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Thermosensing to adjust bacterial virulence in a fluctuating environment

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Abstract

The lifecycle of most microbial pathogens can be divided into two states: outside and inside their hosts. The sudden temperature upshift experienced upon entry from environmental or vector reservoirs into a warm-blooded host is one of the most crucial signals informing the pathogens to adjust virulence gene expression and their host stress survival program. This article reviews the plethora of sophisticated strategies that bacteria have evolved to sense temperature, and outlines the molecular signal transduction mechanisms used to modulate synthesis of crucial virulence determinants. The molecular details of thermal control through conformational changes of DNA, RNA and proteins are summarized, complex and diverse thermo-sensing principles are introduced and their potential as drug targets or synthetic tools are discussed.

Introduction

Bacteria have conquered all conceivable niches and are constantly exposed to changing environmental conditions. Especially pathogens are continuously confronted with nutritional and physical changes and have to cope with often hazardous surroundings. In order to survive stress- and harmful situations, bacteria evolved sophisticated tools to survey their local environment and respond with adaptive and protective measurements. Pathogens generally monitor a variety of physical and chemical parameters to generate environmental profiles that are converted into intracellular signal cascades plugged into control networks. The lifestyle of most pathogens, in particular of those that circulate frequently between environmental/vector reservoirs and warm-blooded hosts are subjected to variations in temperature. Thus, it is not surprising that a temperature upshift is one of the most important decisive signals for mammalian pathogens to turn on their virulence functions [1]. Plant pathogens also respond to thermal changes, but they often favor temperatures for the induction of their pathogenesis programs that are below their optimal growth range [2-4].

The molecular basis for thermoregulation is a precise thermosensing device or moiety. All biomolecules are per se “sensitive” and react to fluctuations of the temperature by conformational changes. However, certain thermosensors have been evolved which exploit this attribute in a more direct, highly accurate and very sophisticated manner. The most specialized case of thermosensing is the heat- and cold-shock response. Several of these systems respond to the consequences of temperature-induced damage, e.g. they detect denatured polypeptides which arouse upon a sudden temperature upshift. This response is transient and irreversible, and is reviewed in other recent articles [5-7]. In contrast, a more persistent but also reversible response to the temperature itself (e.g. 37°C) is required to trigger viru-

lence gene expression as long as the bacteria are located within their hosts. This article focuses on direct and reversible thermosensing strategies used by bacterial pathogens to control virulence-associated traits on the transcriptional and post-transcriptional level. Work over the past years point to three main types of “molecular thermometers”: DNA, RNA and proteins. In addition, other molecules (e.g. lipids) can serve as target of temperature-related stimuli. The underlying principle of these thermosensors is that even small thermo-induced conformational changes provoke major differences in their functional properties. The molecular mechanisms how these biomolecules sense temperature and influence the virulence gene regulatory circuits are described in this review.

Thermosensing through DNA

Bacterial DNA holds all genetic information needed for pathogenesis. Therefore, it is predestined to control access and transcription of virulence-associated loci directly via thermo-induced changes in the DNA. A plethora of examples exists in which temperature has been shown to have a regulatory effect on virulence gene transcription through alterations of the superhelical conformation (supercoiling) or curvature (bending) of chromosomal or plasmid DNA [8-10]. We do not attempt to describe all known examples, we instead selected a number of characteristic DNA-based thermosensors that have been studied in great detail.

Thermo-induced changes of the DNA topology.

Several mechanisms have been identified how temperature-mediated changes in the DNA topology influence the activity of virulence genes. DNA physically responds to temperature shifts with (i) changes of the twist (number of helical turns in the DNA)

and the writhe (number of times the helix is interwound) which results in altered chromatin supercoiling [11], or with (ii) the formation of intrinsic bends most of which occur in AT-rich sequences, in particular phased homopolymeric AT-tracts [12-16]. Loci prone for intrinsic bending have been predominantly identified in the 5'-regulatory sites of genes and thermoinduced modulations of the DNA topology within the promoter regions have been shown to influence transcription efficiency directly by manipulating the binding affinity of the RNA polymerase to the promoter [17].

Thermosensing including nucleoid-associated proteins.

Alternatively, DNA-binding proteins with the ability to manipulate supercoiling or DNA-bending in response to temperature have been shown to modulate RNA polymerase activity. In particular, interaction of the nucleoid-associated proteins (NAPs), such as H-NS, IHF, HU, Hha/YmoA and Fis with promoter regions can induce or enhance pre-existing bends and modulate transcription of downstream genes [18-21]. Likewise, thermoinduced conformational changes can alter the interaction between DNA-binding proteins and their target promoters, thereby affecting virulence gene expression [22]. Most frequently, temperature-dependent control of virulence genes is mediated through the concerted interplay of both - the DNA topology of the target promoter and the DNA-binding protein, in particular H-NS [9,23,24]. Several well-studied examples are illustrated and discussed in the following paragraph.

The histone-like nucleoid-structuring protein H-NS is a universal regulator of the bacterial genome and highly abundant in *Enterobacteriaceae*. It is further the NAP, which is most extensively involved in thermodependent transcriptional control processes. In this context it is also referred to as a high-resolution thermosensor [19,25-27]. In *Escherichia coli*, H-NS was found to control 69% of the thermoregulated

genes, and numerous H-NS-dependent, thermoinduced virulence genes were also identified in many other pathogens [24,28-30]. Evidently, H-NS controls virulence gene expression merely through its ability to bind DNA. The dimeric protein interacts with DNA non-specifically, but prefers binding to bent DNA regions (AT-tracts) from which the molecules polymerize along DNA and form a complex of higher bending rigidity [31-33]. An example includes the *hns* promoter sequences of *E. coli* and *Salmonella* which exhibit features of curved DNA and are preferentially bound by H-NS leading to transcriptional autorepression [34,35]. H-NS can also mediate bridging within and between DNA helices, leading to loop formation, stiffening of the DNA and blockage of transcription initiation by RNA polymerase (Fig. 1A) [30,36-39]. Most notably, ChIP-on-chip experiments revealed that H-NS preferentially represses horizontally acquired DNA regions characterized by a low GC-content. These laterally acquired genomic regions include the pathogenicity islands of *Salmonella enterica* (SPI1-3, SPI5) encoding the majority of its virulence genes [29,40,41]. At low and moderate temperatures, expression of the virulence functions is repressed by H-NS, whereas an increase of the temperature to 37°C decreases the ability of H-NS to bind cooperatively to its target sequences. Thermo-induced transitions of the superhelical state of the target promoters have been assumed to reduce the accessibility of H-NS to DNA [22]. Furthermore, an increase in temperature induces a conformational change in the H-NS dimer that prevents interaction with the high-order oligomers and therefore H-NS no longer binds to DNA in a cooperative manner (Fig. 1A) [30]. However, this matter has not been convincingly resolved since many H-NS-dependent genes remain repressed at 37°C. H-NS oligomerization has also been reported to be constant at temperature between 28°C and 42°C, indicating that changes of the oligomerization properties of H-NS may not play a role in the temperature-dependent control of virulence genes [42].

Participation of H-NS in thermoreception often involves other proteins in the temperature control process. Many H-NS-dependent virulence genes are activated by positive transcription factors, which provide specificity by selecting appropriate operator sites, and/or they are frequently modulated by other NAPs [43-45]. The best-characterized examples include virulence determinants encoded in *Salmonella*, *Shigella*, *Yersinia* species and *E. coli*, but thermo-induced transcription of virulence genes of many other pathogens were also investigated (Fig. **1B**, Tab. **1**).

The virulence regulator VirF is the first transcriptional activator of a multistep regulatory cascade of virulence genes in *Shigella flexneri* and enteroinvasive *E. coli* (EIEC). Temperature-controlled expression of *virF* was one of the first examples demonstrating the close interplay between transcription silencing by H-NS and alleviation of its repression by (i) thermo-induced conformational changes of the local DNA topology and (ii) binding of the Fis protein (factor for inversion stimulation). At moderate temperatures H-NS binds to two sites within an intrinsic bend of the *virF* promoter thereby inhibiting transcription initiation. Transition from a repressed to a derepressed state occurs around 32°C when Fis starts to counteract H-NS by binding to four target sites within the *virF* promoter. The concerted occupancy of these binding sites by Fis and the intrinsic DNA curvature between two H-NS binding sites results in a nucleoprotein complex that favors *virF* transcription [21,22,43].

Over the past years, many other virulence determinants of *E. coli* have been explored which are affected by H-NS in a temperature-dependent manner. Among them are many fimbrial operons, including the Pap pili operon *papBA* of uropathogenic *E. coli* (UPEC), the *daa* operon, which encodes F1845 fimbriae, *fimA*, the gene coding for the phase-variable type 1 fimbrial subunit protein and the *fan* operon, which encodes K99 fimbriae [46-48]. The *papBA* operon has been studied in more detail, and besides H-NS also the regulatory protein RimJ is involved in temperature control

[47,49]. H-NS inhibits transcription initiation of *papBA* at low temperatures by blocking methylation of two GATC sites located upstream of the *papBA* promoter. Methylation of these sites is essential for *papBA* expression since it regulates binding of the global activator protein Lrp [50]. RimJ is an N-terminal acetyltransferase of the ribosomal protein S5, but the functional mechanism how this enzyme contributes to the thermoregulatory response is not yet known [49]. A set of important temperature-regulated virulence loci of enteropathogenic and enterohemorrhagic *E. coli* (EPEC/-EHEC) is encoded on the pathogenicity island LEE (locus of enterocyte effacement) [51]. These crucial virulence genes are required for the intimate attachment and effacement of brush border microvilli (AE lesions), and are only expressed at 37°C [52,53]. Temperature-controlled expression of the five major virulence operons within LEE (LEE1-5) is driven by antagonistic actions of H-NS and the key virulence activator Ler (LEE-associated regulator), a distant homologue of H-NS which itself is encoded on the LEE1 operon [54-57]. H-NS-mediated repression occurs on two levels: (i) H-NS inhibits transcription of LEE1 thereby preventing Ler production and subsequent activation of LEE2-5 transcription, and (ii) H-NS represses LEE2-5 directly [53]. When the temperature rises (37°C), Ler is synthesized, binds to LEE upstream regions and alleviates H-NS repression [53,58,59]. The precise mechanism how Ler antagonizes H-NS function is still unknown, but an H-NS-like *modus operandi* was proposed by which Ler recognizes DNA structure motifs prone to temperature changes [58,60,61]. The structure of a complex between the DNA-binding domain of Ler and a natural DNA fragment revealed that Ler recognizes specific DNA shapes rather than base specific contacts. This explains why Ler is not a general antagonist of H-NS, although the DNA binding domains of Ler and H-NS share a similar indirect DNA readout mechanism [62].

Another important example for H-NS-mediated thermal control includes the virulence genes encoded on the *Salmonella* pathogenicity island SPI-2 which are important for the replication of the bacteria within host cells. Expression of the SPI-2 genes is strictly thermo-regulated by the two component system SsrAB and the two NAPs Hha and H-NS [63,64]. At non-permissive temperatures (<30°C) H-NS abolishes expression of the response regulator SsrB which activates the virulence program. H-NS-mediated temperature-dependent silencing of the *ssrB* gene also requires the transcriptional modulator Hha. This was demonstrated by the deletion of *hha* in an *hns*⁺ strain that displayed a temperature-blind phenotype [64]. Members of the Hha family of NAPs exert their regulatory function by forming heterodimeric complexes with H-NS thereby enhancing its repressive action at some promoters (Fig. 1) [65-67]. Microarray analyses revealed that the main targets of Hha overlap with those of H-NS in *Salmonella*, which suggests that Hha must form complexes with H-NS to silence these DNA regions [68]. Recent studies further demonstrated that relaxation of DNA supercoiling induces the SPI-2 regulator *ssrA* and the SPI-1 regulator *hilC* through a mechanism that requires the two-component system OmpR-EnvZ. In response to the altered DNA topology binding of Fis is reduced whereas OmpR binding is increased. OmpR binding seems to induce a locally supercoiled state, which may assist promoter activation [69-71].

Alteration of the surrounding temperature is recognized as the most important signal to adjust virulence gene expression in *Yersinia*, and multiple molecular thermosensors and temperature-dependent regulatory circuits act in concert to ensure thermoregulation of a large collection of virulence-associated genes [72-74]. All human pathogenic yersiniae cycle between exogenous reservoirs (insects/plants) and mammals (rodents/pigs/humans), and this biphasic lifestyle may explain the presence of such a comprehensive thermotactic response. In most known cases, tem-

perature-associated changes in the DNA topology – both altered supercoiling and induction of DNA bends – act together with H-NS and/or YmoA, a small NAP with homology to the Hha protein of *E. coli* to modulate the induction of virulence genes. *Yersinia* seems to sense changes of the DNA topology to control expression of virulence genes encoded on the *Yersinia* virulence plasmid (pYV) [75,76]. This includes genes of a type III secretion system (*ycs*, *lcr*) essential for the delivery of anti-phagocytic effector proteins, referred to as Yops (*Yersinia* outer proteins) [77]. Expression of these virulence factors is induced by the key transcriptional activator protein VirF/LcrF, which is only synthesized at 37°C, but not at moderate temperatures [78,79]. pYV contains multiple regions of intrinsic curvature, including the *virF/lcrF* locus, which undergoes a conformational transition upon an upshift from 25°C to 37°C. This suggested that *Yersinia* mainly uses changes in the DNA topology as temperature-sensing mechanism to adjust virulence gene expression [75,76]. However, recent studies illustrated that there are several additional thermosensing devices which act in concert. YmoA represses expression of the *virF/lcrF* gene at moderate temperatures by binding downstream of the promoter region, but this repression is alleviated at 37°C [74]. YmoA alone has no DNA-binding capacity and does only interact with DNA as heterodimer together with H-NS [74]. Absence of H-NS is lethal for *Yersinia*, indicating its function is crucial for the biological fitness of this species [80,81]. It is quite reasonable that the ability of both NAPs to bind DNA is influenced by thermo-induced topological changes at or in the vicinity of the binding site(s). In addition, both global regulatory proteins were shown to respond themselves to temperature shifts leading to an altered ability to bind DNA (see also thermosensing by proteins) [30,82].

Thermosensing through RNA

Temperature can further regulate protein synthesis by affecting the structural properties of single-stranded RNA transcripts. Two decades ago the capacity of certain 5'-untranslated regions (5'-UTR) to sense temperature has been discovered, but only recently the molecular structure and function of these **RNA thermometers** and their role in virulence have been receiving growing attention [83-85]. The first thermo-responsive RNA element was identified by Altuvia *et al.* [86] within the transcript encoding bacteriophage λ protein cIII inducing the lysogenic cycle. Depending on the surrounding temperature two alternative *cIII* mRNA structures are formed of which the energetically more stable structure prevents *cIII* translation and leads to the lytic cycle (45°C). Transition into the less stable structure (37°C) liberates the ribosome binding site, allows cIII synthesis and promotes induction of the lysogenic pathway. Nowadays, we know that RNA thermometers are common post-transcriptional regulatory devices among prokaryotes which operate as temperature-dependent zippers or switches controlling translation via RNA structure destabilization or alteration [83-85].

Cis-acting RNA thermosensors

Single-stranded mRNA transcripts basepair intramolecularly and form higher order structures immediately after transcription [87-89]. These RNA structures have a strong influence on the initiation and efficiency of translation. Bacteria have taken advantage of this feature to control virulence genes in response to temperature, but also heat-shock and cold-shock genes are main targets of this regulatory mechanism [83,84]. All RNA thermosensors identified so far are located within the 5'-UTR of genes encoded by the temperature-responsive mRNA. Their underlying thermo-responsive mechanism is usually very simple, highly efficient and allows a very rapid,

precise and low-energy consuming adjustment of virulence factor synthesis to sudden temperature up- and downshifts sensed during host entry or exit [90]. At lower temperatures (like such experienced in the external environments), the thermosensing RNA element is in a “closed” conformation – in which the ribosome-binding site is occluded by a hairpin structure (Fig. 2). A thermal upshift upon entry into a warm-blooded host leads to rearrangements of the intramolecular basepairing and gradual melting/opening of the double-stranded part of the stem-loop (“open” conformation) rendering the ribosome-binding site accessible for the 30S subunit to initiate translation [84]. RNA thermometers including those controlling virulence gene expression convert temperature influence immediately; they function without additional factors and even very small temperature changes (on 1°C scale) can be detected [91,92]. Latter could also be advantageous to sense and adjust to body temperatures of different hosts, e.g. rodents/humans 37°C, pigs 39°C, and birds 40°C.

The thermo-sensitive stem-loop structure of all known RNA thermometers contain internal bulges and/or non-canonical base pairs which generally reduce the thermodynamic stability and make the RNA thermometer prone to thermo-induced zipper-like melting. Their number, quality and position seem to determine the temperature range in which the RNA thermometers switch from the “closed” to the “open” conformation. The accuracy of thermosensing was demonstrated for several individual RNA thermometers implicated in virulence. Even single nucleotide exchanges within the thermosensing stretch of the RNA were sufficient to make the RNA thermometer less or fully thermo-insensitive [74,91,93].

Thermo-responsive RNA zippers.

Up to date several RNA thermometers have been identified in pathogenic bacteria. However, the majority are control elements of heat shock genes (e.g. *groEL* of *Staphylococcus aureus*) and only a small number of them have been shown to be involved in the regulation of virulence genes [74,85,94]. Back in 1993, a thermo-sensing RNA element was postulated to reside upstream of the *lcrF* gene of *Y. pestis* encoding the central virulence gene activator LcrF. LcrF is essential for thermo-induced expression of the antiphagocytic Yop effector-type III secretion machinery [77,95], and all pathogenic *Yersinia* species are highly attenuated/avirulent when *lcrF* (*virF* in *Y. enterocolitica*) is deleted [78,96]. Thermoinduction of these virulence genes occurs only at 37°C due to significantly higher levels of the virulence regulator. Since *lcrF* transcription and RNA levels were not increased, a post-transcriptional control mechanism adjusting ribosomal access to the Shine-Dalgarno site was suggested [97]. Recently, it has been demonstrated that the *lcrF* gene is transcribed from a bicistronic operon (*yscW-lcrF*). Secondary structure predictions using algorithms such as mfold and RNA fold [98-100] predicted the formation of two hairpins within the intergenic region of *yscW* and *lcrF* (Fig. 2). The second stemloop included a stretch of four uridines basepaired with the AGGA sequence of the ribosome-binding site. This motif is referred to as fourU element and was first identified in the small heat shock gene *agsA* (aggregation suppression A) in *S. enterica* serovar Typhimurium [94]. The *in silico* prediction was confirmed by structure probing which demonstrated thermo-induced opening of the second hairpin. Furthermore, toe printing analysis showed that the interaction of ribosomes with the ribosome-binding site occurs at 37°C, but not at 25°C (Fig. 2) [74]. Using translational fusions, it was shown that *lcrF* expression was still thermo-induced when transcription was promoted from the temperature insensitive P_{BAD} promoter, confirming the existence of a thermosensing RNA element. Base substitutions leading to stabilization of the se-

cond stemloop structure (e.g. by replacing an imperfect by a perfect basepair) were found to shift the transcript into the closed conformation at 37°C and completely disrupted its thermosensing function. In contrast, mutations that exchanged a perfect Watson-crick to a non-canonical basepair allowed opening of the thermometer and production of the LcrF protein already at moderate temperatures and increased LcrF production at low and high temperatures [74]. A shortened RNA thermometer composed only of the second hairpin was still thermo-inducible, but exhibited an overall higher expression level, indicating that the first stemloop stabilizes the second. To obtain evidence that the fourU RNA thermometer is essential or contributes to virulence of *Yersinia*, pathogenicity of both, a “closed” and “open” thermometer mutant variant was investigated using a mouse infection model. Although mice were orally challenged with a lethal dose of *Y. pseudotuberculosis*, all mice infected with bacteria encoding the “closed” thermometer variant did not develop any disease symptoms and survived the infection [74]. Intriguingly, the “open” RNA thermometer mutant strain had also no benefit of producing the virulence program at all times. In the contrary, virulence remained unaffected or was even reduced, most likely due to fitness penalties [74]. This illustrates that the conserved architecture of the evolved RNA thermometer is crucial for *Yersinia* to produce the appropriate amount of the virulence regulator that guarantees an ideal infection efficiency.

The second pathogenicity factor known to be thermo-regulated via an RNA thermometer is another key regulator of virulence - the Crp/Fnr-type transcriptional activator PrfA of *Listeria monocytogenes*. This food-borne pathogen causes very severe infections, in particular in pregnant women or immune-compromised individuals. All virulence genes of this pathogen (e.g. adhesins/invasins, immune evasion and suppressing factors) are positively regulated by PrfA [101]. Similar to *Yersinia*, thermoinduction of the *Listeria* virulence program is a consequence of PrfA being

only present at 37°C but not at 30°C, although similar levels of the *prfA* mRNA are produced [93,102,103]. Computational predictions and chemical probing revealed an RNA thermometer consisting of one long hairpin in the 5'-UTR of *prfA* with several internal bulges and loops in which the ribosomal binding site is partially occluded [93]. When an inducible T7 promoter was used to drive *prfA* transcription in the absence of its 5'-UTR equal amounts of PrfA protein were produced at 30°C and 37°C. Furthermore, certain mutations (e.g. T46A, T49A) created to destabilize the hairpin structure resulted in full derepression of the *prfA* RNA thermometer and induction of all PrfA-dependent virulence factors at <30°C that enabled *Listeria* to enter and replicate in mammalian cells [93]. Other substitutions predicted to convert the structure into a “closer” conformation repressed expression of PrfA at 37°C, demonstrating that thermoregulation is solely based on conformational changes of the *prfA* 5'-UTR.

Sensing by RNA thermoswitches.

Although the majority of RNA thermometer prevent ribosome binding at low temperatures and allows translation initiation upon gradual zipper-like melting along a temperature gradient (zipper-like mechanism), other RNA thermosensors have been described that result in an induction of target protein production following a temperature downshift. In this case, other mechanisms were identified which operate in a riboswitch-type manner, favoring temperature-dependent switch-like conversions between two mutually exclusive RNA structures [83]. In more detail: At high temperatures the thermo-responsive element forms basepaired stemloops that sequester the ribosome-binding site, whereas low temperatures enforce an energetical/thermodynamic adaptation of the RNA conformation leading to another set of stemloops with an accessible Shine-Dalgarno sequence. A switch-like RNA thermo-

meter has been described in the 5'-UTR of the cold shock gene *cspA* in *E. coli*. The *cspA* gene encodes an RNA chaperone that interacts with single-stranded RNA and inhibits formation of secondary structures at low temperatures [104,105]. To the best of our knowledge no cold-sensing switch-like RNA thermometer has yet been identified that controls expression of classical virulence genes. Nonetheless, such thermoswitches would be more than conceivable for virulence gene regulation of vector-borne, insect- and plant-associated pathogens which activate their virulence program at low-moderate temperatures. Interestingly, almost all virulence genes of plant pathogenic bacteria studied by Smirnova *et al.* [106] with respect to temperature-dependent regulation exhibit increased expression at temperatures well below their growth optima. This includes virulence determinants directing bacteria-to-plant gene transfer, plant cell-wall-degrading enzymes, phytotoxins, and the type III protein secretion machinery. Similarly, cold-sensing RNA thermometers are suitable for pathogens using insect vectors to infect their mammalian host. For example, flea-specific virulence factors that are necessary for the colonization of the flea intestine by *Y. pestis*, the causative agents of bubonic plague, are highly expressed at moderate temperatures but not at 37°C [107,108]. Although the molecular control mechanisms have not yet been elucidated, low temperature-induced expression of several transmission factors was found to occur on the post-transcriptional level [109].

Thermosensing by trans-acting non-coding RNAs

Fundamentally different to the classical RNA thermometers are trans-acting non-coding RNAs that modulate translation initiation upon temperature changes. An example includes the small RNA DsrA of *E. coli* and *Borrelia burgdorferi* which controls translation of *rpoS*, encoding the general stress sigma factor σ^S in a tempe-

perature-dependent manner [110-112]. RpoS influences the expression of numerous virulence genes in many pathogenic bacteria [113]. In *E. coli*, DsrA binds to a site upstream of the Shine-Dalgarno region of *rpoS* which liberates the otherwise occluded ribosome-binding site and activates RpoS production [111]. Temperature-mediated control is achieved by an increased processing of the full-length DsrA transcript by RNase E and RNase III leading to an increased degradation of the mRNA and reduced RpoS synthesis at higher temperatures [114-117]. In *Borrelia*, the regulatory RNA DsrA folds into a secondary structure that prevents interaction with the *rpoS* mRNA. Melting of the DsrA secondary structure at 37°C enables interaction of the RNA with the anti-Shine-Dalgarno sequence of the *rpoS* transcript and allows translation of the sigma factor under virulence conditions [112]

Thermosensing through proteins

In comparison to nucleic acids, proteins have developed a greater variety of mechanisms to sense and transduce thermal changes into a signal that modulates virulence gene expression. Temperature has been shown to affect the protein conformation on the secondary, tertiary and quaternary level, and can thus be the decisive factor in determining whether a regulatory protein is in an active or inactive state. Protein-based thermosensors are widespread and both structurally and functionally highly diverse [9,118,119]. Temperature shifts can be sensed by chaperones and proteases, two-component systems as well as transcriptional regulators. Likewise, the mechanisms modifying the protein function upon temperature shifts are diverse and can be classified in three main groups: (i) disassembly of oligomeric proteins (ii) conformational changes of oligomers (iii) phosphorylation state of two-component system/integrity of membrane proteins due to alterations in membrane fluidity.

Thermo-regulated disassembly of oligomeric protein sensors

The TlpA protein of *S. enterica* serovar Typhimurium is the first identified proteinaceous thermometer with the extraordinary property that it loses its DNA-binding and repressor activity at higher temperatures. TlpA is encoded on the pSLT virulence plasmid of *Salmonella*. It possesses an N-terminal DNA-binding domain and an extraordinarily long coiled-coil motif implicated in dimer formation [120]. At 28°C, the repressing activity of TlpA is high, but it is strongly reduced when the surrounding temperature is increased. Hurme *et al.* [120] performed the first study that revealed the molecular basis for proteinaceous thermosensing. The TlpA protein forms active dimeric coiled-coils which disassemble more frequently into non-functional monomers at higher temperatures (37°C-43°C). The precise role of TlpA is still unknown, but it was suggested to act as an accessory repressor in the complex gene regulation of *Salmonella* pathogenicity [9]. However, first *in vivo* studies revealed that the transcription regulator is dispensable for virulence in mice [121].

Another example of virulence factors implicating coiled-coil motifs for temperature sensing are the M proteins of group A streptococci. M proteins have antiphagocytic properties as they enhance binding of the bacteria to plasma proteins of their hosts such as immune globulins, plasminogen and fibrinogen. Some M proteins with conserved C-repeats are known to undergo dramatic conformational changes upon temperature upshifts. At 10°C-20°C they form stable coiled-coil homodimers and exhibit a high binding affinity to the corresponding plasma protein. However, at 37°C the surface proteins appear as unfolded random coil monomers with a significantly lower binding activity to the plasma proteins [122]. A recombinant variant of the thermo-insensitive M protein Mrp4 in which the A-repeats were replaced by the C-repeats of the thermo-sensitive M protein Arp4, resulted in the loss of the coiled-coil

structure and fibrinogen-binding at high temperatures (40-45°C), indicating that the structural basis for the thermal stability is at least in part found in the C-repeats [122].

The highly conserved high-temperature requirement protein A (HtrA/DegP in *E. coli*) is a bifunctional polypeptide that displays and switches between two different functions in a temperature-dependent manner. At moderate temperatures, HtrA/DegP acts mainly as a chaperone to guide outer membrane proteins through the periplasm, whereas at temperatures > 30°C it operates predominantly as a serine protease ensuring that misfolded periplasmic proteins are degraded [123,124]. The protein is highly conserved among prokaryotes and plays a crucial role in protein quality control in the periplasmic space, especially upon temperature upshifts [123,125]. HtrA/DegP forms two staggered trimeric rings that harbor the protease domains on the top and bottom with PDZ domains sticking out to the periphery to capture unfolded proteins. In the hexameric state HtrA/DegP is proteolytically inactive. A conversion into catalytically active 12- and 24-meric multimers occurs upon a temperature-dependent switch or by recognition of unfolded substrates. The inner cavity of these large multimers combines the functions of a chaperone and a protease. Encapsulation into the inner cavity of folded protomers of outer-membrane proteins prevents their aggregation and permits their transit through the periplasm, whereas misfolded proteins are eliminated in the molecular reaction chamber [126,127]. HtrA is involved in many virulence processes, e.g. export of autotransporter adhesins and secretion of extracellular factors involved in biofilm formation. Furthermore, HtrA promotes degradation of damaged proteins that accumulate under host-induced oxidative stress. In consistency, *htrA* mutants of many pathogens show attenuated virulence [128-131]. Intriguingly, a novel role has recently been identified for HtrA of *Helicobacter pylori* and *Campylobacter jejuni*: HtrA was found to be secreted during infection and acts as a virulence factor that specifically cleaves the

extracellular domain of E-cadherin on infected gastric epithelial cells [132-135]. As a consequence, the mucosal integrity of the epithelium is destroyed, and the bacteria are enabled to transmigrate across the epithelial layer where they induce severe inflammation and cause disease. While protease activity of HtrA is impaired at low temperatures (5-13°C), it becomes fully induced at 37°C host temperature. This was explained by temperature-dependent disintegration of the HtrA oligomers [136].

Thermo-induced conformational changes abolish DNA-binding

Another important virulence regulator with an in-built temperature-responsive device is the RovA protein identified in all human pathogenic *Yersinia* species. RovA belongs to the SlyA/Hor/Rap family of dimeric winged-helix DNA-binding proteins and controls multiple virulence-associated functions crucial for the colonization and persistence in host tissues [28,137,138]. Among them is invasin the primary adhesion and invasion factor of enteric yersiniae [139-141]. The activity of the virulence regulator RovA is restricted to growth temperatures below 28°C *in vitro*. Under these conditions, RovA activates transcription of the invasin gene (*inv*) by binding to two separate sites upstream of the *inv* promoter region (Fig. 3). However, upon application of increasing temperatures RovA undergoes a gradual and reversible conformational transition (partial defolding) which abolishes DNA-binding and renders the regulator susceptible for degradation by the Lon protease [142]. A superimposed positive autoregulatory loop supports thermocontrol of RovA [80,143]. Recent efforts resolving the crystal structure of the regulator demonstrated that RovA forms highly stable dimers with flexible winged-helix DNA-binding domains that favor temperature-dependent transition between an active and an inactive form [144]. Interestingly, the closely homologous protein SlyA of *S. enterica* serovar Typhimurium was insensitive to thermal changes. In a structural-functional analysis of RovA, single amino acid

substitutions could be identified that affected degradation of the regulator (P98S, S127I/G128K) and thermosensing (G116A - within a loop of the dimerization region) [144]. This provided evidence that thermosensing activity is mainly mediated by a small loop within the RovA dimerization domain and residues in the adjacent C-terminal helix. These determinants allow partial unfolding of RovA upon a temperature upshift without disruption of the dimer. The structural distortion within the dimerization region is transmitted to the flexible DNA-binding domains, which mainly contact the DNA backbone. The low-affinity binding mode seems to enable the immediate release of RovA and allows rapid degradation of the inactive protein. Of the three other known thermosensing regulators (TlpA, and the heat-shock regulators RheA of *Streptomyces albus* and CtsR of *Bacillus subtilis*), the heat-sensing domain is only known for CtsR. Strikingly, in CtsR heat sensing also involves a glycine-containing flexible loop. However, this loop contains four consecutive glycine residues connecting the two β -sheets of its winged helix-turn-helix domain [145,146].

In this context, it should also be noted that another important *Yersinia* virulence regulator – the small nucleoid-associated DNA-binding protein YmoA – is thermoregulated. At body temperature, YmoA is rapidly degraded by Lon and the ClpXP proteases leading to upregulation of the *yop* and the type III secretion system genes important for the defense against the host immune system [82]. The thermosensing mechanism in this process is still unknown. Since the Lon protease of *Yersinia* is somewhat upregulated at 37°C, but still active at lower temperatures [142], thermo-induced conformational changes might also increase the susceptibility of YmoA to degradation. This could involve interaction with accessory proteins (e.g. heterocomplex formation with H-NS) which render them less or more susceptible for proteolysis.

Thermo-induced conformational changes abolish protein-protein interactions

L. monocytogenes' solution to adjust motility gene expression upon transition from ambient to host temperature is a thermo-sensitive repressor-antirepressor system. At moderate temperatures (22-28°C) *Listeria* is flagellated and motile which facilitates entry into host cells [147]. As flagella are easily recognized by the host immune system its synthesis is switched off at 37°C shortly after successful invasion [148,149]. Two proteins contribute reciprocally to the temperature-dependent transcription of flagellar motility genes: the MogR repressor and its anti-repressor GmaR (Fig. 3) [150-152]. MogR binds to the promoter of the flagellar motility genes and prevents their expression at physiological temperatures [152-154]. Below 37°C GmaR is synthesized and antagonizes MogR repression through MogR-GmaR complex formation [153]. The MogR-GmaR heterocomplex is destabilized and disrupted upon a thermal upshift to 37°C due to a conformational change in GmaR. Hence, the actual thermosensor in this process is not the DNA-binding protein MogR, but the interacting protein GmaR [155]. The response regulator DegU has also shown to be involved in the thermo-induced regulation of the flagellar motility genes [156,157]. DegU directly activates *gmaR* transcription and is therefore indispensable for constitutive GmaR expression. However, DegU was excluded from being an additional thermosensing mediator as it activates *gmaR* transcription independently of the temperature and its state of phosphorylation [150,158].

Thermosensing through phosphorylation of sensor kinases

Two-component systems provide a means for bacteria to sense nutritional and physical conditions, including temperature to adjust cell functions and virulence gene expression [159,160]. One prominent example of a thermosensing two-component system was identified in the plant-pathogenic bacterium *Agrobacterium tumefaciens*.

A. tumefaciens infects plants at wound sites and induces tumor formation at temperatures below 32°C. Two gene clusters, both encoded on the large Ti-plasmid of *A. tumefaciens*, are crucial for tumor induction: the so-called T-DNA and the *vir* locus [161,162]. The T-DNA is transferred and integrated into the plant genome, and uncontrolled expression of this gene cluster induces tumor formation [163,164]. The *vir* gene products are responsible for the processing and the transfer of the T-DNA from the bacteria into plant cells [165]. Their expression is only activated at temperatures below 32°C by a two-component system composed of the histidine kinase VirA and the response regulator VirG [166]. VirA is believed to act as the thermosensor of the system based on the following observations: (i) domain switch experiments with VirA rendered the kinase thermo-insensitive, (ii) the temperature optimum for *vir* transcription changed when VirA was modified, and (iii) *vir* expression below 32°C was inhibited when VirA, but not when VirG was constitutively produced. Apparently VirA undergoes a reversible conformational change at temperature higher than 32°C that inactivates the sensor kinase [4,167-169].

A similar membrane-spanning proteinaceous thermosensor was found in the soybean pathogen *Pseudomonas syringae* PG4180, which produces the virulence-enhancing phytotoxin coronatine (COR). COR is produced in a temperature-dependent manner. Large amounts of the molecule are only synthesized at 18°C *in vitro* and *in planta*, whereas at 28°C toxin production is negligible [170-172]. The two gene clusters for COR biosynthesis are separated and controlled by the *corSRP* locus, encoding a modified two-component system [170,173,174]. The histidine kinase (CorS) of the system is believed to respond to temperature and activates the response regulator (CorR) through transfer of its phosphoryl group (Fig. 3) [175]. The membrane-embedded CorS protein is composed of six transmembrane domains, followed by a catalytic H-box which is predicted to be located in the cytoplasm and

partially within a putative 7th transmembrane domain [176]. At 18°C the cytoplasmic portion of the H-box is accessible to autophosphorylation, but at 28°C, it is assumed to be incorporated into the membrane whereby CorS autophosphorylation is abolished (Fig. 3) [177]. Single amino acid substitutions were introduced into CorS to identify residues potentially important for temperature signal perception. Although no variant was found that resulted in a clear conversion of CorS from thermo-sensitive to thermo-insensitive, substitutions were identified that modulated temperature-dependent CorS activity and increased the thermo-responsiveness [178].

Thermosensing control cascades

It has become increasingly apparent that thermoregulation of virulence genes is often not simply mediated by one thermoregulator. Frequently, two or more thermosensing control elements act in concert to fulfill this task. For instance, temperature-dependent expression of the *Yersinia* host cell invasion gene *inv* is not only promoted by the transcriptional activator RovA (Fig. 3); it is supported by the thermo-sensitive binding of H-NS to flexible AT-rich binding sites overlapping the RovA binding region [80,142]. Another prominent example is the expression of the *Yersinia* virulence regulator LcrF which is controlled by the thermo-labile transcriptional repressor YmoA and an intergenic RNA thermosensor (see also above) [74]. The *lcrF/virF* promoter was also shown to contain regions of intrinsic curvature known to modify transcription initiation [76]. However, whether thermoregulation of *lcrF* by YmoA is simply the result of proteolysis of YmoA at 37°C or if altered DNA conformation upon temperature shifts influence YmoA-H-NS nucleocomplex formation at the *lcrF* promoter needs to be clarified. Similar hierarchies of transcriptional and translational thermosensing control elements are also part of the classical heat- and cold-shock re-

sponse. In particular, regulatory cascades composed of an alternative sigma factor (e.g. heat-shock sigma factor σ^{32}) and an RNA thermometer have been reported [118,179-181].

Another highly complex and multifactor regulatory cascade involves the transcriptional activator VirF, H-NS and an antisense RNA modulating the transcription of the virulence gene *icsA* of *S. flexneri*. Intrinsically curved DNA elements are located upstream of *virF* and the VirF-target gene *icsA*. They both respond to temperature shifts and control binding of H-NS whose quarternary structure may also be affected by a thermal increase [21,30,182]. In contrast to *virF*, *icsA* expression is further controlled by the antisense RNA RnaG. RnaG interacts with the 5'-region of *icsA*, including the binding sites of H-NS and VirF which both modulate *rnaG* transcription [182].

The relative contribution of the different thermocontrol mechanisms seems to depend on the actual conditions and appears to vary among the systems. What's the advantage of complex multi-layer control systems? Regulator-based activation of gene transcription is generally delayed compared to post-transcriptional mechanisms that act on the existing transcript. In contrast, both, built-in RNA or protein thermosensors are able to induce an immediate, but also a more cost-intensive response to temperature changes. Furthermore, each cascade can amplify and communicate thermo-control to a large set of different target genes. In summary, multi-layer control systems allow the bacteria to react with a rapid, very precise and economic global response to thermal changes, and enables integration of independent signals (e.g. temperature increase and accumulation of denatured proteins).

Complex multisensors

Circular dichroism spectroscopy illustrated that partial defolding of protein thermometers as well as melting of an RNA thermometer upon a temperature upshift allows the reverse reaction, i.e. refolding into the original protein and RNA structure, when the temperature declines [142,183,184]. Reversibility and speed in thermoregulation ensure an optimal adaptation of pathogens cycling between warm-blooded hosts and environmental niches or insect vectors. However, even within a given host, there might be a need for transient or local gene expression. In fact, recent studies demonstrated that this thermo-regulated reversible switching process can also be manipulated by additional regulatory elements or environmental cues, i.e. presence of certain metabolites. The *prfA* RNA thermometer of *L. monocytogenes* was found to be altered by a small metabolite-sensing riboswitch element. *Listeria* harbors several riboswitches, called SreA-G (SAM riboswitch elements) that are bound by S-adenosyl methionine (SAM) when the metabolite is present at elevated concentrations, e.g. in the host environment. Binding of the metabolite results in the formation of a transcriptional terminator that inhibits further synthesis of the riboswitch. The resulting truncated riboswitch element acts as small non-coding RNA (SreA). At 37°C, but not at 30°C, SreA binds to the distal side of the 5'UTR of *prfA* and inhibits translation of the *prfA* transcript. Although the precise mechanism and the physiological consequences of this process have not yet been elucidated, SreA mediated repression seems only possible under conditions when the RNA thermometer is in the “open” conformation” [185]. An additional level of complexity is established by PrfA. PrfA controls *sreA* expression and thereby includes an autoregulatory control mechanism [185].

Another interesting multisensor constitutes the proteinaceous thermometer RovA of *Yersinia*, a MarR-type transcriptional regulator. Members of MarR family, including RovA, form dimers with a central very flexible DNA-binding domain and an internal

pocket which can be occupied by small anionic lipophilic metabolites such as salicylate [186,187](Quade/Mendonca *et al.* unpublished results). We found that RovA-mediated activation of *inv* transcription was abolished in the presence of salicylate, although Lon-mediated degradation of the regulator was prohibited (Mendonca, unpublished results). Most likely, ligand-binding leads to a conformational change that prevents proper positioning of the winged helix-turn-helix domains and impedes DNA-binding. Superimposition of thermo- and metabolites-dependent control mechanisms might constitute an important strategy to restrict expression of virulence genes to certain host compartments and/or fluids through special metabolites that are indicative for the individual niche.

Future perspectives

Since thermal gradients and fluctuations have profound effects on fundamental cellular processes, molecular thermosensing devices could constitute one of the oldest environmental signal reception systems that evolved to support survival of living organisms [188]. Alterations of the conformational or physico-chemical state of virtually all classes of biomolecules can be used as primary sensor machinery, but so far thermosensitivity has mainly been demonstrated and studied with nucleic acids and proteins. Yet, also the physical state of lipid membranes (e.g. arrangement of amphipathic phospholipids, membrane fluidity) changes in response to temperature shifts. Thus, membranes could act as thermosensors. However, the temperature-induced effects might be less efficient and seem more complex as many different fatty acids, cholesterol-like molecules and membrane-associated proteins influence this process. Nonetheless, there is a small but increasing number of examples in which temperature-induced changes of the membrane fluidity exert high lateral

pressure on membrane-associated proteins involved in intracellular signaling and control of gene expression [189,190]. The expression of cold-inducible desaturases implicated in the control of lipid homeostasis of *Synechocystis* and *B. subtilis* is regulated by classical two-component systems of which the membrane-bound histidine kinases sense and transduce changes in the membrane fluidity [191-194]. Notably in this context is a recent structural analysis of the histidine kinase DesK of *Bacillus subtilis*. DesK is cold-activated through specific interhelical rearrangements in the central four-helix bundle domain which influence the autokinase or phosphotransferase/phosphatase activity of the protein [195]. A similar thermo-induced helix rotation mechanism could be operational in a wide range of sensor histidine kinases. Moreover, the RssAB signal transduction system of *Serratia marcescens* and other bacteria regulate swarming motility and cell envelope architecture in response to temperature and the fatty acid composition/fluidity of the phospholipids [196,197]. Based on these observations, it is highly likely that thermo-induced membrane perturbations also affect the activity of some sensor kinases involved in the control of virulence genes.

Very little is known about the direct influence of thermo-induced alterations of membrane properties on the function of virulence factors [198]. It is worthy to note that host cell invasion of pathogens can be inhibited by a temperature downshift from 37°C to 25°C. In addition, manipulation of the lipid ordering, lipid rafts or membrane microdomain mobility affected the efficiency of receptor-mediated phagocytosis of several pathogens [199-201]. This indicates that fluidity and deformability of the host and pathogen membranes are crucial for proper exposure of adhesins/invasins onto the bacterial cell surface and bacterial phagocytosis. Following on from this, we are convinced that many more lipid thermosensors contribute to temperature control of virulence genes that remain to be discovered.

Many years ago, it has been reported that even water and aqueous solutions change their properties upon a temperature increase from 0 to 60°C, most likely due to altered hydrogen bond interaction [202]. This so-called enthalpy-entropy compensation phenomenon of vicinal water (hydration shell) could influence proteins and nucleic acids and might affect the activity of biological systems [203,204]. In fact, denaturation of RNA hairpin structures is preceded by a change of the RNA-solvent interaction - a melting of the more labile hydration shell [205]. The relevance of this process was demonstrated for the *agsA* fourU RNA thermometer in *S. enterica* where melting of the transcript occurred only after the stabilizing RNA-solvent interaction has vanished completely [92]. Lately, it has also been found that the binding of ions (e.g. Mg^{2+}) to sensory RNAs is regulatory relevant. Applying NMR techniques Mg^{2+} binding site could be identified in the *agsA* fourU RNA thermometer and shown to shift its melting point [91]. To fully comprehend the role of solvent, ion and metabolite interactions, the analysis of additional and functional different thermosensors and their dynamic response to temperature is required.

For thermosensing, it is clear that a number of alternative DNA-, RNA- and protein-based devices exist which sense temperature through conformational changes. A molecular understanding of the structural mechanics of the sensing and signal transduction process of the different molecular thermometers, and analysis of their complexity and role for pathogenesis may allow the development of novel anti-infectives. For instance, small molecule inhibitors could be selected or designed that interact efficiently with a thermosensor and hold it in the “inactive” conformation. Thermally inducible expression systems also represent very cheap and useful tools for biotechnological applications. In fact, recent approaches to construct synthetic RNA thermometers by rational design and from random sequences showed that

even short single stemloops including the Shine-Dalgarno sequence were sufficient to control gene expression in a temperature range of 17-42°C [206,207].

At present, many novel and complex thermosensors are still discovered illustrating the requirement to handle and fine-tune temperature responses in an individual and dynamic fashion. Evidently, there are thermometers in many more biomolecules and novel molecular thermosensing principles are still to be elucidated. We just have to sense them and track them down.

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Executive Summary

Thermosensing through DNA

- Temperature-mediated alterations in the DNA topology (supercoiling or intrinsic bending) influence transcription efficiency by affecting the binding affinity of RNA polymerase or transcriptional regulators
- Nucleoid-associated proteins (H-NS, FIS, Hha/YmoA etc.) bind to DNA and modulate its topology in response to temperature which alters transcription of downstream genes
- Low energy costs: no synthesis of mRNA or protein, speed of response: medium; reversible

RNA thermometer

- At low temperatures the 5'-UTR of target transcripts forms a hairpin structure which sequesters the ribosome-binding site and prevents translation
- Increasing temperature induces „melting“ of the hairpin and renders the ribosome-binding site accessible for ribosomes and permits translation
- Simple and efficient: fourU elements; more complex and integrative: *prfA*
- Medium energy costs: synthesis of mRNA; speed of response: rapid; reversible

Proteinaceous thermosensors

- Temperature modulates conformation of tertiary and quaternary structures
- Several classes of proteins are affected: transcriptional regulators, sensor kinases, repressor/anti-repressor complex, proteases and plasma binding proteins
- Mechanisms that exert thermosensing: (dis)assembly of oligomers, conformational changes leading to (mis)folding, altered phosphorylation capacity of two-component systems
- The variety of affected protein classes together with the diversity of thermo-induced changes in the molecular structure offer numerous regulatory combinations
- High energy costs: synthesis of the protein; speed of response: medium; not always reversible

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Figure Legends

Fig. 1. Thermally controlled mechanisms of gene transcription through the concerted action of the conformational shift in the local DNA topology and the interaction of DNA-bending regulatory proteins, such as H-NS and other NAPs. **(A)** In the left-handed panel (at low/moderate temperatures) H-NS is able to bind and polymerize along the DNA in a cooperative manner. It further mediates bridging between local DNA strands, which results in the formation of a high-order DNA-H-NS oligomer complex and leads to transcriptional silencing. The right-handed panel (37°C) illustrates the thermoinduced conformational change of the promoter region which reduces DNA-bending, and shows the loss in the DNA-binding affinity of H-NS due to changes in the local DNA topology and/or H-NS dimer conformation. Abrogation of H-NS silencing makes the promoter region more accessible to the RNA polymerase, leading to transcription initiation. **(B)** Illustration of the mechanisms by which H-NS modulates virulence gene expression in *Enterobacteriaceae*. (1) Thermo-induced modification of the *virF* promoter topology and binding of Fis abolish H-NS repression in *Shigella*, (2) abrogation of H-NS-mediated DNA-bridging upon a thermal upshift allows antirepression and activation of the major virulence operons within the LEE pathogenicity island by Ler in enteropathogenic and hemorrhagic *E. coli*, (3) thermo-dependent restoration of DNA-bridge formation by H-NS-Hha complexes in *Salmonella* controls expression of the virulence regulator SsrB, and (4) weekend H-NS-mediated DNA-bridging by the H-NS-YmoA complex controlling expression of the virulence regulator LcrF/VirF in *Yersinia* renders the repression complex more sensitive to thermal upshifts.

Fig. 2. LcrF synthesis is post-transcriptionally controlled through a fourU RNA thermometer. **(A)** The 5'UTR of *lcrF* forms a secondary structure with two hairpins

which inhibits translation of the transcript at 25°C. **(B)** At low temperatures (25°C) the ribosomal binding site (rbs) is sequestered by intramolecular basepairing with four uridines (fourU element) in the second hairpin. The binding of the 30S ribosomal subunit to the rbs is hindered leading to translation inhibition. Due to a temperature increase (37°) the hairpin formation starts to open up and liberates the rbs. Now ribosomes gain access and initiate translation.

Fig. 3. Different mechanisms of protein thermosensation. **(A)** At 25°C the homodimeric transcriptional regulator RovA of *Yersinia* binds to two sites in the promoter region of the virulence gene *inv* and activates its expression. At increasing temperatures the dimer conformation changes reversibly which destroys DNA-binding capacity of RovA and leads to *inv* repression at 37°C. **(B)** The repressor/anti-repressor complex MogR:GmaR regulates motility of *Listeria*. At 37°C MogR binds to all flagellar motility gene promoters including that of *gmaR* and represses their transcription. Response regulator DegU can bind upstream of the *gmaR* promoter and activate transcription thermo-independent, but does not prevent repression by MogR. Small amounts of GmaR protein produced at 37°C cannot bind to MogR due to the conformation. When the temperature decreases (30°C) GmaR undergoes a conformational change and is able to bind MogR. GmaR counteracts MogR repression by removing it from target promoters. **(C)** Transcription of the genes for biosynthesis of the phytotoxin COR (e.g. *cma* operon) of *P. syringae* PG4180 is thermo-regulated by the two-component system CorSR. At low temperatures (18°C) the membrane associated sensor kinase CorS autophosphorylates at a conserved histidine residue in the H-box. Signal transfer is provided by subsequent phosphorylation of the response regulator CorR which in turn activates COR gene transcription. Higher temperatures (28°C) may result in rearrangements of CorS transmembrane

domains (TMDs) that force the H-box to localize in the membrane where it is inaccessible for autophosphorylation. As a consequence phosphate transfer to CorR is also abolished and COR gene expression is repressed.

Table 1. The variety of thermo-responsive mechanisms used by pathogens			
organism	thermosensor	affected virulence mechanism	references
DNA			
<i>S. flexneri</i> & <i>E. coli</i> (EIEC)	H-NS/Fis	H-NS inhibits <i>virF</i> transcription <32°C; Fis binds to <i>virF</i> promoter and counteracts H-NS repression >32°C	[21,22,43]
<i>E. coli</i> (UPEC)	H-NS/RimJ	23°C: H-NS and RimJ block <i>papBA</i> transcription independently, H-NS prevents methylation of <i>pap</i> GATC sites; RimJ mechanism is unknown	[46,47,49]
<i>E. coli</i> (EHEC, EPEC)	H-NS/Ler	H-NS represses LEE1-5 transcription (including Ler) <37°C; at 37°C Ler is synthesized and counteracts H-NS	[53,58,60-62]
<i>Salmonella enterica</i>	H-NS/Hha	H-NS represses SsrB transcription thereby blocking expression of SPI-2 virulence genes < 30°C; Hha forms complex with H-NS and enhances repression	[63,64,68-71]
<i>Yersinia</i> spp.	H-NS/YmoA	Intrinsic curvature within virulence plasmid including <i>virF/lcrF</i> ; H-NS/YmoA heterodimeric complex represses <i>lcrF</i> transcription at low temperatures (25°C)	[73-76]
RNA			
<i>Yersinia</i> spp.	<i>lcrF</i>	FourU thermometer, 25°C no translation of <i>lcrF</i> , melting of the secondary structure liberates ribosome-binding site at 37°C Integrative control: <i>lcrF</i> transcriptional regulation by YmoA	[74,97]
<i>Listeria monocytogenes</i>	<i>prfA</i>	5'UTR of <i>prfA</i> forms hairpin <30°C, no translation; melting of the secondary structure liberates ribosome-binding site at 37°C multisensor: truncated riboswitch binds to <i>prfA</i> and inhibits translation at 37°C	[93,185]
protein			
<i>Salmonella enterica</i>	TipA	Coiled coil dimeric transcriptional regulator, represses target genes at 28°C; the regulator dimers disassemble and are inactive at 37°C	[9,120]
<i>Streptococci</i>	M proteins	Class C proteins bind to plasma proteins at low temperatures as coiled coils; homodimers disassemble at 37°C; C-repeats seem to be the thermosensitive unit	[122]
<i>Helicobacter pylori</i> <i>Campylobacter jejuni</i>	HtrA	Secreted virulence factor, cleaves E-cadherin as protease; forms homooligomers and is fully induced at 37°C, disassembly of HtrA oligomers at low temperatures	[132,133,136]
<i>Yersinia</i> spp.	RovA	Transcriptional activator of virulence genes; binds to DNA as homodimer at 20-25°C, defolds partially at higher temperatures and loses its DNA binding capacity; multisensor: salicylate prevents DNA-binding of RovA	[137,139,142]
<i>Listeria monocytogenes</i>	MogR:GmaR	MogR binds to flagella genes and represses transcription at 37°C; anti-repressor GmaR binds MogR at 30°C; in complex no repression; GmaR misfolds at 37°C and MogR:GmaR complex disassembles	[150,152,153,155]
<i>Agrobacterium tumefaciens</i>	VirA	Two-component system VirAG activates transcription of <i>vir</i> genes only below 32°C; sensor kinase VirA changes conformation at high temperatures and is inactive	[4]
<i>Pseudomonas syringae</i>	CorS	Two-component system CorSR activates transcription of phytotoxin biogenesis genes at 18°C; at 28°C: rearrangements of the transmembrane domains of the sensor kinase CorS prevent autophosphorylation and/or phosphotransfer to response regulator	[177,178]

Table 1. The variety of thermo-responsive mechanisms used by pathogens

organism	thermo-sensor	virulence affecting mechanism	reference
DNA			
<i>Salmonella enterica</i>	H-NS/Hha	H-NS represses SsrB transcription thereby blocking expression of SPI-2 virulence genes < 30°C; Hha forms complex with H-NS and enhances repression	Fass and Groisman 2009 Duong 2007 Madrid 2007 Vivero 2008
<i>Shigella flexneri</i> & <i>E. coli</i> (EIEC)	H-NS/Fis	H-NS inhibits <i>virF</i> transcription <32°C; Fis binds to <i>virF</i> promoter and counteracts H-NS repression >32°C	Falconi 1998 Falconi 2001 Prosseda 2004
<i>Yersinia</i>	H-NS/YmoA	Intrinsic curvature within virulence plasmid including <i>virF/lcrF</i> ; H-NS/YmoA heterodimeric complex represses <i>lcrF</i> transcription at low temperatures (25°C)	Rohde 1999 Marceau 2005 Böhme 2012
<i>E. coli</i> (UPEC)	H-NS/RimJ	23°C: H-NS and RimJ block <i>papBA</i> transcription independently, H-NS prevents methylation of <i>pap</i> GATC sites; RimJ mechanism is unknown	Göransson 1990 White-Ziegler 2000 White-Ziegler 2002
<i>E. coli</i> (EHEC, EPEC)	H-NS/Ler	H-NS represses LEE1-5 transcription (including Ler) <37°C; at 37°C Ler is synthesized and counteracts H-NS	Bustamante 2001 Umanski 2002
RNA			
<i>Yersinia pseudotuberculosis</i>	<i>lcrF</i>	FourU thermometer, 25°C no translation of <i>lcrF</i> , liberated rbs at 37°C Integrative control: <i>lcrF</i> transcriptional regulation by YmoA	Böhme 2012
<i>Listeria monocytogenes</i>	<i>prfA</i>	5'UTR of <i>prfA</i> forms hairpin <30°C, no translation ; liberated rbs at 37°C Integrative control: truncated riboswitch binds to <i>prfA</i> and inhibits translation	Johansson 2002 Loh 2009
protein			
<i>Helicobacter pylori</i>	HtrA	Secreted virulence factor, cleaves E-cadherin as protease; forms homooligomers and is fully induced only at 37°C, desintegration of HtrA oligomers at low temperatures	Löwer 2008 Hoy 2010 Hoy 2012
<i>Salmonella typhimurium</i>	TlpA	Repressive activity on virulence genes as coiled coil dimer at 28°C; disrupts to monomers and is inactive at 37°C	Hurme 1996 Hurme 1998
<i>Streptococci</i>	M-like proteins	Class C proteins bind to plasma proteins low temperatures as coiled coils; homodimers disrupt at 37°C; C-repeats seem to be thermosensitive	Cedervall 1995 Cedervall 1997

<i>Yersinia</i>	RovA	Transcriptional activator of virulence genes; binds to DNA as homodimer at 20-25°C, defolds partially at higher temperatures and loses DNA binding capacity	Ellison and Miller 2006 Heroven 2007 Herbst 2009
<i>Listeria monocytogenes</i>	MogR:GmaR	MogR binds to flagella genes and repress transcription at 37°C; anti-repressor GmaR binds MogR at 30°C; in complex no repression; GmaR misfolds at 37°C and MogR:GmaR complex disrupts	Kamp 2009 Kamp 2011 Shen 26 Shen and Higgins 2006
<i>Agrobacterium tumefecians</i>	VirA	TCS VirAG activates transcription of <i>vir</i> genes only below 32°C; sensor kinase VirA changes conformation at high temperatures and is inactive	Jin 1993
<i>Pseudomonas syringae</i>	CorS	TCS CorSR activates transcription of phytotoxin biogenesis genes at 18°C; at 28°C: rearrangements of TMDs of sensor kinase CorS, blocked autophosphorylation and no signal transduction	Smirnova 2008 Braun 2007

A **B**

Change of DNA topology and antirepressor/activator

Modification of H-NS binding by interacting paralogues

Fig. 1 Steinmann & Dersch 2012

A **B**

Fig. 2 Steinmann & Dersch 2012

Fig. 3 Steinmann & Dersch 2012