Concerted Actions of a Thermo-labile Regulator and a Unique Intergenic RNA Thermosensor Control Yersinia Virulence

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Abstract

Expression of all Yersinia pathogenicity factors encoded on the virulence plasmid, including the yop effector and the ysc type III secretion genes, is controlled by the transcriptional activator LcrF in response to temperature. Here, we show that a protein- and RNA-dependent hierarchy of thermosensors induce LcrF synthesis at body temperature. Thermally regulated transcription of lcrF is modest and mediated by the thermo-sensitive modulator YmoA, which represses transcription from a single promoter located far upstream of the yscW-lcrF operon at moderate temperatures. The transcriptional response is complemented by a second layer of temperature-control induced by a unique cis-acting RNA element located within the intergenic region of the yscW-lcrF transcript. Structure probing demonstrated that this region forms a secondary structure composed of two stemloops at 25 °C. The second hairpin sequesters the lcrF ribosomal binding site by a stretch of four uracils. Opening of this structure was favored at 37 °C and permitted ribosome binding at host body temperature. Our study further provides evidence for the biological relevance of an RNA thermometer in an animal model. Following oral infections in mice, we found that two different Y. pseudotuberculosis patient isolates expressing a stabilized thermometer variant were strongly reduced in their ability to disseminate into the Peyer’s patches, liver and spleen and have fully lost their lethality. Intriguingly, Yersinia strains with a destabilized version of the thermosensor were attenuated or exhibited a similar, but not a higher mortality. This illustrates that the RNA thermometer is the decisive control element providing just the appropriate amounts of LcrF protein for optimal infection efficiency.

Introduction

Pathogenic yersiniae, including Y. pestis, the causative agent of the bubonic plague, and the two enteric species Y. enterocolitica and Y. pseudotuberculosis which cause gut-associated diseases (yersiniosis) such as enteritis, diarrhea and mesenterial lymphadenitis express different sets of virulence factors important for different stages of the infection process [1–2]. It is well known that most of the Yersinia virulence genes are tightly controlled in response to temperature [3].

Some of the early stage virulence factors, including the primary internalization factor invasin of both enteric Yersinia species, are mostly produced at moderate temperatures to allow efficient trespassing of the intestinal epithelial barrier shortly after infection [4–6]. These virulence genes are controlled by RovA, an intrinsinc protein thermometer, which undergoes a conformation change upon a temperature shift from 25°C to 37°C, that reduces its DNA-binding capacity and renders it more susceptible to proteolysis [7–9].

Most other known Yersinia virulence genes remain silent outside the mammalian hosts and are only induced after host entry in response to the sudden increase in temperature. One important set of thermo-induced virulence factors is encoded on the 70 kb Yersinia virulence plasmid pYV (pCD1) in Y. pestis [10]. These pathogenicity factors are crucial to avoid phagocytosis or other attacks by the innate immune defense system and comprise a type III secretion system (T3SS), the secreted Yersinia outer proteins (Yops) and regulatory components of the secretion system [11–13].

The Yop secretion genes (ysc) are organized in two operons yscB-L (yscL operon) and yscN-U, or encoded elsewhere (e.g. yscW, yscX, yscT and yscF) on pYV [10] and are required for the formation of the T3S apparatus (injectisome). Body temperature (but not 20–
Author Summary

Many important virulence genes remain silent at moderate temperatures in external environments and are rapidly and strongly induced by a sudden temperature upshift sensed upon host entry. Thermal activation of virulence gene transcription is frequently described, but post-transcriptional control mechanisms implicated in temperature-sensing and induction of virulence factor synthesis are less evident. Here, we present a novel two-layer regulatory system implicating a protein- and an RNA-dependent thermosensor controlling synthesis of the most crucial virulence activator LcrF (VirF) of pathogenic yersiniae. In this case, moderate function of a thermosensitive gene silencer is coupled with the more dominant action of a unique intergenic two-stemloop RNA thermometer. Thermally-induced conformational changes in this RNA element control the transition between a ‘closed’ and an ‘open’ structure which allows ribosome access and translation of the lcrF/virF transcript. This mechanism guarantees optimal virulence factor production during the course of an infection, ideal for survival and multiplication of yersiniae within their warm-blooded hosts. The hierarchical concept combining two temperature-sensing modules constitutes a new example of how bacterial pathogens use complementing strategies to allow rapid, energetically cheap and fine-tuned adaptation of their virulence traits.

25 °C and host cell contact trigger expression and translocation of Yop proteins by the T3S machinery into the cytoplasm of targeted host cells [14–17]. The Yop proteins can be divided into the group of translocators implicated in the formation of the translocation pore and the Yop effector proteins which manipulate numerous signal transduction pathways to prevent phagocytosis and the production of proinflammatory cytokines [18–21].

Expression of the majority of pYV-encoded virulence genes (yadA, yop, lcr and yse genes for T3S and regulation) is induced by temperatures above 30 °C in all pathogenic Yersinia species. Temperature-dependent induction of these genes requires the AraC-type DNA-binding protein LcrF (VirF in Y. enterocolitica) [22–24]. The LcrF protein contains a poorly conserved N-terminal oligomerization domain which is connected to a flexible highly conserved C-terminus with two helix-turn-helix DNA-binding motifs [25]. It exhibits high homology to the main regulator of T3S in Pseudomonas aeruginosa, ExsA and has been shown to bind specifically to TTTaGyCtTat DNA motifs in the promoter regions of yopE, lcrG, virC and yopH [26]. The transcriptional activator LcrF is mainly produced at 37 °C. Hos and Goguen [27] showed that the lcrF mRNA produced in E. coli or Y. pestis could not be translated at 26 °C, but was readily translated at 37 °C. Based on predicted mRNA structure, these authors proposed that translation was dependent on melting of a stem-loop which sequestered the lcrF ribosomal binding site. Calculated thermal stability agreed well with observed translation, but no experimental work testing this hypothesis by manipulating stability of the structure was performed. In contrast, for Y. enterocolitica it has been reported that transcription of the lcrF homologous gene virF is increased at higher temperatures. This activation was shown to depend on topological changes and thermo-induced melting of intrinsically bent DNA identified upstream of the lcrF/virF gene [28–29]. Also chromosomally encoded factors that contribute to the temperature-dependent regulation of yadA and yop transcription have been identified in Y. enterocolitica. Below 30 °C, induction of these virulence genes was only observed in the absence of the Yersinia modulator A (YmoA) [30–31]. YmoA belongs to the superfamily of nucleoid-associated proteins and shares 92% sequence identity with the regulator of “high hemolysin activity” (Hha) in E. coli and Salmonella [32]. The E. coli Hha protein represses the transcription of the hlyGABD operon encoding the pore-forming toxin hemolysin at moderate temperatures [33–34]. YmoA was shown to influence DNA supercoiling and forms heterodimers with the nucleoid-associated protein H-NS [35–36]. However, YmoA or H-NS binding to pYV promoter sequences has never been reported. Hence, the molecular mechanisms by which YmoA controls yadA and yop gene expression is still unclear.

A recent analysis of type III secretion in Y. pestis indicated that regulated proteolysis of YmoA by the ATP-dependent Clp and Lon proteases plays an important role in the temperature-dependent expression of the type III secretion operon [37]. It was shown that YmoA is rapidly degraded at 37 °C, but remains stable at environmental temperatures [37]. Whether the thermo-control mechanisms of LcrF vary between Y. pestis and Y. enterocolitica or whether they are connected, and if so, how they contribute to LcrF production in the different species remained elusive.

In this study, we investigated the molecular mechanism underlying thermoregulated production of the LcrF virulence regulator of Y. pseudotuberculosis. We found that concerted actions of the thermo-labile YmoA regulator protein and an unusual intergenic RNA thermosensor assured best possible production of LcrF for the highest infection efficiency. YmoA repressed lcrF transcription through sequences located within the 5′-UTR of yscW located upstream of the lcrF gene, and contributed moderately to the thermo-dependent production of LcrF. This activity is supplemented by a two-hairpin RNA thermometer composed of four uracil residues (fourU) that pair with the ribosome-binding site (AGGA) within the intergenic region of the yscW-lcrF mRNA. Using a mouse model system we provide evidence that this RNA thermosensor is mainly responsible for thermo-induced LcrF production, and show that its function is relevant for a high pathogenic potential, optimal survival and multiplication of Yersinia during infection.

Results

Temperature and YmoA-dependent regulation of lcrF transcription

The AraC-type transcriptional activator protein LcrF induces the expression of crucial Yersinia pathogenicity factors (e.g. YadA, T3SS and Yop effectors) in response to temperature. Initial efforts in this study to unravel the molecular control mechanisms of lcrF expression in Y. pseudotuberculosis demonstrated that the lcrF gene is organized in an operon with yscW (formerly named virG) located 124 bp upstream of the lcrF coding region on the Yersinia virulence plasmid pYV (Figure 1A). As shown in Figure 1B, a yscW-lcrF-lacZ (pSF4) and a yscW-lacZ (pKB10) translational fusion harboring the yscW regulatory region up to position –572 relative to the yscW start codon were expressed, whereas a construct carrying yscW sequences to position –7 (pSF3) was not. The yscW-lcrF-lacZ fusion was thermo-regulated in dependence of the YmoA protein. Expression was about 2-fold increased in the ymoA mutant strain and showed a significantly higher expression at 37°C than at 25°C (Figure 1C). To confirm this result, western blot analysis was performed to detect the LcrF protein in cell extracts from the Y. pseudotuberculosis wildtype strain YPIII and the isogenic ymoA mutant YP50 grown at 25°C and 37°C. As shown in Figure 1C, the LcrF protein could only be detected in extracts of the ymoA mutant but not in the wildtype strain when the bacteria were
grown at 25°C. In contrast, LcrF production was significantly increased and detectable in both strains at 37°C, whereby the overall level of LcrF was significantly higher in the ymoA-deficient strain. This indicated that lcrF expression occurs from a temperature- and YmoA-dependent promoter located upstream of the yscW gene.

In order to investigate yscW-lcrF transcription in more detail, total RNA of Y. pseudotuberculosis was prepared for Northern blot

Figure 1. Expression of the yscW-lcrF operon in response to temperature. (A) Schematic presentation of the yscW-lacZ and yscW-lcrF-lacZ fusion plasmids. Numbers given in brackets represent the nucleotide positions of the 5’-end of the yscW regulatory region of the fusion constructs with respect to the start codon of yscW. The yscW gene is indicated in grey, the 5’-portion of the lcrF gene is given in black and the lacZ reporter gene is illustrated by a white arrow. (B) Strains YPIII and YP50 (ΔymoA) harboring the yscW-lacZ (pKB10) or the yscW-lcrF-lacZ (pSF3 and pSF4) fusion plasmids ± pAKH71 (ymoA+) were grown overnight in LB medium at 25°C or 37°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min⁻¹ mg⁻¹ for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly from those of YPIII at the same temperature with ** (P < 0.01), and *** (P < 0.001). The activity of all reporter constructs differed significantly between 25°C and 37°C with P < 0.001 (not shown). (C) Whole-cell extracts from overnight cultures of Y. pseudotuberculosis wild type and the mutant strains YP66 (ΔlcrF) and YP50 (ΔymoA) grown at 25°C or 37°C were prepared and analysed by Western blotting with a polyclonal antibody directed against LcrF. A molecular weight marker is loaded on the left. A higher molecular weight protein (c) that reacted with the polyclonal antiserum was used as loading control.

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analysis using an lcrF specific probe (Figure 2). The yscW-lcrF transcript was found to be highly unstable and was rapidly degraded into lower molecular weight transcripts in the wildtype (Figure 2). In contrast, higher concentrations and higher molecular weight transcripts were detectable in the ymoA mutant strain consistent with the conclusion that yscW and lcrF originate from the same promoter. Moreover, as judged from the length of the yscW-lcrF transcript, the transcriptional start site appeared to be about 300 bp upstream of the yscW start codon, leading to the formation of a long 5’-untranslated region (5’-UTR).

To identify the yscW-lcrF promoter we performed primer extension analysis. We found that the transcription of the yscW-lcrF operon starts at a G found 264 nt upstream of the start codon GTG of yscW with a −35 and a −10 region of a typical σ70-dependent promoter (Figure 3) leading to a 264 nt 5’-UTR. Several shorter reverse transcripts were consistent with the Northern results suggesting rapid processing of the yscW-lcrF transcript.

Increased expression of the yscW-lacZ and yscW-lcrF-lacZ fusions in the ymoA deficient Yersinia strain suggested that YmoA influences expression on the transcriptional level (Figure 1B, S1). Continuous deletions of the promoter region showed that elimination of the identified promoter region by a 5’-upstream deletion up to position −2 abrogated transcription of the fusion construct and confirmed presence of a single promoter driving yscW-lcrF expression (Figure S1A, 3C). Further analysis demonstrated that YmoA-dependency was maintained when sequences upstream of the yscW promoter were deleted, but it was lost when the 5’-UTR of yscW was removed (Figure S1A,B). This indicated that YmoA acts through sequences located downstream of the yscW promoter.

Next, we tested whether YmoA influence on yscW-lcrF was direct or involves (an)other regulatory factor(s). Experimental

Figure 2. Analysis of the yscW-lcrF mRNA in wildtype and the ymoA mutant strain. (A) Schematic presentation of the yscW-lcrF operon, the yscW-lcrF mRNA and the lcrF probe used for Northern Blot analysis shown below. (B) Total RNA of YPIII, YP50 and YP66 was prepared, separated on a 1.2% agarose gel, transferred onto a Nylon membrane and probed with Digoxigenin-labelled PCR fragment encoding the lcrF gene. The 16S and 23S rRNAs are shown as RNA loading control. A RNA marker is loaded on the left.

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Figure 3. Mapping of the lcrF transcription start site by primer extension analysis. (A) The 5’-ends of the reverse transcription products are indicated by vertical arrows on the schematic presentation of the yscW-lcrF mRNA. The numbers indicate the position of the nucleotide of the 5’-ends with respect to the start codon of yscW. Location of the annealed primer used for primer extension is indicated by a horizontal arrow. (B) Total RNA of Y. pseudotuberculosis YPIII was prepared and used for primer extension analysis. Primers specific for the yscW coding sequence and a 20 μg template RNA were applied for primer extension and obtained products were separated on a denaturing 6% polyacrylamide/urea gel. Sequencing reactions performed with the same primer are shown on the left. The sequence of the promoter region is shown on the right and identified 5’-end of yscW. (C) The sequence of the promoter region is shown on the right and identified 5’-end of yscW.
evidence support the hypothesis that members of the YmoA(Hha) protein family modulate gene expression by interacting with the nucleoid-structuring DNA-binding protein H-NS or its paralogs [36,38]. However, other studies reported that the Hha/YmoA protein binds specifically to regulatory sequences of virulence genes. Unfortunately copurification of H-NS was not ruled out in these studies [39–41]. In order to test YmoA binding, YmoA was overexpressed and purified from E. coli strain KB4 (Δhns, ΔyphA, Δhha) deficient of all E. coli full-length and partial H-NS family proteins and used for band shift analysis with a yscW promoter fragment harboring the entire 5′-UTR. However, even at very high protein concentrations YmoA was not able to interact specifically with the yscW regulatory region (Figure S2A). In addition, we purified YmoA overexpressed in E. coli strain KB4 also expressing the T. pseudobunellosis hns gene. This YmoA protein sample included copurified H-NS_22_10̆ (data not shown) and was able to interact specifically with the 5′-UTR sequences of the yscW gene (Figure S2C). We further expressed and purified H-NS_22_10̆/YmoA in the absence of YmoA and found that also H-NS_22_10̆ alone is capable to interact with the yscW regulatory sequences (Figure S2B). This indicated that YmoA influences yscW-lcrF expression directly and this involves heterocomplex formation with H-NS.

To confirm these data we also analyzed whether YmoA influence on thermal regulation of LcrF is lost, when the 5′-UTR important for H-NS/YmoA binding is absent. To do so, we compared expression of the yscW-lcrF-lacZ construct and a derived deletion variant of this fusion yscW[A13-241]-lcrF-lacZ at 25°C and 37°C. We found that the expression level is still thermoregulated, but lcrF transcription became independent of YmoA (Figure S2D). This clearly demonstrated that YmoA influences expression of lcrF via the 5′-UTR region of the yscW gene.

Post-transcriptional control of lcrF thermoregulation

The YmoA protein of Y. pestis was shown to be subject to proteolysis by the Lon- and ClpP proteases at 37°C but not at 25°C [37], and this post-translational control was also observed for YmoA in Y. pseudotuberculosis YPIII (K. Böhme, unpublished results). However, expression of the yscW-lcrF-lacZ fusion and LcrF synthesis was still thermoregulated in the ymoA-deficient strain (Figure 1B,C), suggesting that contribution of YmoA to lcrF thermoregulation is rather small and predominantly mediated by an additional YmoA-independent control mechanism.

To localize the region responsible for this type of control, we exchanged the yscW promoter (PycW) against the PBad promoter and analyzed yscW-lcrF-lacZ expression after induction with 0.05% arabinose at 25°C and 37°C. Thermoregulation was maintained when yscW-lcrF was transcribed by PBad independent whether the fusion was expressed in E. coli or in T. pseudobunellosis (Figure 4, S3). In contrast, expression of lacZ fused to the 5′-UTR of the T. pseudobunellosis 6-phosphogluconate dehydrogenase gene (gnd) in the identical vector system was not affected by the growth temperature. These experiments strongly suggested that the temperature control of lcrF expression is mediated by a post-transcriptional mechanism as previously demonstrated in Y. pestis [24] and is independent of Yersinia-specific factors. Deletions removing different portions of the yscW locus or the entire yscW gene further demonstrated that presence of the yscW gene is dispensable and that the intergenic region of the yscW-lcrF operon is sufficient for temperature control of lcrF translation (Figure 4, S4).

To investigate whether the intergenic region of yscW-lcrF forms a functional RNA thermometer we deleted hairpin I (Δhha: −111/−57) or parts of hairpin II (Δhha: −44/−25) and introduced stabilizing (AG-46/−45CC; UU-28/-27CC) and destabilizing (AUA-36/-34CCC; GUU-30/-28AAA) point mutations into the PBad-lcrF-lacZ fusion construct and in the yscW-lcrF intergenic region of the virulence plasmid pYV (Figure 5A). Absence of hairpin I (Δhha) resulted in a significant reduction of lcrF thermo-induction from 5- to 2-fold (Figure 5B,C). Expression was already high at 25°C and induction was lost when sequences implicated in the formation of hairpin II (Δhha) were deleted. Similarly, thermo-induced expression of lcrF was strongly decreased in both mutations designed to destabilize hairpin II, whereas expression of variants with stabilizing mutations remained repressed upon a temperature upshift and only very small amounts of the LcrF protein were produced at both 25°C and 37°C (Figure 5B,C). Increase of LcrF levels in the destabilized mutant from 25°C and 37°C demonstrates a two-layer regulation by the thermo-labile YmoA protein and the RNA thermometer. In the following experiments the stabilizing mutation UU-28/-27CC and the destabilizing GUU-30/-28AAA are also referred to as ‘closed’ and ‘open’, respectively. In summary, our data demonstrate that the intergenic region of the yscW-lcrF operon contains a thermo-responsive RNA element composed of two hairpins that mediate post-transcriptional control in an RNA thermometer-like manner.

Temperature-dependent structural changes of the intergenic, non-translated yscW-lcrF mRNA

In order to examine the architecture of the predicted RNA structure experimentally, we determined the structure and the nature of thermo-induced conformational changes of the intergenic yscW-lcrF mRNA by enzymatic probing at 25°C and 37°C using RNase T1 (cleaves 3′ of unpaired guanines) and double-strand specific RNase V1. Due to the large size of the full-length yscW-lcrF transcript, the structure of a shorter RNA fragment including the entire yscW-lcrF intergenic region (5′-UTR of lcrF) was probed (Figure 6A, B). The cleavage pattern at 25°C was in full agreement with the predicted two hairpin structure (Figure 5A). RNase T1 digestion at positions −81 to −85 and positions −101 to −102 as well as the sensitivity of adjacent regions to RNase V1 detected primer extension products are given in bold. (C) The regulatory region of the yscW-lcrF operon is shown. The broken arrows indicate the 5′-end points of the promoter deletion constructs and straight arrows show the 5′-end of the degradation products. The −35 and −10 region of the identified promoter is underlined, the transcriptional start site is given in bold, and the Shine-Dalgarno sequence (SD) is indicated.

References:
[1] K. Böhme, unpublished.
[2] K. Böhme, unpublished.
[3] Y. pestis, Y. enterocolitica, and secondary structure predictions by Mfold [42] revealed a potential RNA structure composed of two stemloops (hairpin I and II) with a free energy of −19.67 kcal mol⁻¹ (Figure 5A). The first hairpin (57 nt) consists of three base-pairing stretches interrupted by two internal loops and is separated from the second hairpin (hairpin II, 46 nt) by 11 nt. In hairpin II, the ribosomal binding site (RBS) of lcrF pairs with a stretch of four uracil residues (fourU) located 26 to 29 nt upstream of the translation initiation site of lcrF. This structure resembles a fourU thermometer identified in the 5′-untranslated region of the Salmonella agsA gene [43]. Presence of two small loops (C-5/A-6/A-38 and A-12/A-31/A-32) and three imperfect base-pairs in the RBS region (G-15/U-28; G-16/U-27; U-19/G-24) in hairpin II suggested a temperature-labile structure prone to melting at increasing temperatures.

Using RNase T1 and RNase V1 the 124 nt yscW-lcrF intergenic region was subjected to enzymatic probing at 25°C and 37°C using the identical vector system was not affected by the growth temperature. These experiments strongly suggested that the temperature control of lcrF expression is mediated by a post-transcriptional mechanism as previously demonstrated in Y. pestis [24] and is independent of Yersinia-specific factors. Deletions removing different portions of the yscW locus or the entire yscW gene further demonstrated that presence of the yscW gene is dispensable and that the intergenic region of the yscW-lcrF operon is sufficient for temperature control of lcrF translation (Figure 4, S4).

Systemic inspection of the 124 nt yscW-lcrF intergenic region, comparison with related bacteria (Y. pestis, Y. enterocolitica), and secondary structure predictions by Mfold [42] revealed a potential RNA structure composed of two stemloops (hairpin I and II) with a free energy of −19.67 kcal mol⁻¹ (Figure 5A). The first hairpin (57 nt) consists of three base-pairing stretches interrupted by two internal loops and is separated from the second hairpin (hairpin II, 46 nt) by 11 nt. In hairpin II, the ribosomal binding site (RBS) of lcrF pairs with a stretch of four uracil residues (fourU) located 26 to 29 nt upstream of the translation initiation site of lcrF. This structure resembles a fourU thermometer identified in the 5′-untranslated region of the Salmonella agsA gene [43]. Presence of two small loops (C-5/A-6/A-38 and A-12/A-31/A-32) and three imperfect base-pairs in the RBS region (G-15/U-28; G-16/U-27; U-19/G-24) in hairpin II suggested a temperature-labile structure prone to melting at increasing temperatures.
cleavage (positions 271 to 268; 278 to 275; 287 to 290; 294 to 299; 310 to 318) confirmed the predicted secondary structure of hairpin I containing three loop segments. Also hairpin II seems to form the predicted structure (protection to RNase V1 at positions 224 and 220). Consistent with its function as a temperature sensor, this stemloop is more dynamic and adapts a thermo-sensitive conformation that seems to open after a shift to 37°C. As shown in Figure 6, the stem region including the imperfect UUUU/AGGA base pairs with the RBS and flanking regions is more resistant to RNases T1 at 25°C than at 37°C. Temperature-induced melting of the stemloop II at 37°C is also supported by digest with the RNase V1, which is less active at 25°C. To confirm that RNase T1 cleavage at the RBS is the result of structural changes and not the result of an induced activity of
Figure 5. Predicted secondary structure of the lcrF RNA thermometer. (A) The Mfold program [81] was used for the prediction of the secondary structure of the yscW-lcrF 124 nt intergenic region. The most probable prediction with the lowest free energy is shown. The blue dots represent base pairing. The start of the protein synthesis at the AUG start codon (START) and the ribosome binding site (RBS) paired with the fourU motif are labelled. Deletion of the hairpin I and II are indicated. Nucleotide exchanges leading to increased complementarity are marked in red, mutations impairing base pair formation are given in green. Numbers indicate the nucleotides relative to the lcrF start codon. (B) Strains YPIII harboring the P_{BAD}:lcrF(-124)-lacZ, including the different hairpin deletions or nucleotide exchanges were grown overnight in LB medium at 25°C or 37°C supplemented with 0.05% arabinose. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly from those of the wildtype at the same temperature with * (P<0.05), ** (P<0.01), and *** (P<0.001). (C) Y. pseudotuberculosis strains harboring the deletion and nucleotide substitution illustrated in (A) in the pYV were grown at
Ribosomes bind to the lcrF transcript at 37°C but not at 25°C

To demonstrate temperature-dependent interaction of the 30S ribosome with the RBS in the intergenic region of the yscW-lcrF mRNA, we performed toeprinting analysis. Ribosomal subunits and the initiator tRNA^30S were added after annealing of the lcrF-specific reverse primer to the yscW-lcrF template and incubated at 25°C or 37°C. The primer extension reaction was not inhibited at 25°C and/or in the absence of the 30S ribosome. However, at 37°C a toeprint (prematurely terminated product) was detected at position +14/+18 relative to the translational start site, demonstrating the formation of a ternary translation initiation complex composed of the yscW-lcrF mRNA, the 30S ribosome and tRNA^30S (Figure 7). More prominent toeprint signals were observed when the destabilizing GUU-30/-28AAA exchanges were introduced, whereas significantly read-through up to the full length transcript and less toeprint signals were found with the stabilizing UU-28/-27CC under the same growth conditions. Strikingly, although lcrF synthesis and the LcrF-dependent yopE gene expression are already induced in the derepressed strain YP95 (GUU-30/-28AAA) at 25°C, no Yop secretion was detectable after Ca^{2+} depletion, indicating that a temperature-dependent mechanism blocks YopE production and/or secretion at low temperatures.

In order to define the influence of the lcrF RNA thermometer on bacterial pathogenesis, we compared survival and dissemination of the Y. pseudotuberculosis wildtype YPIII and the repressed and derepressed mutant strains YP90 (UU-28/-27CC) and YP95 (GUU-30/-28AAA) in the mouse model. Presence of each strain was examined three days after intragastrically infection of a group of BALB/c mice (n=12) with 5·10^9 bacteria by quantifying the number of bacteria that reached and survived in the Peyer’s patches (PP), the mesenterial lymph nodes (MLN), liver and spleen. Significantly reduced numbers of the repressed YP90 (UU-28/-27CC) mutant strain were recovered from the Peyer’s patches and organs (Figure 9), similar to the lcrF mutant strain YP66 (Figure S5). We also introduced the ‘closed’ and ‘open’ mutation into the more virulent Y. pseudotuberculosis strain IP32953. Oral infections with IP32953 and the isogenic ‘open’ variant YPIP02 led to a higher organ burden three days post infection. However, the number of bacteria was similarly reduced in the host tissues with the ‘closed’ mutant (Figure S6). This demonstrated that a repression of the fourU RNA thermometer reduced virulence and showed that the structural rearrangements of the 5′-UTR of the lcrF mRNA affects pathogenesis of both Yersinia strains. Our results also showed that introduction of derepressing nucleotide exchanges (GUU-30/-28AAA) had no or only a minor effect on the colonisation of host tissue (Figure 9, S6).

To complement the infection experiments, the potential of the different lcrF RNA thermometer mutant strains to cause a lethal infection was determined. Groups of BALB/c mice (n=10) were infected intragastrically with 2·10^9 bacteria of each mutant, YP90 or YPIP01 (UU-28/-27CC) and YP95 or YPIP02 (GUU-30/-28AAA) and the wildtype strains YPIII or IP32953. Survival of the mice was followed over 14 days and date of death was recorded (Figure 10). All mice infected with the wildtype strain showed visible signs of infection by day three post infection (e.g. lethargy, rough fur) and succumbed to infection between day three to six post challenge. Strikingly, none of the mice infected with the repressed mutant strain YP90 or YPIP01 (UU-28/-27CC) developed disease symptoms and all were still alive 14 days after infection, similar to the ΔlcrF mutant strain YP66 (Figure 10). This indicated that stabilization of hairpin II renders the bacteria avirulent. In contrast, the destabilizing mutations in the lcrF RNA thermometer had no apparent effect on the initial rate of death, and did not cause a higher mortality than the wildtype over a 14-day period (Figure 10). The average day to death of mice challenged with the ‘open’ mutant variants YP95 or YPIP02 (GUU-30/-28AAA) was similar or increased from four to seven days. Taken together, this illustrates that the RNA thermometer is
Figure 6. Enzymatic probing of the *yscW-lcrF* intergenic region variants. (A) Enzymatic hydrolysis of the *yscW-lcrF* wildtype sequence with endonucleases T1 (0.001 U/µl) and V (0.0002 U/µl) performed on the 5′-end labelled intergenic region between the *yscW* and *lcrF* gene on the pYV at 25°C and 37°C. The G nucleotides in single stranded regions are indicated. (B) Magnification of the enzymatic probing pattern of the four U/Shine-Dalgarno region. (C) Enzymatic probing of the *yscW-lcrF* of the repressed UU-28/-27CC variant, and (D) enzymatic probing of the *yscW-lcrF* of the derepressed GUU-30/-28AAA variant. The RNA fragments were separated on 8% polyacrylamide gels. Lane L: alkaline ladder; lane C: controls without RNase. The Shine-Dalgarno sequence and the nucleotide exchanges are indicated.

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crucial for virulence, as it plays an important role adjusting the appropriate amounts of LcrF for maximal pathogenicity.

**Discussion**

Many environmental signals are sensed by enteric pathogens such as *Y. pseudotuberculosis* in order to induce and adjust expression of virulence factors upon host entry and during ongoing infections. Temperature is among the most important decisive parameters for an intestinal pathogen, indicating that it successfully invaded a warm-blooded host. A prerequisite for an appropriate response to temperature changes is precise thermosensing, and different principles governing the temperature-sensing mechanism have been uncovered for a variety of macromolecules [47–48]. Thermo-induced structural changes in supercoiled or intrinsically curved DNA have long been known to manipulate gene expression by altering the accessibility of promoter elements [49–50]. Recently, also regulatory proteins were shown to act as intrinsic thermosensors to adjust their DNA-binding properties [8,51–53], and experimental evidence accu-

|                | wildtype | UU -28/-27 CC | GUU -30 to -28 AAA |
|----------------|----------|----------------|-------------------|
| Protein        |          |                |                   |
| 30S ribosomal subunits |          |                |                   |
| full length transcript |          |                |                   |

**Figure 7. Temperature-dependent binding of ribosomes to the yscW-lcrF intergenic region.** Toeprinting analysis was performed with the wildtype, the repressed (UU-28/-27CC) and derepressed (GUU-30/-28AAA) variants as described in material and methods. The presence (+) and absence (−) of the 30S ribosomal subunits are indicated. The terminated primer extension products (toeprints) are marked. The sequencing ladder (ACGU) generated with the same lcrF-specific primer is loaded on the left. The positions of the fourU motif, the Shine-Dalgarno sequence and the start codon AUG are indicated.

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mulated that also RNA plays a fundamental role in temperature sensing [54–56].

Although control of translation initiation by limiting the access to the ribosome-binding site has been reported earlier, the full dimension to which structured mRNAs contribute to thermosensing has only recently been recognized. Several distinct and structurally unrelated RNA sensors have been identified in bacteria, but almost all control the synthesis of heat shock proteins. To our knowledge only one RNA thermometer located upstream of the virulence regulator gene prfA of Listeria monocytogenes has been described to regulate virulence gene expression and invasion into cultured cells [57]. However, its impact for pathogenesis, e.g. initiation or progression of the infection has not been investigated. In this study, we report the existence of an unusual intergenic, two-stem-loop RNA thermometer and provide first experimental evidence that its function is crucial for Y. pseudotuberculosis virulence in a mouse model.

Two temperature-sensing modules, the thermo-sensitive virulence modulator protein YmoA and the RNA thermosensor, act in concert to optimize temperature perception and fine-tune virulence gene expression during infection (Figure 11).

A comprehensive expression analysis revealed, that the lcrF gene is transcribed from a single σ70-specific promoter of the yscW gene (formerly named virG) which is located 124 bp upstream of lcrF. Cotranscription is consistent with the observation that the yscW-lcrF locus is similar to the last two genes of the exsC-exsB-exsA operon of Pseudomonas aeruginosa required for the ExoS effector synthesis [58]. It also reconciles previous contradictory models for temperature control of lcrF (virF) expression in Y. pestis and Y. enterocolitica. Cornelis et al. showed that virF of Y. enterocolitica itself is thermoregulated at the transcriptional level [44]. In that study, virF::cat fusions and virF Northern blots demonstrated transcription activation at elevated temperature. Based on the present analysis, thermo-dependent virF expression can be explained by YmoA-dependent repression of the yscW promoter that is eliminated at higher temperatures due to increased degradation of YmoA by the Lon and Clp proteases [37].

Figure 8. lcrF thermosensor-dependent expression of the yadA and yopE genes. (A) Strains YPIII (wildtype), YP66 (∆lcrF) and the repressed and derepressed yscW-lcrF variants YP90 (UUG-27CC) and YP95 (GUU-30-28AAA) harboring the yadA-lacZ fusion plasmid pSF1 were grown in LB medium at 25°C or 37°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min⁻¹ mg⁻¹ for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly from those of the wildtype at the same temperature with ** (P<0.01), and *** (P<0.001). Whole-cell extracts from overnight cultures of Y. pseudotuberculosis YPIII (wildtype) and the repressed and derepressed yscW-lcrF variants YP90 (UUG-27CC) and YP95 (GUU-30-28AAA) grown at 25°C or 37°C were prepared, and analysed by Western blotting with a polyclonal antibody directed against LcrF and YadA. A higher molecular weight protein (c) was used as control the protein content of the cell extracts. (B) Strains YPIII (wildtype) and the repressed and derepressed yscW-lcrF variants YP90 (UUG-27CC) and YP95 (GUU-30-28AAA) harboring the yopE-luxCDABE plasmid pWO34 were grown in LB medium at 25°C and 37°C. Bioluminescence emitted by the cultures was monitored and is given as relative luminescence units (RLU) and represents the mean of three independent experiments done in triplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly from those of the wildtype at the same temperature with ** (P<0.01), and *** (P<0.001). The panel below shows TCA-precipitated supernatants of YPIII (wildtype), the repressed and derepressed yscW-lcrF variants YP90 (UUG-27CC) and YP95 (GUU-30-28AAA) grown at 25°C and 37°C in the presence (+) or absence (−) of Ca²⁺. The secreted Yop proteins are indicated.

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In contrast, lcrF-lacZ reporter fusions in Y. pestis, including only 206 bp upstream of the lcrF start codon, were found to be insensitive to temperature changes, although much higher levels of the LcrF protein were produced in Y. pestis with raising temperature [24,27]. This implied that a different post-transcriptional mechanism modulates LcrF levels in response to temperature in this organism. A simple model for lcrF thermal regulation has been suggested in which a predicted thermo-labile stem-loop (identical to the upper part of hairpin II) sequesters the lcrF ribosomal binding site [27,43], but its function has never been proven.

Reporter gene assays and a detailed structure-function analysis of the isolated intergenic region in this study provide evidence for a functional RNA thermometer in which temperature regulation of lcrF is mediated in the absence of the natural promoter. Structural probing experiments demonstrated the formation of two hairpins of which hairpin II includes a consecutive stretch of four uridine nucleotides (fourU motif), which base pair with the RBS, and two internal unpaired bulges. Mutational analyses and toeprinting experiments further showed that this RNA structure is sufficiently stable to resist melting at moderate temperature (25°C), but it allows partial unfolding at body temperature (37°C) which permits access of ribosomes and initiation of lcrF translation. Hairpin I was not essential for thermosensing, but it seems to support proper folding and/or the stability of the ‘closed’ RNA thermometer structure, as generally higher amounts of the LcrF protein were

Figure 9. Influence of the lcrF RNA thermometer on tissue colonization by Y. pseudotuberculosis. Strains YPIII (wildtype) and the yscW-lcrF variants YP90 (UUU-28/-27CC) and YP95 (GUU-30/-28AAA) were infected intragastrically (5-10⁸ CFU/mice) into BALB/c mice (n = 12/strain). After three days of infection, mice were sacrificed and the number of bacteria in homogenized host tissues and organs was determined by plating. Solid lines indicate the means. The statistical significances between the wildtype and the repressed and derepressed lcrF RNA thermometer variants were determined by Student’s t test. P-values: *: <0.05; **: <0.001.

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detectable in Δloop1 mutant variants. Importance of this RNA structure is further supported by the fact that the RNA thermosensor sequence is 100% identical in all human pathogenic Yersinia species, although the homology between Y. pseudotuberculosis and Y. enterocolitica is less than 70% and several nucleotide substitutions are detectable in the adjacent yscW and lcrF genes (Figure S7). In fact, a PrAD-lcrF-lacZ reporter in Y. enterocolitica 8081 exhibited a similar thermo-dependent expression pattern, indicating that the RNA thermometer is also functional in this Yersinia species (R. Steinmann, K. Böhme, unpublished results).

The intergenic position of the Yersinia RNA thermosensor is unique. All previously known RNA thermometers are positioned at the 5′-end of heat shock or virulence transcripts [54]. Also sequence and structure of the Yersinia thermometer deviate significantly from the thermosensor controlling virulence genes of L. monocytogenes. The listerial RNA thermometer is positioned within the untranslated region (5′-UTR) of the prfA mRNA and forms one extended stemloop structure (130 nt) in which the ribosome binding site and the start codon locate in two small and unpaired bulges within the long hairpin structure. This overall structure prevents translation at moderate temperature but is

**Figure 10. lcrF RNA thermometer variants affect survival of Y. pseudotuberculosis infected mice.** (A) 2×10⁸ CFU of Y. pseudotuberculosis YPIII (wildtype), the yscW-lcrF variants YP90 (UU-28/-27CC) and YP95 (GUU-30/-28AAA), and YP66 (ΔlcrF) were used to orally infect BALB/c mice (n = 10/strain). (B) 1×10¹⁰ CFU of Y. pseudotuberculosis IP32953 (wildtype), the yscW-lcrF variants YPIP01 (UU-28/-27CC) and YPIP02 (GUU-30/-28AAA) were used to orally infect BALB/c mice (n = 10/strain). Survival of the mice was monitored up to 14 days.

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The hairpin II of the lcrF transcript is blocked through the formation of a two-stemloop structure within the intergenic region which sequesters the RBS and prevents enhanced transcription of the yscW-lcrF operon. Furthermore, thermally-induced conformational changes allow access of ribosomes and translation of the lcrF transcript leading to LcrF synthesis and induction of all LcrF-dependent virulence genes of Yersinia.

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Figure 11. Model of thermoregulated expression of LcrF synthesis. At moderate growth temperature, transcription of the yscW-lcrF operon is repressed by the regulatory protein YmoA through sequences located downstream of the transcription initiation site. In addition, translation of the lcrF transcript is blocked through the formation of a two-stemloop structure within the intergenic region which sequesters the RBS and prevents access of the ribosomes. After a sudden temperature upshift upon host entry, YmoA is rapidly degraded by the ClpP and Lon proteases, leading to an enhanced transcription of the yscW-lcrF operon.
temperature not only prevents the opening of the lcrF RNA thermometer, but also impedes Yop secretion which might be explained by the fact that some T3S genes are only activated by temperature and not by LcrF [31].

Similar to other RNA thermometers, the yscW-lcrF intergenic region accounts only in part for the drastic induction of LcrF after temperature upshift. Apparently, very rapid and efficient activation of LcrF production is achieved by the combination of separate regulatory modules. Thermal induction of LcrF translation mediated by the RNA thermometer is combined with temperature-regulated proteolysis of YmoA repressing yscW-lcrF expression. Regulatory cascades composed of an alternative sigma factor and an RNA thermometer have been reported [55,63,66]. They might be able to integrate several independent signals (e.g. heat and unfolded proteins), whereas the two-layered control by a thermo-sensitive regulator and an RNA thermometer discovered in Yersinia presents a novel strategy to strictly adjust virulence gene expression to the presence in the warm-blooded host.

Both thermosensing mechanisms appear to be complemented by yet another control level. The yscW-lcrF RNA is highly unstable in Y. pseudotuberculosis, which is consistent with a previous study reporting that lcrF mRNA degradation was so fast in Y. pestis that the transcript could not reliably be detected [24]. Recently, it has been shown that the translocator pore protein YopD recognizes the 5'-ends of transcripts of all type III secretion genes and facilitates their degradation [67]. A negative YopD effect was also observed for LcrF synthesis (R. Steinmann, unpublished results) which could be explained by YopD-mediated stabilization of hairpin II or YopD-induced degradation of the yscW-lcrF transcript. Translocation of the YopD protein upon host cell contact would then result in stabilization and increased translation of the LcrF virulence activator, adjusting injectisome and Yop production according to effector translocation.

Presence of highly homologous 5'-UTRs and structurally conserved RNA thermometers in the yscW-lcrF intergenic region of all pathogenic Yersinia species suggests that they are highly important to adjust biological fitness and virulence. RNA-based regulation of LcrF synthesis is very rapid, highly efficient and less energy-consumptive as it allows fast induction upon host entry and tissue contact, and permits immediate shut-off when the initiating signals are removed. This is particularly important as failure to perform type III secretion results in avirulence due to rapid clearance by the host. On the other hand, uncontrolled secretion of Yops was found to be highly detrimental and cause a severe growth defect of the bacteria, which would also be disadvantageous for their survival and host persistence [68–70]. As a consequence a complex feedback mechanism must be responsible for perfect adaptation. The molecular mechanism coupling transcription and translation with Yop export is currently under investigation.

Material and Methods

Ethics statement

Animal work was performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit: animal licensing committee permission no. 33.9.42502-04-055/09.

Cell culture, media and growth conditions

E. coli and Yersinia strains were routinely grown under aerobic conditions at 25°C or 37°C in LB (Luria Bertani) broth on solid or in liquid media if not indicated otherwise. The antibiotics for bacterial selection were as follows: ampicillin 100 µg ml⁻¹, chloramphenicol 30 µg ml⁻¹, tetracyclin 10 µg ml⁻¹, and kanamycin 50 µg ml⁻¹.

Strain and plasmid constructions

All DNA manipulations, polymerase chain reactions, restriction digestions, ligations and transformations were performed using standard genetic and molecular techniques [71–72]. Plasmid DNA was purified using the Qiagen Plasmid Mini or Midi Kits. Restriction and DNA-modifying enzymes were obtained from Roche, Fermentas, Promega or New England Biolabs. The oligonucleotides used for amplification by PCR, sequencing and primer extension were purchased from Metabion. PCR reactions were performed routinely in a 100 µl mix for 25 cycles using Tag polymerase or Phusion High-Fidelity DNA polymerase (New England Biolabs) according to the manufacturer’s instructions. PCR products were purified with the QiAquick PCR purification kit (Qiagen) before and after digestion of the amplification product. Site-directed mutagenesis was performed using the QuikChange mutagenesis kit (Stratagene, LaJolla, USA) with plasmids harboring the yscW-lcrF wildtype sequence as template and the mutagenic primers listed in Table S1. Sequencing reactions were performed by GATC (Konstanz, Germany) or by the in-house facility.

Strains and plasmids used in this study are listed in Table 1 and primers for plasmid generation are listed in Table S1. The ymaq4 fragment of Y. pseudotuberculosis of pAKH71 was generated by PCR using primers 1 and 2, digested with BamHI and SalI, and inserted into pACYC184. To construct pAKH77 a DNA fragment carrying the ymaq4 gene was amplified by PCR using the primer pair 79/80. The fragment was digested with EcoRI and Xhol and inserted into the corresponding sites of pASK-IBA5plus. A fragment carrying the yscW-lcrF intergenic region and the first 10 nt of the lcrF gene under control of the T7 promoter was amplified with primer pair 3/4. The resulting fragment was cloned into the Smal site of pUC18 to generate plasmid pBO1817. Plasmid pBO1823 and pBO1855 were derived from pBO1817 by site-specific mutagenesis using the mutagenesis primer pairs 5/6 and 7/8, respectively. For toetprinting analysis pBO1818 was constructed by insertion of a PCR fragment amplified with primer pair 9/10 into the Smal site of pUC18. Plasmids pBO1824 and pBO1855 were derived from pBO1818 by site-specific mutagenesis using the mutagenesis primer pairs 11/12 and 13/14, respectively. Primer 15 and 16 were used for amplification of the 5'-untranslated region of the gnd gene of E. coli from chromosomal DNA of MC4100 and the resulting fragment was cloned into the NdeI/EcoRI sites of pBAD18-ib-lacZ(481) to generate pED05. For construction of plasmids pED06–pED08 and pED12–pED13 harboring different mutations within the yscW-lcrF intergenic region upstream of the lcrF-lacZ fusion on pKB14, a two-step PCR was performed. The first PCR reaction was always performed with primer 17 and mutagenesis primer I, and the second PCR with primer 18 and mutagenesis primer II. The following listed mutagenesis primer I/II were used for plasmid: pED06 (19/20), pED07 (21/22), pED08 (23/24), pED12 (25/26), and pED13 (27/28). The two generated PCR fragments for each plasmid were used as templates, amplified with primer pair 17 and 18, and cloned into the NdeI/EcoRI site of pBAD18-ib-lacZ(481). Plasmids pED10 and pED11 contain lcrF-lacZfusions with different portions of the yscW locus located upstream of the lcrF gene, starting from position +5 and +246 relative to the yscW start.
| Table 1. Bacterial strains and plasmids. |
|----------------------------------------|
| **Bacterial strains**                  |
| *E. coli* K-12                         |
| CC118 λ::pir                           |
| F^- Δ(ara-lex)7697 Δ(lacZ)74 ΔphoAΔ20 araD139 |
| galE galK thi rpsL rpoB artF^reA1, λ::pir |
| BL21 λDE3                              |
| F^- ompT gal dcm lon hisD81(r^- mthan) gal λ::DE3 |
| KB1                                    |
| BL21 λDE3 (ΔstnA)                      |
| KB3                                    |
| KB1 (Δhns)                             |
| KB4                                    |
| KB3 (Δhha)                             |
| Y. pseudotuberculosis                   |
| IP32953                                |
| pYV, wildtype                          |
| YP1P01                                 |
| IP32953, yscW-lcrF (UU-28/-27CC)        |
| This work                              |
| YP1P02                                 |
| IP32953, yscW-lcrF (GUU-30/-28AAA)      |
| This work                              |
| YP1II                                  |
| pKB1, wildtype                         |
| YP50                                   |
| YP1II, ΔymoA, Km^R                     |
| YP66                                   |
| YP1II, ΔlcrF, Ap^R                     |
| YP82                                   |
| YP1II, yscW-lcrF (AG-46/-45CC), Km^R   |
| This work                              |
| YP83                                   |
| YP1II, yscW-lcrF (UU-28/-27CC), Km^R   |
| This work                              |
| YP84                                   |
| YP1II, yscW-lcrF (GUU-30/-28AAA), Km^R |
| This work                              |
| YP85                                   |
| YP1II, yscW-lcrF (AU-36/-34CCC), Km^R  |
| This work                              |
| YP86                                   |
| YP1II, yscW-lcrF (Δloop1−111−57), Km^R |
| This work                              |
| YP90                                   |
| YP1II, yscW-lcrF (UU-28/-27CC)          |
| This work                              |
| YP94                                   |
| YP1II, yscW-lcrF (Δloop2−44−25)         |
| This work                              |
| YP95                                   |
| YP1II, yscW-lcrF (GUU-30/-28AAA)        |
| This work                              |
| YP96                                   |
| YP1II, yscW (∆271−651)-lcrF             |
| This work                              |
| **Plasmids**                           |
| pACYC177                                |
| cloning vector, p15A, Ap^R, Km^R        |
| [86]                                   |
| pACYC184                                |
| cloning vector, p15A, Cm^R, Tet^R       |
| [86]                                   |
| pAKH11                                  |
| pET28, hns^+, Km^R                     |
| [78]                                   |
| pAKH71                                  |
| pACYC184, ymoA^+, Cm^R                  |
| This work                              |
| pAKH74                                  |
| pACYC184, hns-, Cm^R                    |
| [74]                                   |
| pAKH77                                  |
| pASK-IBA5plus, ymoA^+, Ap^R             |
| This work                              |
| pASK-IBA5plus                           |
| tetR, tet p/o, N-terminal-Strep-Taq-fusion, Ap^R |
| IBA GmbH                               |
| pBAD18-lacZ(481)                        |
| pBAD18, P_BAD::lacZ, Ap^R               |
| [55]                                   |
| pBO1817                                 |
| pUC18, T7-lcrF^- (−123 to 10), Ap^R     |
| This work                              |
| pBO1818                                 |
| pUC18, T7-lcrF^- (−123 to 63), Ap^R     |
| This work                              |
| pBO1823                                 |
| pUC18, T7-lcrF^- (−123 to 10, UU-28/-27CC), Ap^R |
| This work                              |
| pBO1824                                 |
| pUC18, T7-lcrF^- (−123 to 63, GUU-30/-28AAA), Ap^R |
| This work                              |
| pBO1833                                 |
| pUC18, T7-lcrF^- (−123 to 63, UU-28/-27CC), Ap^R |
| This work                              |
| pBO1855                                 |
| pUC18, T7-lcrF^- (−123 to 10, GUU-30/-28AAA), Ap^R |
| This work                              |
| pCP20                                  |
| FLP recombinase expression vector, Ap^R, Cm^R |
| [75]                                   |
| pDM4                                   |
| R6K derivative, sacB, Cm^R              |
| Debra Milton                           |
| pED05                                  |
| pBAD18-lacZ(481), P_BAD::gnd::lacZ, Ap^R |
| This work                              |
| pED06                                  |
| pKB14, P_BAD::lcrF-lacZ (AG-46/-45CC)^R, Ap^R |
| This work                              |
| pED07                                  |
| pKB14, P_BAD::lcrF-lacZ (GUU-30/-28AAA)^R, Ap^R |
| This work                              |
| pED08                                  |
| pKB14, P_BAD::lcrF-lacZ (UU-28/-27CC)^R, Ap^R |
| This work                              |
| pED10                                  |
| pBAD18, yscW-lcrF-lacZ (+269^-74+), Ap^R |
| This work                              |
| pED11                                  |
| pBAD18, yscW-lcrF-lacZ (+510^-74+), Ap^R |
| This work                              |
| pED13                                  |
| pKB14, P_BAD::lcrF-lacZ (AG-36/-34CCC)^R, Ap^R |
| This work                              |
| pET28a                                  |
| T7 overexpression vector, Kn^R          |
| Novagen                                |
| pFU68                                  |
| pSC101*, lacZ, Ap^R                     |
| [87]                                   |
Table 1. Cont.

| Strains, Plasmids | Description | Source and reference |
|-------------------|-------------|---------------------|
| pGP20             | pSC101, promoterless lacZ gene, Tet<sup>R</sup> | P. Gerlach |
| pKB10             | pGP20, yscW-lacZ (~310 to +281)<sup>a</sup>, Ap<sup>R</sup> | This work |
| pKB12             | pGP20, yscW(Δ1378–564)-lcrF-lacZ, Tet<sup>R</sup> | This work |
| pKB13             | pBAD18, lcrF-lacZ (Δloop2 – 44/–25)<sup>a</sup>, Ap<sup>R</sup> | This work |
| pKB14             | pBAD18-lacZ(481), P<sub>BAD</sub>lcrF-lacZ (~124/+74)<sup>a</sup>, Ap<sup>R</sup> | This work |
| pKB18             | pKB14, P<sub>BAD</sub>lcrF-lacZ (Δloop1 – 111/–57)<sup>a</sup>, Ap<sup>R</sup> | This work |
| pKB34             | pT502, yscW-lcrF-lacZ (~310)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB39             | pT502, yscW-lcrF-lacZ (~211)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB40             | pT502, yscW-lcrF-lacZ (~103)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB41             | pT502, yscW-lcrF-lacZ (~2)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB42             | pT502, yscW-lcrF-lacZ (~94)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB84             | pFU68, yscW-lacZ (~1016 to ~265)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB85             | pFU68, yscW-lacZ (~1016 to ~15)<sup>b</sup>, Ap<sup>R</sup> | This work |
| pKB90             | pT502, yscW-lcrF-lacZ (~310, Δ131–241), Ap<sup>R</sup> | This work |
| pKD4              | kanamycin cassette template, Km<sup>R</sup> | [75] |
| pKD46             | recombination vector, λ RED recombinase, Ap<sup>R</sup> | [75] |
| pKOBEG-sacB       | recombination vector, sacB<sup>C</sup>, Cm<sup>R</sup> | [73] |
| pRS29             | pDM4, yscW(Δ271–651), Cm<sup>R</sup> | This work |
| pRS41             | pDM4, lcrF (AG-46/-45CC<sup>C</sup>), Cm<sup>R</sup> | This work |
| pRS42             | pDM4, lcrF (GUU-30/-28AA<sup>A</sup>), Cm<sup>R</sup> | This work |
| pRS43             | pDM4, lcrF (UU-26/-27CC<sup>C</sup>), Cm<sup>R</sup> | This work |
| pRS44             | pDM4, lcrF (AU-36/-34CC<sup>C</sup>), Cm<sup>R</sup> | This work |
| pRS45             | pDM4, lcrF (Δloop2 – 44/–25)<sup>a</sup>, Cm<sup>R</sup> | This work |
| pRS46             | pDM4, lcrF (Δloop1 – 111/–57)<sup>a</sup>, Cm<sup>R</sup> | This work |
| pSF1              | pGP20, yadA-lacZ, Tet<sup>R</sup> | This work |
| pSF3              | pGP20, lcrF-lacZ (+257<sup>b</sup>, +74<sup>b</sup>), Tet<sup>R</sup> | This work |
| pSF4              | pGP20, yscW-lcrF-lacZ (~310<sup>b</sup>; +74<sup>b</sup>), Tet<sup>R</sup> | This work |
| pT502             | pPG20, Ap<sup>R</sup> | Tatjana Stolz |
| pWD34             | pSC101<sup>a</sup>, yopE-lacCDABE, Cm<sup>R</sup> | [87] |

*The number indicates the nucleotide of lcrF, relative to the lcrF start codon.
*The number indicates the nucleotide of yscW, relative to the yscW transcriptional start site.

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start site, a two-step PCR reaction was performed. Two fragments containing the region upstream and downstream of the yscW 3’-UTR were amplified from chromosomal DNA of YPIII using primer pairs 87/89 and 88/90. Subsequently a third PCR was performed with primers 87 and 86 using the upstream and the downstream fragments as templates. The product was digested with PstI introduced into pTS02. To engineer a deletion of yscW from nucleotide position +271 to +651 two fragments containing the region upstream and downstream of the yscW deletion were amplified from chromosomal DNA of YPIII using primer pairs 75/77 and 76/78. Subsequently, a third PCR was performed with primers 77 and 78 using the upstream and the downstream fragments as templates. The product was digested with SpeI and SphI and introduced into pDM4 resulting in pRS29.

For construction of plasmids pRS41–46 harboring different mutations within the yscW-lcrF intergenic region upstream of the lcrF gene, a two-step PCR was performed and the resulting fragments were cloned into the SpeI/SphI sites in pDM4. For the construction of pRS41, first two PCR reactions were performed with primers 46/50 and 47/51, and the two PCR fragments were used as templates to amplify the yscW-lcrF (AG-46/-45CC) mutant version with primer pair 44/45. Plasmids pRS42–44 were constructed by insertion of SpeI/SphI fragments amplified with primers 44/45 from different PCR fragments used as templates. For the production of the template fragments the two primer pairs 44/52 and 45/53, 44/54 and 45/55 as well as 44/56 and 45/57 were used for the synthesis of the GUU-30/-28AAA, UU-28/27CC and AUA-36/-34CCCC fragments. To engineer plasmid pRS45, (Δloop2 –44/–25) both template fragments were obtained by amplification with primers 48/49 and 64/49. After annealing of the template fragments, the yscW-lcrF fragment harboring the Δloop2 –44/–25 mutation was amplified with primer pair 63/64 and cloned into the SpeI/SphI sites of pDM4. For the construction of pRS46, first two PCR reactions were performed with primers 48/43 and 49/42, and the two PCR fragments were used as templates to amplify the yscW-lcrF (AG-46/-45CC) mutant version with primer pair 48/49. The yadA-lacZ translational fusion encoded by pSF1 was constructed by insertion of a yadA promoter fragment amplified with the primer pair 91/92 into the PstI site of pGP20. The lcrF-lacZ and yscW-lcrF-lacZ fusion plasmids pSF3 and pSF4 were constructed by insertion of PCR fragments amplified from \( \textit{F. pseudotuberculosis} \) YPIII genomic DNA with primer pairs 37/58 and 37/32 into the PstI site of pGP20.

Construction of the \( \textit{E. coli} \) and \( \textit{F. pseudotuberculosis} \) deletion mutants

\( \textit{F. pseudotuberculosis} \) strain YP50 was constructed by insertion of a kanamycin cassette into the locus of wildtype strain YPIII using the RED recombinase system as described previously [73]. First, the kanamycin resistance gene was amplified using the kan\(_{\text{mut}}\) primers (Table S2) and plasmid pACYC177 as template. Next, the \( \textit{Yersinia} \) genomic DNA was used as a template to amplify 500-bp regions flanking the target gene. The upstream fragment was amplified with a primer pair of which the reverse primer contained additional 20 nt at the 5’-end which were homologous to the start of the kanamycin resistance gene. The downstream fragment was amplified with a primer pair of which the forward primer contained additional 20 nt at the 3’-end which were homologous to the end of the kanamycin resistance gene (for primer see Table S2). In the next step, a PCR reaction was performed with the forward primer and the reverse primer using the upstream and downstream PCR products of the target gene and the \( \textit{kan} \) gene fragment as templates. The PCR fragment was transformed into \( \textit{F. pseudotuberculosis} \) YPIII pKOBE-G-sacB and chromosomal integration of the fragments was selected by plating on LB supplemented with kanamycin. Mutants cured of pKOBE-G-sacB were proven by PCR and DNA sequencing.

For the construction of the lcrF knock-out mutant strain YP66, a lcrF::AmpR PCR fragment was generated using an AmpR-resistant plasmid as a template with primers composed of 55 nucleotides which are homologous to the upstream and downstream region of the lcrF gene followed by 20 nucleotides homologous to the 5’- or 3’-end of the ampicillin resistance gene (for primer see Table S2). The resulting PCR fragment was integrated into the lcrF locus of \( \textit{F. pseudotuberculosis} \) YPIII on pYV by the RED recombinase system (Derbise et al., 2003). Selection of the mutant strain was performed as described [74]. One strain, YP66, harboring the lcrF::AmpR mutation in the lcrF locus, as proven by PCR and DNA sequencing, was used for further studies.

All mutant strains with deletions or nucleotide substitutions in the yscW-lcrF intergenic region (YP82–86, YP90, YP95, YP96, YPIP01 and YPIP02) were constructed by homologous recombination using suicide plasmids pRS41–46 and pRS29. Plasmids were mated from \( \textit{E. coli} \) S17-1 λpir (λ+) into \( \textit{F. pseudotuberculosis} \) YPIII or IP32953 and transconjugants were selected on \( \textit{Yersinia} \) selective agar (Oxoid) supplemented with chloramphenicol. The recombinant of the plasmid into the \( \textit{Yersinia} \) virulence plasmid pYV yielded a merodiploid strain, including a wildtype and the mutant copy of yscW-lcrF. Subsequently, the resulting strain was plated on 10% sucrose and fast growing, large colonies were selected. Because sucrose induces the expression of the \( \textit{bab} \) gene on the integrated plasmids and leads to the production of a toxic substance that prevents growth, a spontaneous second recombination process resulting in the excision of the integrated plasmid is advantageous. 50 selected fast-growing strains were screened for chloramphenicol sensitivity to prove the loss of the integrated plasmid. One strain (Table 1), harboring the desired yscW-lcrF mutation, as proven by PCR and DNA sequencing, was taken for further analysis.

The \( \textit{E. coli} \) mutant strains were constructed with the RED recombinase system as described previously [75]. \( \textit{E. coli} \) strain KB1 was constructed by introducing a \( \textit{stpA} \) deletion into strain BL21DE3 and used to generate KB3 (BL21DE3 \( \textit{stpA}^{\text{his}} \)). Subsequently, KB3 was used to construct KB4 (BL21DE3 \( \textit{stpA}^{\text{his}} \textit{hns}^{\text{bla}} \)). First, a kanamycin cassette was amplified by PCR with primers homologous to the resistance gene encoded on pKD4 followed by homologous sequences of adjacent regions of the target gene (for primer see Table S2). The PCR fragment was transformed into \( \textit{E. coli} \) BL21 pKD16. Chromosomal integration of the fragment was selected by plating on LB supplemented with kanamycin. Subsequently, mutant derivatives were cured of the temperature-sensitive plasmid pKD46 by cultivation at 37°C. To remove the resistance gene at its FLP recognition sites the mutants were transformed with the helper plasmid pCP20 encoding the FLP recombinase. For thermal induction of FLP synthesis and subsequent removal of the temperature-sensitive plasmid pCP20, mutants were incubated at 37°C.

RNA isolation and Northern detection

Overnight cultures were diluted 1/50 in fresh medium and grown to stationary phase (OD\(_{600}\) of 3). 2.5 ml culture were withdrawn, mixed with 0.2 volume of stop solution (5% water-saturated phenol, 95% ethanol) and snap-frozen in liquid nitrogen. After thawing on ice, bacteria were pelleted by centrifugation (2 min, 14,000 rpm, 4°C), and RNA was isolated using the SV total RNA purification kit (Promega) as described by the
manufacturer. RNA concentration and quality were determined by measurement of A260 and A280.

Total cellular RNA (20 µg) was separated on MOPS agarose gels (1.2%), transferred by Vacuum Blotting for 1.5 h onto positively charged membranes (Whatman) in 10 × SSC using a semi-dry blotting system and UV cross-linked. Prehybridization, hybridization to DIG-labelled probes and membrane washing were conducted using the DIG luminescent Detection kit (Roche) according to the manufacturers instructions. The yscW-lcrF transcript was detected with a DIG-labelled PCR fragment (DIG-PCR nucleotide mix, Roche) with primer pair 59 and 60 (Table S1).

Expression and purification of the Y. pseudotuberculosis YmoA and H-NS protein

KB4 transformed with pAKH77 or pAKH11 was grown at 37°C in LB broth to an A600 of 0.6. Anhydrotetracycline was added (0.2 µg/ml) to induce the expression of YmoA-Strep-Tag or 2 mM IPTG was used to induce H-NS-His6 expression. For purification of the YmoA-H-NS heterodimer KB4 transformed with pAKH74 and pAKH77 was used for overexpression of the YmoA in the presence of the Yersinia H-NS protein. The cells were grown for an additional 3 h before being harvested. The purification procedure for the Strep-tagged YmoA protein was performed according to the manufacturers instructions (IBA GmbH, Germany). H-NS purification was performed as described [76]. The purity of the YmoA and the H-NS protein was estimated to be >95%.

Gel retardation assays

For DNA-binding studies the purified YmoA and H-NS proteins were dialysed against the DNA-binding buffer (10 mM Tris-HCl pH 7.5, 3 mM DTT; 7.5% glycerol; 100 mM KCl; 100 mM MgCl2). The yscW fragment (−2 to +272) for DNA band shift analysis was obtained by PCR using primer pair 68/69 with chromosomal DNA of Y. pseudotuberculosis YPIII and the csiD1 and csiD2 control fragments were amplified by PCR from chromosomal DNA of E. coli strain MC4100 with primer pairs 71/72 and 70/71 (see Table S1). The DNA fragments and increasing concentrations of purified YmoA or H-NS were incubated for 30 min in DNA-binding buffer at room temperature and immediately loaded on 4% polyacrylamide gels.

In vitro transcription and structure probing of the lcrF RNA

The lcrF RNA for structural probing was obtained by run-off transcription with T7 RNA polymerase from linearized plasmids pBO1818, pBO1824, and pBO1833. Toeprobing experiments were performed with 30S ribosomal subunits, lcrF mRNA and RNA75-Me, mainly as described previously [43]. The 5′-[32P]-labelled lcrF-specific primer 61 (2 pmol) was used for reverse transcriptase reaction. About 1 pmol of the lcrF mRNA was annealed to the radioactive primer and incubated for 20–30 min at 25°C and 37°C in a 20 µl reaction mix with 16 pmol of uncharged rRNA75-Me (Sigma-Aldrich, USA) in the presence or absence of 6 pmol of the 30S ribosomal subunits isolated as described [78]. To initiate the primer extension reaction, 2 µl Vd-Mg2+ buffer (0.05 M Tris-HCl pH 7.4, 0.3 M NH4Cl, 30 mM β-mercaptoethanol, 0.05 M MgO acetate) containing 80 µl of the MMLV reverse transcriptase (USB, USA), dNTPs, and BSA was added and incubated for 10 min at 25°C. cDNA synthesis was stopped by the addition of 20 µl formamide stop solution. In parallel, sequencing reactions using the same lcrF specific primer was performed with the Thermo Sequenase cycle sequencing Kit (USB, USA) according to the manufacturer’s instructions. All samples were denatured at 95°C for 5 min, and separated on a denaturating 8% polyacrylamide/urea gel.

Primer extension analysis to identify the yscW-lcrF transcriptional start site

Primer extension analysis was performed to determine the transcriptional start site of the yscW-lcrF mRNA from strain YP111. At an OD600 of 2.0 (early stationary phase), total RNA was extracted of the samples using the SV total RNA purification kit (Promega) as described by the manufacturer. Annealing was performed with 20 µg extracted RNA and the 5′-Dig-labelled oligonucleotide 62 for yscW in 20 µl of 1× First Strand Buffer (Invitrogen) by slow cooling of the sample (0.01°C/sec). 8 mM dNTPs and 5× FS Buffer (Invitrogen) with 200 U Superscript II reverse transcriptase (Invitrogen) was added to the reaction mix and incubated for 1 h at 42°C. The size of the Dig-labelled reaction products was determined on a denaturing 6% DNA sequencing gel by a detection procedure as described [79].

Analysis of the reporter gene expression

The activity of the β-galactosidase activity of the lacZ and the phoA fusion constructs was measured in permeabilized cells as described previously [71,80]. The activities were calculated as follows: β-galactosidase activity OD120 × 6.75 × OD600−1 × Δt (min)−1 × Vol (ml)−1; alkaline phosphatase activity OD120 × 6.46 × OD578−1 × Δt (min)−1 × Vol (ml)−1. Reporter fusions emitting bioluminescence were measured in non-permeabilized cells with a Varioskan Flash (Thermo Scientific) using the SkanIt software (Thermo Scientific) for 1 s per time point. The data are given as relative light units (RLU/OD600) from three independent cultures performed in duplicate. The level of statistical significance for differences in reporter gene expression was determined by the Student’s t test.

Gel electrophoresis, preparation of cell extracts and Western blotting

For immunological detection of the LcrF and YadA proteins, Y. pseudotuberculosis cultures were grown under specific environmental conditions as described. Cell extracts of equal amounts of the bacteria were prepared and separated on a 15% (LcrF) or 10% (YadA) SDS-PAGE [72]. Subsequently the samples were transferred onto an Immobilon-P membrane (Millipore) and probed
with polyclonal antibodies directed against YadA or LcrF (generous gift of Greg Plano) as described [74]. The cell extracts used for Western blotting were also separated by SDS-PAGE and stained with Coomassie blue to ensure that the protein concentrations in the different cell extracts are comparable; about 10 mg protein was applied of each sample.

Yop secretion assay

The Yop secretion assay was performed as described previously [69]. Bacteria were grown overnight at 25°C in LB medium, diluted 1:50 in fresh LB medium and grown at 25°C until the culture reached an OD_{600} of about 0.4–0.5. Subsequently, the cultures were shifted to 37°C for 3–4 h in the absence or presence of 20 mM Mg^{2+} and 20 mM Naoxalate, a Ca^{2+} chelator. Proteins in the medium supernatant were harvested, filtered and precipitated with TCA. Precipitated proteins were resuspended in equal amounts of sample buffer, separated on 15% SDS polyacrylamide gels and visualized by Coomassie brilliant blue staining.

Mouse infections

In order to assess the impact of the RNA thermometer on Y. pseudotuberculosis virulence, groups of 7-week-old female BALB/c mice were orally infected with 5×10^8 bacteria using a ball-tipped feeding needle. To prepare the inocula, the Y. pseudotuberculosis wildtype strain YPIII and isogenic mutant strains were cultured overnight in LB at 25°C. The bacteria were harvested by centrifugation, washed and resuspended to the appropriate concentration in PBS. Three days after infection of the mice the colony forming units (CFU) per gram tissue were determined in the Peyer’s patches, mesenterial lymph nodes, liver and spleen. Isolated Peyer’s patches were rinsed with sterile PBS and incubated with 100 μg/ml gentamicin in PBS in order to kill the bacteria on the luminal surface. After 30 min, gentamicin was removed by extensive washing with PBS for three times. Subsequently, all organs were weighted, homogenized in PBS, and plated in three independent serial dilutions on Yersinia selective agar (Oxoid, Germany). For the survival assays, groups (n = 10) of 7-week-old BALB/c mice were orally infected with a lethal dose of 2×10^8 bacteria (Y. pseudotuberculosis strains YPIII and mutant strains cultured overnight in LB at 25°C). The infected mice were monitored for 14 days every day to determine the survival rate.

Supporting information

**Figure S1 Identification of regulatory sequences important for yscW-lcrF expression.** (A) Strains YPIII and YP50 (∆ymoA) expressing yscW-lcrF-lacZ fusions with different 5’-deletions of the yscW regulatory region were grown overnight in LB medium at 25°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly between the different 5’-deletion constructs in the wildtype or the ymoA mutant strain with ** (P<0.01), and *** (P<0.001). (B) Influence of YmoA on the 5’ UTR of yscW. Strains YPIII and YP50 (∆ymoA) expressing yscW transcriptional fusions either with the promoter region and the 5’ UTR (pKB85) or only the promoter region (pKB84) were grown overnight in LB medium at 25°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly between the different 5’-deletion constructs in the wildtype and the repressed and Student’s t test. Stars indicate the results that differed significantly between the different 5’-deletion constructs in the wildtype or the ymoA mutant strain with ** (P<0.001).

**Figure S2 Gel retardation experiments using purified YmoA, H-NS and YmoA copurified with H-NS.** Individual DNA fragments comprising the yscW upstream region and the different portion of the E. coli cisD gene as negative control (cisD1 and cisD2) were incubated without protein or with increasing amounts of purified Y. pseudotuberculosis YmoA (A), H-NS (B) or YmoA purified in the presence of H-NS (YmoAH-NS) protein (C). The samples were separated on a 4% polyacrylamide gel, a molecular weight standard (M: 100 bp ladder) was loaded, and the corresponding molecular weights are indicated on the left. The positions of the DNA fragments and the higher molecular weight protein-DNA complexes are indicated. (D) Strains YPIII and YP50 (∆ymoA) expressing a yscW-lcrF-lacZ (pKB34) or a yscWΔ13–241-lcrF-lacZ fusion (pKB90) with a deletion of the 5’-UTR of the yscW regulatory region were grown overnight in LB medium at 25°C and 37°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three different experiments each done in duplicate. Data were analyzed by the Student’s t test. Stars indicate the results that differed significantly between the wildtype and the ymoA mutant strain with *** (P<0.001).

**Figure S3 The intergenic region of the yscW-lcrF operon is implicated in the temperature control of LcrF production.** YPIII harboring the different P_{yscW}::yclcW-lcrF-lacZ reporter plasmids pED10, pED11 and pKB14 or the P_{yscW}::gnd-lacZ control plasmid (pED05) were grown overnight in LB medium at 25°C or 37°C in the presence of 0.05% arabinose. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three experiments each done in duplicate. Stars indicate the reporter activity that differed significantly between 25°C and 37°C with *** (P<0.001).

**Figure S4 Influence of yscW on LcrF production.** (A) YPIII harboring the different yscW-lcrF-lacZ or the yscWΔ378–564-lcrF-lacZ reporter plasmids pSF4 and pKB12 were grown overnight in LB medium at 25°C or 37°C. β-Galactosidase activity from overnight cultures was determined and is given in μmol min^{-1} mg^{-1} for comparison. The data represent the average ± SD from at least three experiments each done in duplicate. (B) YPIII and YP96 harboring a yscWΔ271–651 were grown overnight in LB medium at 25°C or 37°C. Whole-cell extracts from overnight cultures were prepared and analysed by Western blotting with a polyclonal antibody directed against LcrF. A molecular weight marker is loaded on the left. A higher molecular weight protein (c) that reacted with the polyclonal antiserum was used as loading control.

**Figure S5 Influence of the lcrF RNA thermometer on tissue colonization by Y. pseudotuberculosis.** Strains YPIII (wildtype), the ‘closed’ yscW-lcrF variant YP90, and YP66 (ΔlcrF) were infected intragastrically (5×10^8 CFU/mice) into BALB/c mice. After three days of infection, mice were sacrificed and the number of bacteria in homogenized host tissues and organs was determined by plating. Solid lines indicate the means. The statistical significances between the wildtype and the repressed and...
deregressed lcrF RNA thermometer variants were determined by the Student’s t test. P-values: ***: <0.001.

Figure S6 Influence of the lcrF RNA thermometer on tissue colonization by *Y. pseudotuberculosis*. Strains IP32953 (wildtype), the ‘closed’ and open yscW-lcrF variant YPIP01 and YPIP02 were infected intragastically (5×10⁶ CFU/mice) into BALB/c mice. After three days of infection, mice were sacrificed and the number of bacteria in homogenized host tissues and organs was determined by plating. Solid lines indicate the means. The statistical significances between the wildtype and the repressed and derepressed lcrF RNA thermometer variants were determined by the Student’s t test. P-values: ***: <0.001.

Figure S7 Multiple DNA sequence alignment of the yscW-lcrF operon of pathogenic *Yersinia* species. The nucleotides identical in the sequences are indicated with dots. The alignment was created using ClustalW. The coding sequence of *yscW* are given in green, the intergenic region (RNA thermometer sequence) is given in blue. The promoter regions are bold and underlined, the transcriptional and translational start sites as well as the stop codons are indicated in bold. *Y. pest*: *Y. pseudotuberculosis*; *Y. pestis*: *Y. pestis* and *Y. ent.:* *Y. enterocolitica*.

Table S1 Oligonucleotides used in this study. The corresponding restriction sites are underlined, the T7 promoter region is given in italic, and introduced basepair substitutions are indicated in bold, the deletion of nucleotides is indicated by a short line, the sequence of kan resistance cassette is given in bold and underlined.

Table S2 Primers used for the generation of deletion mutants. * The *Y. pseudotuberculosis* mutants were constructed by adding a kanamycin resistance cassette (Kan). Underlined bases correspond to the homologous nucleotides of the resistance gene. Rev: reverse primer; for: forward primer.

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Author Contributions

Conceived and designed the experiments: KB RS JK HWW FN PD. Performed the experiments: KB RS JK SS AKH EB FP TT. Analyzed the data: KB RS JK AKH HWW FN PD. Contributed reagents/materials/analysis tools: HWW FN. Wrote the paper: KB RS PD.
Two-Layered Thermo-Control of Yersinia Virulence

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