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Thermal control of virulence factors in bacteria: A hot topic

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Pathogenic bacteria sense environmental cues, including the local temperature, to control the production of key virulence factors. Thermal regulation can be achieved at the level of DNA, RNA or protein and although many virulence factors are subject to thermal regulation, the exact mechanisms of control are yet to be elucidated in many instances. Understanding how virulence factors are regulated by temperature presents a significant challenge, as gene expression and protein production are often influenced by complex regulatory networks involving multiple transcription factors in bacteria. Here we highlight some recent insights into thermal regulation of virulence in pathogenic bacteria. We focus on bacteria which cause disease in mammalian hosts, which are at a significantly higher temperature than the outside environment. We outline the mechanisms of thermal regulation and how understanding this fundamental aspect of the biology of bacteria has implications for pathogenesis and human health.

Introduction

Pathogenic bacteria must sense and respond to changes in their local environment in order to successfully colonise and invade their hosts. Microbes achieve this by continually monitoring multiple characteristics of their surroundings, including the ambient temperature, pH, osmotic pressure and nutrient availability. These signals are then integrated to fine-tune gene expression and protein production. Bacteria possess specific virulence factors that enable them to attach to host cell surfaces, evade immune defenses or obtain nutrients which might be otherwise inaccessible. Unregulated expression of these virulence factors can be detrimental for bacteria, through wasteful expenditure of metabolic resources and inappropriate induction of inflammatory and immune responses. Therefore, bacteria possess regulatory mechanisms that restrain production of virulence factors until they detect appropriate environmental cues present in their hosts.[1]

As the core temperature of mammalian hosts is usually higher than that of their surroundings, an increase in temperature is a change consistently experienced by pathogens when they migrate from the external environment to their host. As a result, many bacterial virulence factors are thermally regulated. The various mechanisms which the bacteria employ to sense host temperatures and control their virulence factors are usually considered distinct from the heat shock response, which is a homeostatic collection of processes shared by both commensal and pathogenic bacteria. However, there are examples of heat shock proteins also playing a role in virulence, which suggests there is a significant degree of overlap between these responses.

Here we review recent advances in understanding the underlying mechanisms of how bacterial pathogens regulate virulence factors in relation to temperature and provide examples of how this contributes to the development of disease (**Fig. 1**). We focus on examples of how microbes sense the elevated temperatures once they enter a mammalian host, even though pathogens of plants and insects can regulate gene expression in response to lower temperatures.[2-4]

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Mechanisms of thermo-sensing: exerting multi-level control

Regulation at the level of DNA

Changes in the local temperature are detected by bacteria through multiple mechanisms; temperature can influence the topology of DNA, affect RNA structuring and metabolism, and change the activity and processing of proteins (**Fig. 2**). At the DNA level, temperature influences the extent of DNA supercoiling which can affect the rate of transcription,[5] providing a mechanism for thermal regulation of gene expression.[6] For example, super-coiling is involved in the expression of genes encoding Type I fimbriae in *Escherichia coli*[7] and a type III secretion system (T3SS) in *Salmonella enterica*,[8] which are both required for virulence. Additionally, alterations in DNA curvature can modulate gene expression in a temperature-dependent manner. This is illustrated by the transcription of *virF*, which encodes a regulator of genes on the large virulence plasmid in *Shigella* spp.[9] At non-permissive lower temperatures, DNA at the *virF* promoter assumes a bent configuration, which is facilitated by the histone-like nucleoid structuring protein, H-NS. This impairs binding of RNA polymerase at the promoter, resulting in reduced transcription of genes encoding virulence factors outside the host.

H-NS is found in several Gram-negative pathogens[10] in which it can repress expression of virulence genes.[11-14] H-NS binds AT-rich, curved stretches of DNA,[15] and silences genes by oligomerising to form a repression complex that blocks access of RNA polymerase to promoters.[16] As formation of the active, tetrameric form of H-NS does not change at temperatures above 28°C,[17]

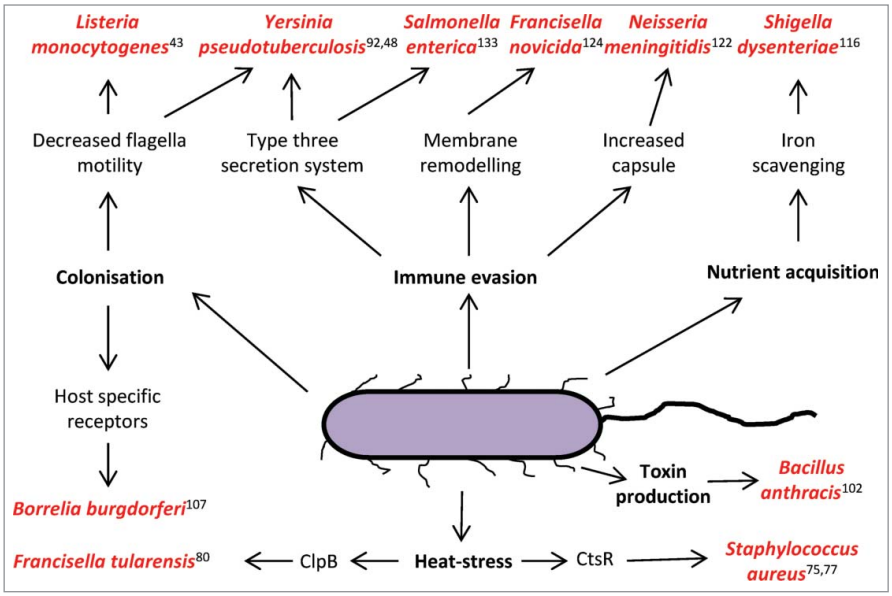

Figure 1. Thermo-regulation of important virulence factors in pathogenic bacteria species. An increase to 37°C (mammalian body temperature) is a universal invasion signal to the bacteria and enables fine tuning of virulence factor expression, promoting survival and proliferation in its host.

the oligomerization state of H-NS cannot fully account for the de-repression of gene expression above 30°C. The importance of temperature-dependent H-NS regulation in bacteria is illustrated by *Salmonella enterica* in which over 200 genes are upregulated at 37°C in an H-NS dependent fashion; many of these genes are within *Salmonella* SPI-1 pathogenicity island.[18] Furthermore, the binding of H-NS at the *hilC* promoter decreases between 25°C and 37°C,[18] allowing expression of this activator of SPI-1 genes at higher temperatures;[19] similar H-NS dependent thermoregulation also occurs at the *E. coli* hemolysin (*hly*) locus.[20] In both instances, local changes in DNA conformation might contribute to the increased affinity of H-NS for its target sequence at lower temperatures.

Regulation at the level of RNA

mRNA can assume a variety of secondary and tertiary structures which have consequences on protein translation by altering interactions of the mRNA with ribosomes.[6,21] For example, RNA thermosensors are typically found in the 5′-untranslated region (5′-UTR) of an mRNA molecule and control translation of that specific message, in which they are said to be *cis*-acting.[22] *cis*-acting RNA thermosensors were initially discovered in genes encoding heat shock proteins (HSP), and include the *hspA* ROSE1 element in *Bradyrhizobium japonicum*,[23] the *agsA* fourU element in *Salmonella enterica*,[24] and the *hsp17* bi-partite hairpin found in *Synechocystis* spp[25] In each case, the RNA thermosensor is located in the 5′-UTR of the mRNA with the 3′-end of the stem-loop sequestering the ribosome binding site (Shine-Dalgarno sequence, SD) and preventing translation at lower temperatures. In a stem-loop structure, the length of the hairpin and the presence of non-canonical base pairing define the thermodynamic properties of melting and annealing, and thus determine the relationship between protein translation and temperature.[22,26] RNA thermosensors offer a particularly rapid response to a change in temperature, as they do not require a signaling pathway or *de novo* mRNA transcription. RNA thermosensors can also induce protein expression following cold shock.[27] This occurs through the formation of two discrete structures: one which sequesters the SD site from ribosomes, while another structure forms at lower temperature that exposes the SD, enabling translation. Given the complexity of RNA structures, predicting RNA thermosensors using genome-wide bioinformatics has become a significant obstacle and requires further understanding of the dynamics of RNA secondary structures,[28] computer modeling,[29] and extending databases of known RNA thermometers.

Alternatively, RNAs can regulate other transcripts, when they are *trans*-acting.[30] In this case, a regulatory small RNA (sRNA) is transcribed independently from the target mRNA. Base pairing between the two RNA molecules can result in gene silencing through targeted degradation by ribonucleases (RNases) or sequestration of the ribosome binding site. Alternatively, base pairing can alter the secondary structure of the mRNA to expose the ribosome binding site.[31] Another mechanism of thermal regulation at the level of mRNA occurs by changes in the stability of the message. Degradation of specific RNAs is an efficient way of adjusting their abundance and this is brought about by RNases.[32,33] For example, RNase E is autoregulated by cleaving a stem-loop structure in its own 5′ UTR,[34] and can also be involved in silencing genes by cleaving mRNAs paired with a sRNA.[33,35] For example, in pathogenic *E. coli*, the gene encoding the AfaD adhesin is regulated by the temperature-dependent expression of sRNA, AfaR.[36] Approximately 42 bases of AfaR bind to the 5' UTR of the *afaD* transcript. Binding is promoted by Hfq, and leads to RNase E degradation of the message. Similarly, in *Yersinia pestis* and *Yersinia pseudotuberculosis*, many small RNAs are expressed at 37°C and not at 28°C and have thus been suggested to have roles in regulating virulence.[37] The targets of these sRNA have not yet been defined but their function can be modulated by the RNA chaperone Hfq, whose ability to bind RNA is higher at lower temperatures.[37,38] Hfq facilitates binding of sRNAs to target mRNAs, leading to their subsequent degradation,[39] and has been shown to play an important role in pathogenesis in several organisms.[38-41]

Regulation at the protein level

At the level of protein activity and processing, thermo-regulation usually results from a conformational change in a thermosensitive domain of a protein.[6,42] This subsequently modulates

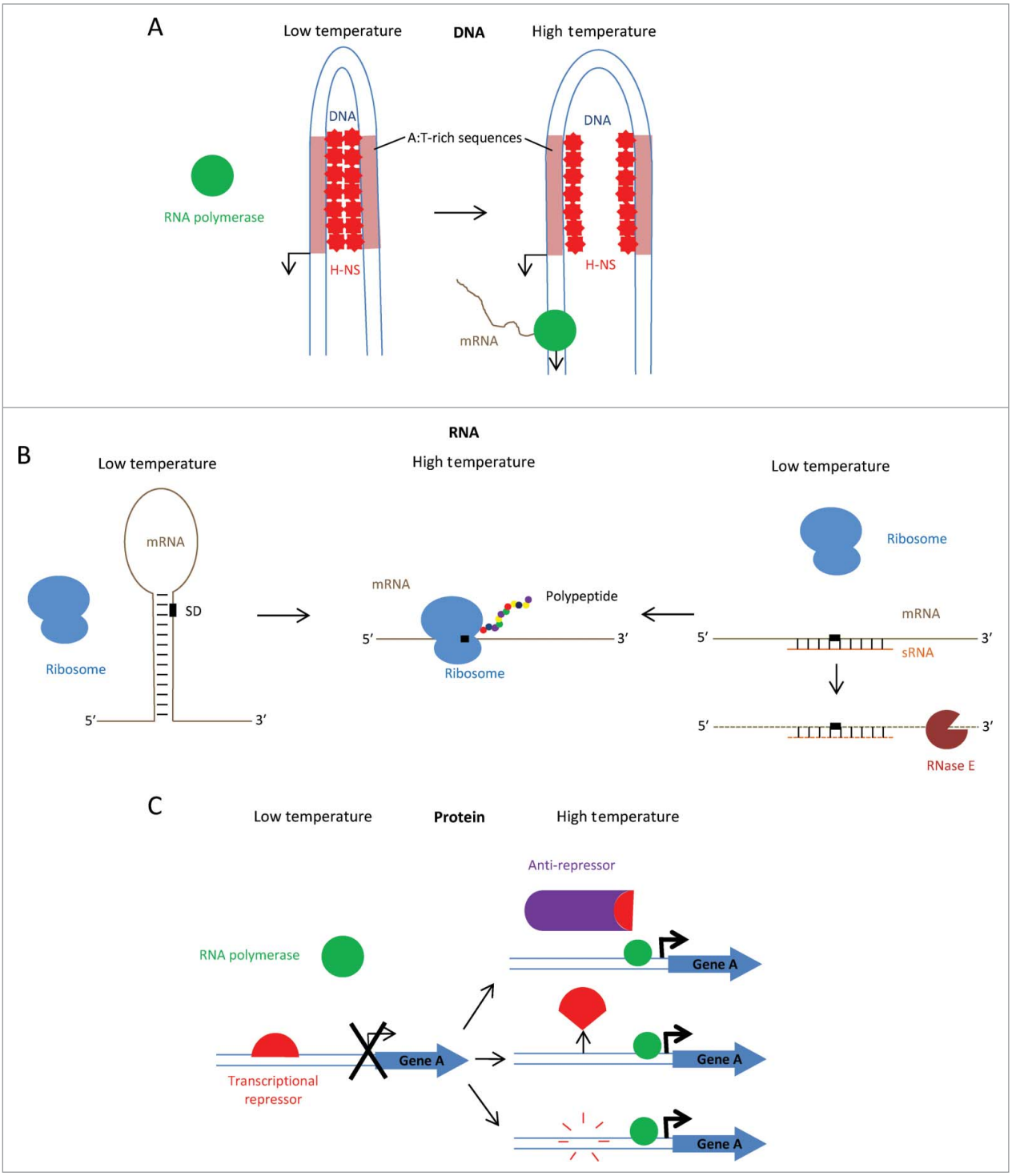

Figure 2. Mechanisms of thermo-regulation (**A**) At low temperature, the DNA at the promoter region of some virulence genes favors a bent conformation and is stabilised by H-NS, which binds curved DNA at A:T rich sites, blocking access of RNA polymerase to the promoter. At high temperature, homotypic H-NS binding is reduced by relaxation of the DNA bend, enabling access of RNA polymerase to the promoter (e.g. virF in Shigella). (**B**) At low temperature, mRNA thermometer adopts a stem-loop structure at the 5' position, sequestering the Shine-Dalgarno (SD) sequence from ribosome binding and preventing translation. At high temperature, stem-loop melts to expose the Shine-Dalgarno sequence to ribosome binding and translation occurs. Alternatively, trans-acting small RNAs act by sequestering the SD sequence at low temperature but not at high temperature. Double stranded RNAs are targeted for degradation by RNases. (**C**) At low temperature, repressor is bound to promoter of Gene A and transcription is prohibited. At high temperature, either: the anti-repressor is stabilised and removes the repressor from the promoter (e.g., GmaR in Listeria monocytogenes), a conformational change causes the repressor to dissociate from the DNA (e.g. RovA in Yersinia spp.) or the repressor is degraded by temperature-dependant proteases which permits the transcription of Gene A (e.g., RovA in Yersinia spp).

the function of the protein or changes its susceptibility to degradation by proteases.[43] For example, GmaR, an anti-repressor in *Listeria*, undergoes a conformational shift at 37°C and is then degraded; loss of GmaR allows MogR to repress genes involved in flagellar based motility within the mammalian host.[42] The interaction between the function and stability of several proteins in a network allows fine tuning of virulence factor expression, as well as the integration of inputs from several environmental stimuli,[44] permitting a more dynamic response.

Temperature affecting global gene expression through a single transcriptional regulator

Co-ordinating regulation of multiple virulence factors can be efficiently achieved through a single transcription factor that modulates expression of a suite of genes. By placing a single transcription factor under thermal control, a pathogen can adapt to the new environments it encounters when it enters its host. An exemplar is provided by PrfA, a transcriptional regulator in *Listeria monocytogenes*, that is controlled by an RNA thermosensor.[45] At temperatures below 30°C, a stem-loop structure is predicted to form in the 5′-UTR of the *prfA* mRNA, which occludes the ribosome binding site and prevents protein translation. However, at the higher temperatures found in mammalian hosts, this hairpin melts enabling efficient translation of PrfA, which orchestrates expression of genes involved in intracellular invasion and survival. Analysis of the dynamic response of the PrfA thermosensor demonstrates that it acts as an abrupt, on/off switch; this could be explained by the abundance and clustering of G:C bonds at the base and top of the predicted stem loop.[26] Furthermore, a *trans*-acting riboswitch in the *prfA* transcript binds S-adenosyl methionine. Binding of this metabolite to the mRNA also prevents translation,[30] enabling integration of cues from the local temperature and nutritional state of the bacterium to optimise the expression of virulence factors.

Yersinia spp. provide another example of the diversity of regulatory mechanisms found in bacteria that respond to host temperatures.[46,47] LcrF is a global regulator in *Y. pseudotuberculosis*, the cause of a scarlet fever-like illness in humans. LcrF is regulated by temperature both at the level of transcription and translation.[48] This transcriptional activator controls expression of genes encoding a T3SS and secreted proteins, that contribute to pathogenesis.[49] The 5′-UTR of the *lcrF* mRNA harbours a RNA thermosensor similar to *prfA*. Furthermore, transcription of *lcrF* is also repressed by the nucleoid-associated protein YmoA, which is selectively degraded at 37°C.[48] *Yersinia* spp also possess RovA which represses the expression of several virulence factors,[50-53] and is required for colonisation and adaptation to host-associated stress.[54] A shift from external to host physiological temperatures (i.e. to 37°C) results in a conformational change in the DNA-binding domain of RovA, leading to its dissociation from target promoters, and de-repression of gene expression.[42,55] Additionally, proteolytic degradation of RovA is enhanced at higher temperatures, further reducing RovA repression regulator within the host.[55]

In *Bordetella* spp. many important virulence factors are controlled by the BvgAS two-component system. This system is composed of the histidine kinase BvgS, which is found in the inner membrane of the bacteria, and BvgA, a response regulator. Auto-phosphorylation of the cytoplasmic domain of BvgS in response to specific environmental stimuli leads to phosphorylation and activation of BvgA, which subsequently binds promoter regions and activates transcription.[56] Upon shift to 37°C, BvgA also autoregulates its own expression and also controls genes required in the early stage of infection, such as those for cell adhesion.[57] Additionally, BvgA activates other genes required later in the infection process, such as ptx encoding pertussis toxin.[58] Although the precise mechanism for the temperature dependent activation of BvgAS has yet to be characterized, the linker region between the transmembrane and kinase domains of BvgS is crucial for thermal sensing.[59]

Feeling the heat in the host: Integration of thermal regulation with stress responses

Pathogens encounter several forms of stress once in a mammalian host including a change in pH, osmotic gradients, exposure to reactive oxygen and nitrogen species, as well as nutrient limitation. As previously discussed, heat shock responses in bacteria are usually considered as distinct from specific, temperature-triggered adaptations for survival in hosts. However overlapping networks of regulation often found in bacteria, and indirect effects of heat shock and other stress responses can influence the production of virulence factors.[60,61]

In *E. coli*, the heat shock response is orchestrated by the sigma factor, σ^{32} (RpoH), which controls the expression of a diverse range of heat shock proteins with homeostatic function.[62] This transcription factor is subject to several hierarchies of control and functional homologues of RpoH are present in a range of Gram-negative bacteria.[63] Translation of *rpoH* mRNA itself is modulated in *E. coli* by an RNA thermometer[64] which, following an increase in temperature to 42°C, enhances the efficiency of ribosome binding leading to an increase in translation. The activity of RpoH is also regulated post-translationally by the chaperones DnaK and GroEL,[65] and its turnover is controlled by specific proteases such as FtsH.[66] RpoH indirectly influences the transcription of virulence gene regulators.[67,68] In uropathogenic *E. coli*, RpoH controls expression of *leuX*[68] which encodes tRNA5leu which is important for the synthesis of virulence factors encoded on the pathogenicity island, PAIII356.[69]

Gram-positive bacteria possess heat shock proteins, but the way in which the heat shock response is co-ordinated is distinct. In *Bacillus subtilis*, for example, there are three distinct classes of heat-inducible proteins.[70] Class I heat shock genes are the most highly induced following heat stress, and include those encoding the major chaperones DnaK and GroEL.[71] Class II heat shock genes are subject to regulation by σ^B which is upregulated in response to several types of stress.[71,72] Finally, class III heat shock genes include those encoding ATP-dependent proteases, with expression occurring independent of σ^B.[71] Of note, σ^B positively regulates expression of *sar*, which encodes winged helix-turn-helix transcriptional regulator which influences the production of

many virulence factors in *Staphylococcus aureus*.[73,74] Additionally, CtsR, which is a transcriptional repressor that is part of the σ^B-independent heat shock response in several Gram-positive pathogens,[75] also controls a wide range of target proteins involved in virulence.[76] CtsR exhibits high affinity DNA binding to target promoters at low temperature (30°C) but lower affinities at normal host temperature (37°C) and, in addition, is preferentially degraded at the higher temperature.[77]

Several ATP-dependent proteases that are integral to heat shock responses also contribute to virulence.[61] One example is ClpB, an ATP-dependent chaperone part of the Clp family of proteins.[78,79] ClpB is crucial for replication of the human pathogen *Francisella tularensisis* in host tissues[80] and involved in pathogenesis of *L. monocytogenes*.[81] Finally, there a examples of chaperones contributing to virulence which are induced during heat shock.[61] In particular, the major chaperone DnaK has been shown to be important for during phagocytosis of *Listeria* by macrophages but not for intracellular survival.[82]

Temperature influencing bacterial motility

Motility is a common feature of many pathogens and permits migration to a specific niche at mucosal surfaces. Thermotaxis, i.e., motility in response to temperature, has been observed in several bacteria including *E. coli*.[83,84] Using flagellar based motility, *E. coli* swims along temperature gradients to warmer sites at low culture densities.[85] This is achieved by varying swimming behavior at different temperatures: at warmer temperatures, the bacteria generally swim undirectionally, but at low temperature the bacteria tumble and change direction.[85] Mutants which are incapable of chemotaxis are generally found to be devoid of thermotaxis,[86] indicating that these two responses share some common mechanism. Chemotaxis has been well-characterized and requires membrane bound receptors which transduce environmental stimuli into a phosphorylation cascade that modifies flagellar assembly and rotation.[87,88] Of note, the direction of migration reverses at higher bacterial densities,[83] and this reversal of motility is affected by the nutritional state of the microbe.[89] As a consequence, in situations where there is nutrient limitation, *E. coli* migrates to cooler areas where the metabolic demands on the bacterium may be reduced.[90]

L. monocytogenes also possesses a switch-like mechanism which ensures that motility is limited to environments which are below physiological temperatures in hosts (i.e. below 37°C).[43] In this case, a thermo-sensing anti-repressor protein (GMaR) binds to and inhibits the activity of the flagella motility transcriptional repressor MogR at 30°C, but not at 37°C. Similarly a proteomic analysis of *Clostridium difficile* demonstrated that flagellin levels are reduced in response to heat shock,[91] which could affect motility.

Yersinia is non-motile at 37°C,[92] and the expression of genes involved in flagellar biogenesis is orchestrated by several different proteins including the sigma factor FliA (σ^{28}), its anti-sigma factor FlgM, and the chaperone FliS.[93] At 37°C, transcription of both *fliA* and *flgM* is arrested, resulting in a flagella-null phenotype.[94] The mechanism is unknown, but might be related to changes in DNA supercoiling.[95] In *Campylobacter coli* the flagellum is composed of two structural proteins, FlaA and FlaB.[96] The gene encoding FlaB is regulated by a σ^{54}-dependent promoter, and its transcription is higher at 42°C than 37°C which correlates with an increase in flagellar motility at the higher temperature.[97] In *Campylobacter*, the interaction of the anti-sigma factor FlgM with the sigma factor FliA (σ^{28}) is temperature dependent and prevents excessive elongation of the flagellum, allowing higher motility at 42°C than 37°C.[98] Furthermore FlaA biosynthesis is highest at 42°C, which may contribute to increased motility at 42°C,[98] the core temperature of the avian host of *Campylobacter* spp.

Thermoregulation of exotoxin production

The foodborne pathogen enterohemorrhagic *E. coli* (EHEC) secretes Shiga-like toxins that contribute to the development of hemolytic uraemic syndrome and hemorrhagic colitis in humans.[99] Shiga-like toxin 2 (Stx-2), the more potent of the two toxins produced by EHEC, is subject to thermoregulation,[14] although the precise mechanism(s) for this are unknown. Thermoregulation of toxin production is also seen in other pathogenic bacteria including *B. pertussis* (see above) and *Y. enterocolitica*.[100] In *Yersina*, production of the enterotoxin, Yst, is higher at 28°C (i.e., sub-host temperature).[101] The reasons for this thermal regulation in light of this remain unclear. Furthermore in *Bacillus anthracis*, synthesis of the Anthrax Toxin Activator (AtxA) is temperature-dependent.[102] AtxA regulates genes required for synthesis of both the edema and lethal toxins, which are necessary for virulence[103] and have highest expression at 37°C.[102] This corresponds to an increase in the transcription of all three toxin structural genes at the permissive temperature.[104] To date, the mechanisms by which AtxA regulates the expression of other genes or by which its synthesis is thermally regulated it unknown.[105]

Temperature as a signal for adaptation between different hosts

Some bacteria colonise both a vertebrate and an invertebrate host, and so are exposed to entirely different sets of conditions including temperature. *Borrelia burgdorferi*, which causes Lyme disease, must deal with conditions within both its tick vector and its mammalian host. One of the ways this is accomplished is by expressing the outer surface protein OspA or OspC which bind distinct ligands in the invertebrate and vertebrate host, respectively. The alternative sigma factor RpoS (σ^{38}), which regulates several virulence factors under heat stress,[106] is responsible for the differential expression of OspA/C via a temperature-dependent small RNA, $DsrA_{Bb}$.[107] $DsrA_{Bb}$ was predicted to bind to the 5′-UTR of *rpoS* mRNA through complementary base-paring and was hypothesized to be a *trans*-acting sRNA that liberates the SD site of *rpoS* mRNA, permitting translation.[107] This finding is supported by translational fusion studies *in vivo* demonstrating a higher translational efficiency of RpoS mRNA at 37°C compared to the low temperature 26°C,[108] comparable to the conditions experienced by the bacteria in its tick vector. However the level of $DsrA_{Bb}$ transcript was found to be similar at both temperatures, suggesting that $DsrA_{Bb}$ is not the key temperature-

regulatory molecule.[107] Recent evidence in *E. coli* suggests that the RNA chaperone Hfq is required for efficient translation of RpoS in conjunction with $DsrA_{Bb}$[109] and may confer thermal regulation.[37,38] Other virulence factors in *Borrelia* spp., such as the complement regulator-acquiring surface proteins (CRASPs) are also regulated in response to temperature[110] and the mammal-tick infection cycle,[111] indicating that temperature is the primary cue by which this pathogen senses transmission between their hosts.

The haemin uptake system (Hms) in *Y. pestis* is another example of thermoregulation that occurs during transmission from vector to host.[46] Interestingly, the Hms is not essential for pathogenesis in mammals[112] but allows iron acquisition from a blood meal and proliferation of bacteria in fleas that leads to blockage of the proventricular valve,[113] regurgitation and the spread of infection when the flea feeds on a human. Hms has been shown to be thermoregulated at the level of protein stability,[114] with bacteria containing higher levels of Hms at temperatures found in fleas (i.e. below 26°C).

Thermal control of nutrient acquisition

The availability of nutrients within hosts is dramatically different from the external environment. Several micronutrients, for example iron, are sequestered from invading microbes in mammalian hosts, limiting bacterial proliferation. Many pathogens have developed strategies that allow them to acquire nutrients which would otherwise be inaccessible in their host.[115] Enteropathogenic *E. coli* and *Shigella dysenteriae* both scavenge iron in the form of heme present in the human gastrointestinal tract using the TonB-dependent receptors, ChuA and ShuA respectively. Two distinct regulatory mechanisms permit iron scavenging within the host where there is both physiological temperature (37°C) and low levels of free iron.[116] There is a fourU-type RNA thermosensor located in the 5′-UTR region of the mRNA of *chuA* and *shuA*, which forms a hairpin enabling the 4 U nucleotides to pair the RBS, and preventing translation at lower temperatures.[117]

Similarly, a recent transcriptome analysis of *Pseudomonas aeruginosa* highlighted that genes encoding enzymes required for the biosynthesis of the siderophores, pyochelin and pyoverdine,[118] are differentially regulated at 22°C compared to 37°C.[119] In general, genes involved in pyoverdine biosynthesis are upregulated at the low temperature, while those for pyochelin biosynthesis are upregulated at 37°C.[119] Expression of *ppyR*, which encodes an activator of the genes required for pyoverdine synthesis, is also upregulated at 22°C[119] by an unknown mechanism. These findings suggest that the bacterium switches strategies for iron utilization from outside to inside its host.

Immune evasion mechanisms

Once inside their mammalian host, bacteria must defend themselves against the immune system as molecules secreted or localized on the surface of microbes can elicit potent inflammatory and immune responses. Therefore bacteria carefully control expression of immune-stimulatory molecules to avoid being detected and eliminated. There are several instances in which temperature plays a key role in regulating bacterial immune evasion.

Neisseria meningitidis is a major cause of meningitis and septicaemia in children and young adults that normally resides as a commensal of the human nasopharynx. It has been found that temperature acts as a danger signal for this important pathogen, as an elevation in temperature leads to an increase in its suite of immune evasion molecules. Production of factor H binding protein, fHbp (which recruits the human complement regulator human factor H at high affinity),[120,121] lipopolysaccharide (LPS) sialylation, and the polysaccharide capsule (made by the *css* operon) protect the pathogen from complement-mediated lysis; these factors appear to be controlled by independent RNA thermosensors.[122] This strategy is distinct from those seen in other pathogens, in which multiple effector molecules are controlled by thermo-regulation of a pleiotropic transcription factor. In the meningococcus, these three mechanisms of immune evasion have their own dedicated RNA thermosensor; this may reflect the constant association between the meningococcus and its human host, and its habitat in the thermal gradients of the nasopharynx. The meningococcal capsule thermosensor shows a gradual response to a rise in temperature unlike the Listeria PrfA thermosensor which acts like an on:off switch, and common polymorphisms in the 5′ UTR of the *css* mRNA do not affect thermoregulation.[122] In both the *css* and *fHbp* thermosensors, the SD sequence is predicted to be included in a hairpin structure at lower temperatures. Following entry of the bacterium across the epithelial barrier into the bloodstream, increased deployment of immune avoidance mechanisms at the elevated temperature in the body will promote survival in the systemic circulation and thence virulence. However, as virulence is an evolutionary dead end for this microbe, thermosensing of immune defense will be an adaptation for survival in the upper airway. Here, temperature may be acting as a danger signal for the meningococcus, by indicating the inflammatory status of the nasopharyngeal mucosa.

LPS is an essential component of the outer membrane of Gram negative bacteria, and required for membrane integrity and protection against exogenous bactericidal agents, such as anti-microbial peptides.[123] LPS also acts as a microbe associated molecular pattern molecule (MAMP), with the lipid A portion of LPS being a potent stimulator of inflammatory responses by recognition by Toll-like receptor-4 (TLR-4) on host cells. The human pathogen *Francisella novicida* can cause septicaemia and remodels its LPS in response to temperature.[124] At environmental temperatures (i.e., 18°C), lipid A is acetylated at the 3-OH C16 position by LpxD2. However at host temperatures (37°C), LPS is acetylated at the 3-OH C18 position by LpxD1 which confers increased resistance to anti-microbial peptides, antibodies and *in vivo* killing. Transcriptional and post-translational regulation of the LpxD enzymes increases their activity at corresponding temperatures but these mechanisms have not been characterized. This demonstrates that subtle changes to

components of bacteria, such as modification of acyl chains in LPS in response to temperature, are sufficient to alter innate immune signaling.

Another example of changes in LPS in response to temperature is in *Y. enterocolitica*; modification with of lipid A with aminoarabinose and palmitate is reduced at 37°C, which contribute to antimicrobial peptide resistance at low temperature (21°C).[123] Genes involved in these modifications are transcriptionally regulated the two-component systems, PhoPQ and PmrAB. The expression of both these two component systems is temperature dependent, through relief of H-NS repression.[123] Additionally another important component of LPS, the O-antigen, which also plays a role in host-pathogen interactions, is regulated by temperature in *Y. enterocolitica*.[125] The activity of the two promoters, P_{Wb1} and P_{Wb2}, which control expression of the genes required for O-antigen synthesis, are repressed at 37°C.[125]

Polysaccharide capsules offer bacteria protection from harsh external environments and host immunity. However capsule expression can also be detrimental by impairing interactions between microbial outer membrane ligands and their host cell receptors for attachment and invasion.[126] Capsule biosynthesis in *Streptococcus pyogenes*, a cause of tonsillitis in humans, has been shown to be thermoregulated.[127] At lower temperatures (i.e. 25°C) capsule expression increases though an undefined, post-transcriptional mechanism; this is thought to confer anti-phagocytic properties to the bacterium. Since the main route of infection is through the skin where the temperature is lower than core body temperature, a thicker capsule may condition the bacterium prior to penetrating the epithelial layer. The membrane-associated endoribonuclease, CvfA (which regulates virulence genes in response to nutrient availability)[128] is responsible for the thermoregulation at a post-transcriptional level although the mechanism is still to be elucidated.

T3SSs are complex multi-protein machineries which allow secretion of proteins directly from the cytoplasm of bacteria into host cells; T3SSs can contribute to intracellular invasion, and thence immune evasion. Temperature can be an important cue to modulate the assembly and activity of T3SSs to precise sites in the host where they are required.[129] In *Salmonella enterica*, there are two T3SSs encoded on two distinct pathogenicity islands, SPI-1 and SPI-2. SPI-1 contains a set of virulence genes specific for invasion into host cells, whereas SPI-2 contains genes required for intracellular replication and survival.[130] The expression of genes encoding the SPI-2 T3SS has been shown to be dependent upon local pH,[131] concentrations of phosphate and magnesium[132] and, more recently by temperature.[133] Activation of SPI-2 T3SS genes requires the SsrAB two-component regulatory system, although the response to temperature is governed by two nucleoid-associated proteins, H-NS and Hha which contribute to the absence of this T3SS on the surface of bacteria located outside the host.[133] This highlights the importance of integrating temperature as a regulatory stimulus in virulence factor expression, since other environmental factors (nutrient availability, osmolality and pH) are alone, not host-specific. Similarly, the regulator InvE in *Shigella sonnei* is required for the transcription of T3SS components.[134] *invE* mRNA has recently been demonstrated to be negatively regulated at the post-transcriptional level by the RNA chaperone Hfq, which, as mentioned previously, is dependent on temperature.[37,38] At 37°C there is reduced binding of Hfq to *invE* mRNA compared to 30°C, leading to increased stability of *invE* mRNA at the higher temperature and increased activity of the T3SS. Since VirF is the positive regulator of *invE*[135] and its synthesis is up-regulated at higher temperatures,[9] this is an elegant example in how temperature mediates exquisite control of virulence gene expression through multiple integrated mechanisms.

Leptospira interrogans causes a zoonotic infection, transmitted to humans through broken skin upon contact with infected animal urine, which can cause serious kidney failure and liver damage. The two cell surface lipoproteins LigA and LigB can bind to a multitude of host proteins, contribute to avoidance of the complement system[136] and to colonisation by binding extracellular matrix proteins.[137] Recently, mRNA thermosensors have been identified in the 5′-UTR of both *ligA* and *ligB*,[138] which are predicted to form a double stem loop structure, with the second hairpin occluding the SD sequence at 30°C and melting to enable translation at 37°C.

Conclusions

Understanding how temperature and other environmental cues act on regulatory networks highlights key aspects of the evolution in pathogenic bacteria, and provides insights into the habitats they occupy during steps in the disease process. While cold and heat shock proteins protect bacteria from sudden fluctuations in temperature through a homeostatic mechanism, many virulence factors use the signal provided by temperature, in addition to other cues, to enhance production of virulence factors within their hosts. Besides shedding light on how pathogens cause disease, understanding the molecular mechanisms underlying thermosensing has potential benefits for human health. For example, the production of many molecules that have been exploited as vaccine antigens is under temperature control. Examples include bacterial capsules and fHbp (from the meningococcus), and several toxins; modified Ptx is a component of the licensed acellular pertussis vaccines, while there have been efforts to use detoxified toxin from anthrax as an immunogen. Additionally components of T3SS have been proposed as a vaccine candidate against *Shigella* spp. Therefore, defining the sequences and motifs that allow thermosensing in microbes and how these signals are linked with other host signals, should allow the rapid identification of other thermally regulated molecules. This could provide the next generation of vaccine candidates and potentially drug targets.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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