNPase was expressed in the *pnp* mutant. Notably, T3SS expression, but not T3SS activity, was identical in Δ*pnp* strains expressing active or inactive S1 variants, indicating that PNPase influence on T3SS may involve an RNA intermediate (Rosenzweig et al. 2005, 2007; Rosenzweig and Chopra 2013). Likewise, T3SS functioning is impaired in *Y. pseudotuberculosis* with a reduced RNase E activity, and these bacteria further resemble the *pnp* mutant, indicating that they act via a common pathway, e.g., via the degradosome (Yang et al. 2008). In *Yersinia*, the single-strand-specific RNase Y (YbeY) processing 3′-ends of the 16S RNA was also found to control T3SS expression (Leskinen et al. 2015). The precise mechanism is unknown, but the CsrB and CsrC RNAs are among its target, indicating that the influence of YbeY on T3SS occurs through regulation of the Csr system.

S. typhimurium strains harboring a deletion of the 310 nt 3′-untranslated region (3′-UTR) of *hilD* or lacking the RNase E gene *rne* or the PNPase gene *pnp* exhibit increased *hilD* mRNA levels. This resulted in SPI-1 gene overexpression, impaired *Salmonella* growth and uncontrolled invasion of epithelial cells, suggesting that the *hilD* 3′-UTR is a target for degradation by the bacterial degradosome (Fig. 1) (Lopez-Garrido et al. 2014). In contrast, the RNA chaperone Hfq interacts with the

3′-UTR of the *hilD* mRNA and has a positive influence on its stability. Therefore, it has been assumed that this effect occurs through competitive inhibition of the degradosome (Lopez-Garrido et al. 2014; Sittka et al. 2007; Chao et al. 2012).

The degradosome is also implicated in the regulation of T3SS gene expression in pathogenic *E. coli*. In EHEC, the *espADB* translocon genes are controlled by an RNase E-dependent mechanism, in which a small six-codon mini-open reading frame at the 5'-end of the common transcript is recognized and preferentially degraded by RNase E (Fig. 3). However, translation of the mini-open reading frame by ribosomes protects the mRNA and allows a more efficient production of the translocon proteins (Lodato et al. 2012, 2017).

3.3 Regulatory RNAs

Over the past years, a plethora of regulatory RNAs have been identified in many bacterial pathogens that are implicated in virulence regulation. Besides the Csr/Rsm-type RNAs (see also Sect. 3.1.2), several others of them have also been found to control T3SSs. For instance, the small non-coding RNA Spot42 regulates the expression of the chaperone protein VP1682 of components of the T3SS-1 of *Vibrio parahaemolyticus* by basepairing with the RBS and the initial codons of the *vp1682* transcript (Tanabe et al. 2015). Another example constitutes the *Yersinia*-specific non-coding RNA Ysr141. In the absence of Ysr141, a selection of the T3SS components and the effectors YpkA, YscF, YopE, YopK and YopJ and the regulator LcrF were 30–70% decreased (Schiano et al. 2014). Although the molecular mechanism how Ysr141 exerts this effect is largely unknown, initial experiments demonstrated that Ysr141 interferes with *yopJ* mRNA translation. Schiano et al. (Schiano et al. 2014) proposed that all observed changes could be based on the dysregulation of YopJ production as *Yersinia* T3SS was shown to be sensitive to changes in Yop protein levels. However, the observed regulatory influence on YopJ translation was rather small suggesting that other mechanisms contribute to the overall influence of Ysr141 on T3SS regulation. A recent study revealed that the copy number of the *Yersinia* virulence plasmid pYV encoding the Ysc/Yop T3SS increases during infection (Wang et al. 2016). This upregulation is caused by an increased expression of the pYV replicase RepA. It was shown that levels of an antisense RNA, CopA, overlapping with the upstream region of the *repA* gene are strongly decreased under secretion conditions and during infection, leading to a marked decrease of the CopA/*repA* mRNA ratio and an increase of the copy number (Nuss et al. 2017; Wang et al. 2016). The precise regulatory mechanism still needs to be elucidated, but first evidence exists that the secreted translocon protein YopD is involved in this process (Wang et al. 2016). Finally, the unique bacterial translational control system, including the small protein B (SmpB) and the regulatory RNA SsrA/tmRNA, is required for efficient expression of Yop effectors and the flagellar T3SS of *Yersinia* (Okan et al. 2006).

A study by Gruber and Sperandio (2015) identified multiple non-coding EHEC-specific RNAs of which sRNA56, sRNA103 and sRNA350 activate T3SS genes of the LEE pathogenicity island (Fig. 3). sRNA350, encoded in the 3′-UTR of the *cesF* transcript, activates all LEE operons via its influence on the master regulator gene *ler*, whereas sRNA56 and sRNA103 target only the LEE4-encoded *espA* mRNA. Besides these EHEC-specific non-coding RNAs, the well-characterized, paralogous non-coding RNAs GlmZ and GlmY also influence expression of EHEC T3SS genes (Fig. 3). Both RNAs destabilize the LEE4- and LEE5-encoded mRNAs and increase translation of the *espF* transcript (Gruber and Sperandio 2014). GlmZ interacts with the LEE4 mRNA and selectively destabilizes the downstream part of the transcript encoding the translocon *espADB* genes, but not the upstream part encoding *sepL*, which acts as regulatory switch by binding effectors until the T3SS is formed (Gruber and Sperandio 2014). In addition, GlmY also represses LEE4 on the post-transcriptional level. However, this occurs by binding to and sequestering the protein RapZ from GlmZ, which, when released from GlmZ, can destabilize the LEE4 transcript (Gruber and Sperandio 2015). The antagonistic control of LEE4/ LEE5 and *espF* by both RNAs is not easy to interpret, as all targeted genes are required for pedestal and A/E lesion formation. However, as suggested by the authors, it is possible that GlmY/GlmZ limits overexpression of the LEE4/LEE5 T3SS components and synchronizes their production with the non-LEE *espF* gene to enable a precise ratio of the structure components and secreted effectors (Gruber and Sperandio 2014). Moreover, a *cis*-encoded antisense RNA (Arl) located downstream of the *ler* gene, covering the last codons and the 3′-UTR, controls T3SS gene expression from the LEE pathogenicity island (Tobe et al. 2014). Interaction of Arl with the *ler* transcript destabilizes the LEE1 mRNA and also hinders final elongation of the Ler protein synthesis.

3.4 Feedback Control Mechanisms Through Secreted Effectors or Anti-sigma Factors

T3SS gene expression in several pathogens is strongly induced upon host cell contact and effector secretion. This includes the T3SS systems of *Yersinia, Salmonella, Shigella* and *Pseudomonas* (Pettersson et al. 1996; Brutinel and Yahr 2008; Zierler and Galan 1995). In *Yersinia*, T3SS gene expression is coupled to secretion via the translocon protein YopD, the YopD chaperone LcrH and the effector LcrQ (YscM1/YscM2 in *Y. enterocolitica*) (Anderson et al. 2002; Anderson and Schneewind 1999). LcrQ and YopD are secreted upon host cell contact reducing their concentration in the bacterial cytoplasm. The role of LcrQ is still unclear, but it is assumed that it assists the YopD–LcrH complex (Cambronne and Schneewind 2002; Li et al. 2014). In addition, to its translocon function, YopD is able to interact with RNA (alone or in complex with the chaperone LcrH) by for example binding to the 5′-UTR of multiple *yop* transcripts and the *lcrF* mRNA

(Fig. 2) (Cambronne and Schneewind 2002; Chen and Anderson 2011). Absence of the YopD protein under secretion conditions strongly decreases their degradation and/or prevents their translation (Chen and Anderson 2011). The RNA-binding mode of the protein is not known, but AU-rich regions in the proximity of the RBS seem important for YopD-mediated repression of the targeted T3SS mRNAs. Another important information shedding more light on the molecular mechanism of cell contact-mediated induction of T3SS genes was obtained by an analysis of Kopaskie et al. (2013). They report that the YopD protein is able to interact with the 30S particles of the bacterial ribosome in an LcrH-dependent manner and could show that YopD in association with YscM1 and LcrH is able to repress YopQ translation. However, how YopD–LcrH interaction with the 30S ribosomal particle is mediated and how this influences the translation of other transcripts of the Ysc/ Yop T3SS is still unclear. Yet, it is likely that transient interaction perturbs the formation of the 30S complex before the 50S ribosomal particle associate with the transcript, as no binding of YopD to the assembled 70S ribosome could be detected (Kopaskie et al. 2013).

Another sophisticated feedback control mechanism is promoted by secreted anti-sigma factors and was found for the flagellar T3SS and virulence-associated T3SSs, e.g., from *Bordetella* spp. (Chevance and Hughes 2017; Ahuja et al. 2016). The flagellar master regulator FlhDC activates *fliA*, encoding the alternative sigma factor σ^{28}. This sigma factor promotes expression of the motor force generator and the filament, and its function is inhibited when bound by an anti-σ^{28} factor (FlgM). Upon formation of the hook basal body, the anti-sigma factor is secreted and σ^{28} is released to activate σ^{28} target genes (Chevance and Hughes 2017). Similarly, the secreted antagonist BtrA of the sigma factor BtrS establishes a feedback loop that couples the activity of the T3SS with expression of the T3SS genes. BtrA differentially controls nearly 300 genes, including many T3SS genes, which define six distinct regulatory virulence modules in *Bordetella* (Ahuja et al. 2016).

4 T3SS Regulation on the Protein Level

4.1 Changing Binding Partners

Another mechanism promoting cell contact- and secretion-induced T3SS gene expression is 'partner-switching.' One important control step of this process is the interaction of a T3SS chaperone with its T3SS substrate or a cytoplasmic regulatory factor. Without host cell contact, the secreted T3SS effector remains bound to its chaperone that keeps the substrate in a transport-competent stage and inhibits the activating function of the chaperone (Schulmeyer and Yahr 2017). Upon host cell contact, the chaperone is released from the secreted binding partner (effector or translocon proteins) and interacts with transcriptional activators or suppressors of the transcriptional activators (anti-repressor) to induce T3SS gene transcription.

This changing of partner(s) can implicate multiple co-, anti- or anti-anti-activators that form a complex feedback control cascade linking T3SS gene expression with the secretory activity of the system (Dasgupta et al. 2004; Urbanowski et al. 2005). This partner-switching mechanism was characterized in detail in *P. aeruginosa* by the Yahr group and was shown to include four different proteins (Urbanowski et al. 2005; Schulmeyer and Yahr 2017; Vakulskas et al. 2009). In the absence of host cell contact, the effector protein ExsE interacts with chaperone ExsC. The master T3SS transcriptional activator ExsA is bound to ExsD. Upon cell contact, the released ExsC chaperone, which has a higher affinity to ExsD than ExsA, sequesters the ExsD protein, whereby the ExsA activator is liberated and can activate T3SS gene transcription (Brutinel et al. 2010; Zheng et al. 2007; McCaw et al. 2002; Rietsch et al. 2005; Dasgupta et al. 2006). Interestingly, *Vibrio parahaemolyticus* and *Vibrio cholerae* appear to have functional orthologues of their *Pseudomonas* counterparts to control the T3SS-1 (Zhou et al. 2010). A similar mechanism with a very different set of partners has been identified in *S. flexneri*. In this pathogen, the translocon proteins IpaB and IpaC are bound by the chaperone IpgC. The activator MxiE is inactivated through interaction with the anti-activator OspD1 and Spa15 protein, which acts as OspD1 chaperone under non-secretion conditions. IpaB, IpaC and OspD1 translocation into host cell allows released MxiE and IpgC to interact and activate T3SS gene expression (Parsot et al. 2005).

4.2 Modulation or Modification of T3SS Regulatory Components

Besides interaction with other regulatory proteins, activity of T3SS master regulators can be manipulated either by small compounds or through modification, such as phosphorylation. An exciting recent work demonstrated that long-chain fatty acids (e.g., oleate) prevent expression of the T3SS-1 genes in *S. enterica*. This inhibition is independent of the long-chain fatty acid degradation pathway and occurs solely through direct binding and inhibition of the DNA-binding activity of the T3SS activator HilD (Golubeva et al. 2016). Long-chain fatty acids are present in the intestinal tract and inhibit HilD-mediated T3SS gene expression until the bacteria reach the distal ileum of the small intestine. There, the metabolites are absorbed and the concentration falls below a critical threshold, which relieves the blockage in order to activate T3SS gene expression.

Another mechanism used to change the activity of T3SS regulators is through protein modification, i.e., tyrosine phosphorylation. However, only a few examples are known, and how the modification influences the activity of the modified proteins is often unclear. One example is *Shigella*, in which the second master regulator VirB (besides MxiE) is unable to stimulate T3SS gene expression when phosphorylated at residue 247 (Standish et al. 2016).

5   Conclusions and Future Perspectives

The long list of transcriptional, post-transcriptional and post-translational control mechanisms implicated in T3SS control in the different pathogens illustrates that T3SS regulation is highly complex. A large intervening network has been evolved that integrates various environmental signals, nutrient/ion availability and physiological conditions to control regulators, structural components and secreted effectors of the T3SS in a fine-tuned and concerted manner. This occurs through a plethora of sensory and regulatory factors, which can be RNA or proteins that modulate specifically or globally, and the players in the network can be modulated on different regulatory levels.

It is evident, that common players, e.g., conserved AraC/XylS regulators and transcriptional modulators, sensory and regulatory RNA elements, RNases, and common two-component systems are part of the control circuit. However, their interactions, targets and arrangements within the regulatory cascades and feedback cycles can vary significantly between the different pathogens. Moreover, even very small changes in the genetic information were found to provoke fundamental changes of T3SS control. This set of possible variations defines the different T3SS control variants, which adapt and optimize the expression of the secretion system to the distinct needs and lifestyles of the different pathogens.

Ongoing discovery of new regulatory factors and different T3SS control variants further illustrates that our understanding of T3SS regulation is still far from being complete. There is a dire need to explore the molecular mechanisms and the role of regulatory RNAs, controlled RNA degradation and translation changes under non-inducing and secretion conditions during the infection. However, this is challenging due to the complexity of the controlling signals and components. One major difficulty in the analysis of T3SS expression is the identification of appropriate in vitro growth conditions mimicking the different steps of T3SS production under infection conditions.

Another unsolved question is, how the detected molecular mechanisms of one representative strain extend to other strains or members of the family or species. In particular, post-transcriptional control elements such as sensory and regulatory RNAs appear more malleable to intrinsic changes and regulatory rewiring than transcriptional regulators. This promotes individual, strain-specific variations—a trait that is particularly advantageous for the bacteria to adapt to frequently changing host niches. Thus, it would be ill-advised to automatically extrapolate their role to others. For instance, it is known that GlmY/GlmZ promoted control of *espF* expression, which is essential for EHEC to form pedestals, is not encoded in the genome of closely related EPEC, and the DsrA RNA that activates transcription of *ler* in EHEC does not affect LEE in EPEC (Bhatt et al. 2016). Moreover, Hfq has a very strong influence on the Ysc/Yop T3SS in *Yersinia pestis*, but not in *Y. enterocolitica*, suggesting that T3SS regulation relies on different post-transcriptional mechanisms (Kakoschke et al. 2014, 2016; Schiano et al. 2010). Consequently, the function of

these control elements must be experimentally investigated in additional members of the species and family.

An additional future challenge is to unravel the dynamics of the multicomponent and multilayered network of T3SS regulation during different phases of the process. This includes the transition from the repressed stage (e.g., 25 °C, outside host), to the preparing phase (host entry, 37 °C), and the different secretion phases upon host cell contact that allows the bacteria to carefully balance nutrient and energy use to maintain their biological fitness and competiveness.

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