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A Proteinaceous Gene Regulatory Thermometer in Salmonella

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Summary

Novel utilization of the coiled-coil motif is presented that enables TlpA, an autoregulatory repressor protein in Salmonella, to sense temperature shifts directly and thereby to modulate the extent of transcription repression. Salmonella cells shifted to higher temperatures, such as those encountered at host entry, showed derepressed *tlpA* activity. *tlpA::lacZ* fusions indicated that the promoter itself is insensitive to thermal shifts and that transcription control was exerted by the autorepressor TlpA only. In vitro studies with highly purified TlpA showed concentration and temperature dependence for both fully folded conformation and function, indicating that the thermosensing in TlpA is based on monomer-to-coiled-coil equilibrium.

Introduction

Entry from the "cold" into the "warm" host environment is believed to be one of the central cues triggering virulence factors in pathogenic bacteria (Maurelli, 1989; Miller et al., 1989; Mekalanos, 1992). Several studies on thermoregulation of virulence genes in bacteria have focused on changes in DNA topology (Dorman, 1991). The histone-like protein H-NS with the ability to constrain supercoiling of DNA has often been proposed to take part in this activity (Higgins et al., 1990; Hulton et al., 1990). H-NS has been implicated in virulence regulation of uropathogenic Escherichia coli (Göransson et al., 1990) and Shigella flexneri (Dorman et al., 1990; Hromockyj et al., 1992; Tobe et al., 1993). In Yersinia enterocolitica, a histone-like protein, YmoA, acts in a manner similar to that of H-NS (Cornelis et al., 1991; Mikulskis and Cornelis, 1994). The direct effect of temperature on mRNA topology has also been put forth as one way to turn on needed genes. For example, in Yersinia pestis, a study suggests a sensing mechanism based on mRNA secondary structure where a stem loop is "melted" by a shift to 37°C, allowing translation to proceed (Hoe and Goguen, 1993). Despite these documented efforts, it is fair to claim that, so far, studies on temperature regulation of virulence genes have not clearly identified the thermosensing moiety or its molecular mechanism of action.

Another much-investigated process where temperature sensing is required is the heat-shock response. In the current model of heat-shock regulation in bacteria temperature stress is detected by sensing the presence of denatured or misfolded polypeptides by way of the DnaK chaperone system and σ^{32} binding and release cycles (Craig and Gross, 1991; Yura et al., 1993; Gamer et al., 1996).

Representative examples with "thermometer" and gene regulator activity combined into a single molecule are not common. However, we hypothesize that it would not be unexpected to find such proteinaceous thermosensing transcription factors. A possible candidate is TlpA, a Salmonella typhimurium virulence plasmid-encoded protein, previously shown to be a sequence-specific DNA-binding autoregulator (Hurme et al., 1996). In deciphering the role of *tlpA* in Salmonella, increased temperature was the only tested condition, producing a derepression of this gene. The *tlpA* gene product, TlpA protein, contains an N-terminal DNA-binding region and a long coiled-coil domain (Hurme et al., 1996). Coiled coils are formed by sequences containing amino acid heptad repeats (a-b-c-d-e-f-g), where "a" and "d" residues are hydrophobes that form an apolar stripe, providing the driving force for this conformation (Crick, 1953; Pauling and Corey, 1953; McLachlan and Stewart, 1975). The heptad repeat-containing subunit α helices wind around each other and pack their side chains in a "knobs-into-holes" manner, producing the left-handed superhelical coiled-coil structure (Crick, 1953; Cohen and Parry, 1990; Lupas, 1996).

The evidence for a coiled coil in TlpA is based on a number of observations. In line with this proposal, TlpA has been shown to have a circular dichroism (CD) spectrum with a high α-helical content (85%), as expected for a long coiled coil (Hurme et al., 1996). Two available coiled-coil prediction algorithms, COILS2 (Lupas et al., 1991) and PAIRCOIL (Berger et al., 1995), a recent algorithm that has been successfully tested on known structures, give TlpA a high score for most of its sequence (Hurme et al., 1996; unpublished data). Furthermore, the appearance in electron microscopy (EM) of smallest visible units is consistent with the predicted dimensions of a two-stranded rod-like coiled coil (Hurme et al., 1994). Oxidation experiments, which make use of the single cysteines in TlpA monomer chains, showed a rapid and quantitative covalent dimer formation, suggesting that the subunits are parallel and unstaggered in the coiled coil (Hurme et al., 1994). Some coiled coil–containing proteins are capable of dynamic switching of monomer subunits between dimers (Lehrer and Qian, 1990; Jancso and Graceffa, 1991; Wendt et al., 1995). TlpA is also capable of such behavior in vitro and in vivo, where transcription experiments with a dominant negative mutant dTlp5 are based on such chain exchange (Hurme et al., 1994, 1996).

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In our previous work, we suggested that the extensive coiled-coil dimerization domain in TlpA might also be involved in modulating the extent of transcription repression of *tlpA* (Hurme et al., 1996). Indeed, in vivo experiments presented here suggest that TlpA-mediated autorepression is a dynamic system that can sense temperature shifts in the physiologically relevant range. The analysis of both in vitro DNA-binding and TlpA biophysical characteristics provide the basis for understanding this behavior at the molecular level. We propose that the molecular principle of thermosensing in TlpA is the dynamic monomer-to-coiled-coil equilibrium, the stability of which is well known to be both temperature and concentration dependent for its fully folded state (O'Shea et al., 1989a; Greenfield and Hitchcock-DeGregori, 1995). Our results establish TlpA as a protein capable, without accessory factors, of coupled temperature sensing and gene regulation.

Results

TlpA Senses Elevated Temperature and Derepresses *tlpA* Transcription

From previous studies, we knew that the TlpA coiled coil can rapidly exchange monomer chains with partner molecules at 37°C and higher temperatures (Hurme et al., 1994, 1996). Therefore, we assayed *tlpA::lacZ* transcription fusion activity at 43°C and 45°C, reasoning that the higher temperatures would lead to increased dimer dissociation, yielding monomers incapable of DNA binding.

For the sake of clarity, let us first briefly summarize characteristics of the *tlpA* system from a recent study (Hurme et al., 1996). The *tlpA* promoter fused to *lacZ* is constitutively active, as opposed to a fusion to the full length *tlpA*, which is repressed. Fusion to the bare promoter can be repressed in *trans* by the wild-type protein but not by a mutant TlpA (dTlp5). dTlp5 is a TlpA deletion protein that lacks 13 amino acid residues in the amino-terminal domain. Consequently, this mutant is unable to bind DNA, but is otherwise physically indistinguishable from the wild type (Hurme et al., 1996). Interestingly, a fusion to the full-length *tlpA* reading frame can be derepressed by bringing in *trans* dTlp5, which, we interpret, results from heterodimerization via chain exchange and leads to a dominant negative effect, allowing transcription to proceed (Hurme et al., 1996).

A *lacZ* fusion, either to the full-length *tlpA* expressing the functional repressor (pRHTF01), to the promoter itself with no TlpA production (pRHTF02), or to the mutated *tlpA* gene expressing dTlp5 protein derivative (pRHTF03), was used in a *tlpA*$^{-}$ S. typhimurium, 1275-1 (Figure 1). All fusion-carrying strains were grown at 37°C to the exponential phase and then divided into aliquots incubated for 2 hr at 37°C, 43°C, or 45°C, whereafter β-galactosidase activity was assayed and reported in Miller Units (Miller, 1972). The full-length fusion, pRHTF01, showed increasing activity upon a shift from 37°C to 43°C or 45°C (Table 1). This 4.2- and 13.2-fold induction, for 43°C and 45°C, respectively, is in striking contrast to the bare promoter itself in pRHTF02 and pRHTF03, which showed hardly any increase at higher temperatures, pointing to the fact that it is not the promoter that is temperature activated (Table 1). As expected, pRHTF03 is active regardless of temperature, since it expresses dTlp5, which does not bind DNA and therefore is unable to cause repression (Table 1). At 43°C, the transcription repression of pRHTF01 begins to leak, and at 45°C a full derepression is evident, as now the activity is equivalent to the naked promoter in pRHTF02 and pRHTF03 (Table 1). Two control fusions, pOF11, a vector control without transcriptional terminators preventing plasmid readthrough, and pOF70, which is a *lacZ* fusion to the *spvR* promoter (a virulence plasmid gene; Rhen et al., 1993), did not respond to elevated temperature (Table 1).

To rule out any accessory factor involvement, additional control experiments were carried out. *tlpA* fusions were tested in H-NS mutant background (*osmZ*::Tn10; CH 1701; Hulton et al., 1990) and a topoisomerase I mutant (CH582; Richardson et al., 1984). In both cases, *tlpA::lacZ* fusions were unaffected by the respective genetic backgrounds, from which it was concluded that DNA supercoiling is an unlikely factor in controlling *tlpA* repression. Furthermore, the *tlpA* promoter was not derepressed by ethanol stress, an activator of the heat shock genes (VanBogelen et al., 1987; data not shown). These controls are consistent with the idea that in our experiments, temperature alone is the signal for derepressing transcription via impairing TlpA action.

The Autoregulatory Repressor TlpA Accumulates Only at Elevated Temperature, Whereas the Mutant-Protein dTlp5, Impaired in DNA Binding, Is Produced Constitutively

Clear induction of *tlpA* at a transcriptional level could mean that the amount of TlpA protein also increases, if the protein is stable enough to accumulate. If so, the amount of the dTlp5 protein should be independent of temperature, since this mutant protein is unable to interact with DNA. The amount of polypeptide produced was

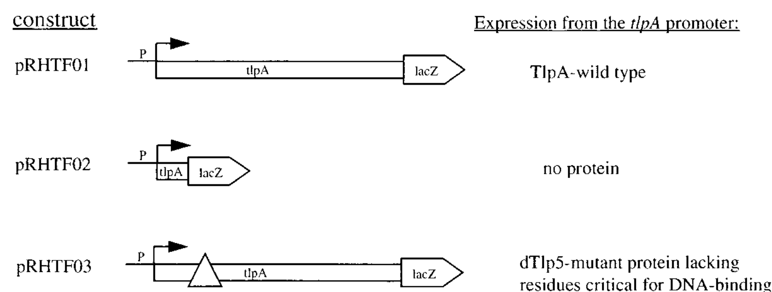

Figure 1. Schematic Representation of *lacZ* Transcriptional Fusions to *tlpA* and Truncations Thereof

P marks the promoter as delineated in Hurme et al. (1996). Arrows mark the first translated codon ATG of the *tlpA* reading frame, which is complete in pRHTF01 but in pRHTF03 has an in-frame deletion corresponding to 13 amino acids marked by an open triangle.

Table 1. Transcriptional Activity of *lacZ* Fusions at 37°C, 43°C, and 45°C in Miller Units[a]

Fusion Construct in 1275-1	37°C	43°C	45°C
pRHTF01[b]	68 ± 16	294 ± 116 (4.2)[c]	923 ± 465 (13.2)[d]
pRHTF02	1405 ± 281	1607 ± 256 (1.1)	1000 ± 226 (0.7)
pRHTF03	1482 ± 191	1790 ± 464 (1.2)	1140 ± 220 (0.8)
pOF11	482 ± 56	503 ± 65 (1.0)	367 ± 52 (0.8)
pOF70	49 ± 7	49 ± 15 (1.0)	43 ± 6 (0.9)

[a] Mean values from four experiments where cells grown to the exponential phase were incubated for 2 hr.
[b] *lacZ* fusion are to: full-length *tlpA* (pRHTF01), *tlpA* promoter (pRHTF02), *tlpA* gene deleted in frame for a region coding for 13 amino acid region critical to DNA binding (pRHTF03), vector without transcriptional terminators (pOF11), spvR (pOF70).
[c] In parentheses, ratio 43°C/37°C of measured activity.
[d] In parentheses, ratio 45°C/37°C of measured activity.

assayed utilizing the transcription fusion vectors expressing TlpA (pRHTF01) and dTlp5 (pRHTF03), respectively (Figure 1). As an internal control, we probed for chloramphenicol acetyl transferase (CAT) expressed from the very same vectors. Strains carrying the appropriate fusions were analyzed by Western blotting with antibodies recognizing TlpA and CAT (Figure 2A). By scanning the Western blot film (Figure 2A), we quantified the signal and determined that dTlp5 was produced in amounts close to 10-fold higher as compared to TlpA at 37°C, indicating a striking autorepression by TlpA (Table 2). This autorepression is interrupted efficiently by temperature shifts whereby the amount of TlpA increases 7-fold (Table 2 and Figure 2A), which is now closer to that of the constitutively active construct expressing dTlp5 (Table 2). Temperature induction was not a global phenomenon, since CAT serving as the internal control blotted for the same samples was negligibly affected (Figure 2A; Table 2).

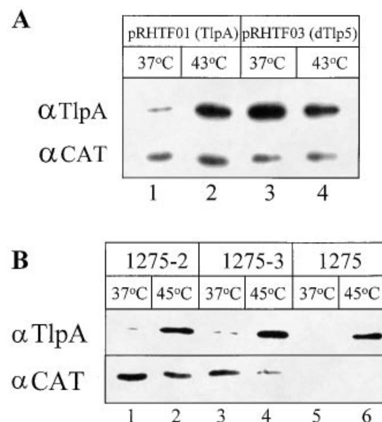

Figure 2. Temperature Induced Accumulation of TlpA Protein

(A) Western blot analysis of TlpA and dTlp5 expression at elevated temperature. Cell lysates of Salmonella typhimurium 1275-1 (*tlpA*−), carrying *lacZ* fusion plasmid either to the intact *tlpA* reading frame expressing TlpA (pRHTF01) or one expressing a DNA binding mutant dTlp5 (pRHTF03), incubated at 37°C and 43°C for 2 hr, were probed with TlpA and CAT antibodies (indicated as α).

(B) Western blot of Salmonella incubated at 37°C and 45°C for 2 hr. 1275 is the wild type; 1275-2 and 1275-3 are Mud transductants of 1275 (see Table 6). Samples were probed with TlpA and CAT antibodies. CAT signal is derived from the chromosomal Mud element carrying the *cat* gene, whereas the TlpA signal originates from the *tlpA* gene carried on a single copy virulence plasmid.

tlpA in Wild-Type Salmonella Is Derepressed by Elevated Temperature

Induction as a function of temperature in previous experiments has relied on transcription from a 10–15 copy vector in a transformed S. typhimurium *tlpA*$^{-}$ strain (1275-1). Temperature induction should also be reproducible in wild-type bacteria. Thus, wild-type S. typhimurium 1275 was tested for derepression of its single copy of *tlpA* at 45°C to obtain maximal effects as predicted from transcription data in Table 1. For a reliable control, we used the CAT protein expressed by strains 1275-2, -4, and -5. Strains 1275-2, 1275-3, and 1275-4 are the result of transducing 1275 with lysates of strains carrying the *cat* gene in Mud/P22 P/Q transposon elements at three different locations on the chromosome (see Experimental Procedures). Western blotting with TlpA and CAT antibodies was utilized again (Figure 2B). Strains 1275-2, 1275-3, 1275-4 (not shown), and the wild type all showed accumulation of TlpA at 45°C (Figure 2B; Table 3). Strains carrying the *cat* gene exhibited no temperature-mediated induction of CAT protein (Figure 2B; Table 3). As expected, in the wild-type Salmonella, *tlpA* is also prone to derepression owing to temperature effects on the protein TlpA.

The temperature shift effects were also studied by isolating RNA from the wild-type strain 1275. Cells were grown at 28°C to the exponential state, incubated for 30 min at 43°C, and finally shifted back to 28°C for another 30 min. As a control, we used *ompA* mRNA (outer membrane protein A; von Gabain et al., 1983). *tlpA* mRNA was scarcely detectable prior to the temperature upshift (Figure 3, lane 1); however, incubation at 43°C caused a marked accumulation of the mRNA (Figure 3, lane 2). As expected, the temperature downshift led to an efficient repression and virtual disappearance of

Table 2. Quantitative Analysis of Thermoinduced Protein Accumulation: Figure 2A

	Construct/Protein Expressed			
	pRHTF01/T1pA		pRHTF03/dT1p5	
Quantitated Protein	37°C	43°C	37°C	43°C
TlpA	2.8[a]	19.8 (7.1)[b]	24.6	15.7 (−)
CAT	7.9	11.2 (1.4)	6.1	7.1 (1.2)

[a] Relative signal of the protein band in the Western blot film.
[b] Induction as a ratio of values for 43°C/37°C.

Table 3. Quantitative Analysis of Thermoinduced Protein Accumulation: Figure 2B[a]

	1275-2		1275-3		1275	
	37°C	45°C	37°C	45°C	37°C	45°C
TlpA	3	185	3	233	0	145
CAT	153	72	82	14	NA[b]	NA

[a] Relative signal of the protein band in the Western blot film.
[b] NA, not available.

the *tlpA* mRNA (Figure 3, lane 3). *ompA*-mRNA blotted for the exact same samples did not display a similar pattern of regulation (Figure 3, lanes 1–3). A temperature upshift always leads to accumulation of newly synthesized TlpA, and therefore we were curious to see whether the amount of TlpA initially present in the cell, prior to the temperature upshift, would remain viable during the temperature stress and be able to repress efficiently again at a downshift. Samples were processed as above except chloramphenicol was added 5 min prior to the temperature shift in order to block protein synthesis, i.e., the production of new TlpA (Figure 3, lane 4–5). Chloramphenicol had a general stabilizing effect on mRNA, making it difficult to reach a conclusive answer by this approach. Nevertheless, when the amount of mRNA between *tlpA* and *ompA* is compared at a temperature downshift, we see that *tlpA* mRNA is decreased 2-fold (Table 4) whereas *ompA* mRNA is decreased only 1.3-fold. This difference was reproducible in repeat experiments and indeed suggests that *tlpA* is able to exert repressive influence after

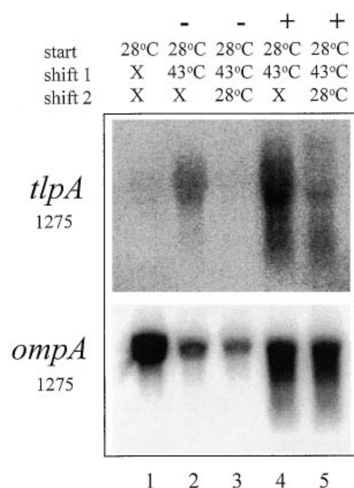

Figure 3. Northern Blot Analysis of *tlpA* mRNA at Temperature Shifts

Same panel of RNA samples was analyzed with either *tlpA* or the *ompA* (control) probe. 1275 was grown to exponential state at 28°C (start; lane 1), and the culture was divided into (+) and (−), indicating the addition of protein synthesis inhibitor chloramphenicol or the lack thereof. Incubation was continued for 30 min at 43°C (shift 1; lanes 2 and 4) and then for an additional 30 min at 28°C (shift 2; lanes 3 and 5). RNA was isolated immediately at the end of each 30 min incubation. X indicates that the sample was not incubated further.

Table 4. Induction Ratios Derived from Scanned Northern Blots of 1275 from Samples Taken at Different Temperature Shifts[a]

mRNA	→43°C	→28°C
tlpA (−)	+14.4[b]	−17.8[c]
tlpA (+)	+52.3	−2.2
ompA (−)	−7.8	−1.3
ompA (+)	−1.7	−1.2

[a] Strain 1275 was grown to the exponential phase at 28°C, culture was divided in two parts indicated by (+) and (−) signs indicative of addition of chloramphenicol or the lack thereof, respectively. Cultures were incubated in a static water bath at 43°C for 30 min and then at 28°C for an additional 30 min. Samples were withdrawn prior to and after each 30 min period.
[b] Signal in lane 1 used as a reference point.
[c] Signal in lane 3 (−) or lane 5 (+) used as a reference point.

the temperature challenge, even when the de novo repressor synthesis is blocked.

As Salmonella Are Shifted from 28°C into Physiological Temperatures of 37°C–42°C, Efficient Repression by TlpA Is Impaired

The above data indicate that TlpA is sensitive to temperature changes in the range 37°C–45°C. Is TlpA also sensitive in a lower temperature window, which is more physiologically relevant? To test this, 1275-1 transformed with the appropriate transcription fusions was grown at 28°C to exponential phase and then divided into four portions incubated for 1 hr at 28°C, 37°C, 39°C, or 42°C. Indeed, pRHTF01 was induced 2-, 2.7-, and 4.8-fold compared with values ranging from 1.3 to 1.6 for the promoter alone (Table 5). The values for two other controls, pOF11 and pOF70, ranged from 1.0 to 1.5, indicating they are not significantly induced by the heat treatment. The induction of pRHTF01 also shows a pattern where it is induced by increasing increments at each successively higher temperature, whereas other fusions do not display such a pattern (Table 5).

TlpA-Mediated DNA Binding Is Controlled by Temperature

The notion that TlpA senses temperature shifts should also be evident in in vitro experiments utilizing only the essential components. For this purpose, the highly purified protein and a 223 bp DNA fragment containing the target sequence (Hurme et al., 1996) were subjected to gel-mobility-shift assays (GMSA). In GMSA with TlpA,

Table 5. Induction Ratios for Transcriptional *lacZ* Fusions at Various Temperature Shifts[a]

Fusion Construct in 1275-1	28°C→37°C	28°C→39°C	28°C→42°C
pRHTF01	2.0 ± 0.2	2.7 ± 0.4	4.8 ± 1.6
pRHTF02	1.4 ± 0.1	1.4 ± 0.2	1.3 ± 0.2
pRHTF03	1.5 ± 0.2	1.6 ± 0.3	1.6 ± 0.4
pOF11	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
pOF70	1.4 ± 0.4	1.5 ± 0.4	1.2 ± 0.3

[a] Exponentially growing cell culture was shifted to the indicated temperature and incubated for 1 hr in a water bath before β-galactosidase activity was determined. Ratios are based on Miller Units. Standard deviation is indicated in each case (n = 4).

one should look for the disappearance of the free probe rather than a complex formation, because the elongated size of TlpA impairs the penetration of the gel matrix. This behavior is common to rod-like proteins, as demonstrated by their difficulty in entering the particles of a gel permeation chromatography matrix (Hodges et al., 1981). TlpA-mediated binding is nevertheless specific, since it competes only with DNA containing the target sequence and shows a distinct footprint in DNase I protection assays (Hurme et al., 1996).

TlpA was tested at three different monomer concentrations (0.66 μM, 0.33 μM, and 0.17 μM) and was subjected to three different temperatures (22°C, 37°C, and 43°C) at which differential behavior could be seen (Figure 4). At the highest protein concentration (Figure 4, lanes 2–4), binding is equal at 22°C and 37°C, but at 43°C not all of the probe is shifted (Figure 4, lanes 2–4). At the intermediate and the lowest TlpA concentrations, the effects are even more pronounced, showing less binding also at 37°C, and using the lowest protein concentration even at 22°C (Figure 4, lanes 7–9 and 12–14).

A specificity control was also included, which showed that the addition of excess unlabeled target leads to severely reduced binding (Figure 4, lanes 5, 10, and 15). Finally, to show that the higher temperature of 43°C does not irreversibly damage the function of this protein, a control was carried out in which TlpA was preincubated at 43°C and then allowed to cool to room temperature. At the higher concentrations, such a mixture also showed clear binding, demonstrating that TlpA is not irreversibly affected by heating (Figure 4, lane 6).

TlpA Conformation Is Temperature and Concentration Dependent

A number of proteins containing coiled-coil oligomerization domains can be described by a single two-state equilibrium between unfolded monomers and folded, highly α-helical coiled coils, as noted in the equation below:

$$nU \leftrightarrow F \tag{1}$$

The number (n) of monomers (U) in the coiled coil (F), is typically two to four (Lupas, 1996). Consequently, both the structure and stability of the folded coiled coil should display a concentration dependence. Since TlpA is a coiled-coil protein, it was obviously important to explore these characteristics. We were interested in whether conformational changes at temperatures used in GMSA and in experiments utilizing *lacZ* transcriptional fusions could be observed.

The CD spectrum of TlpA at 25°C (Figure 5A) is characteristic of a highly α-helical protein (Hurme et al., 1996). TlpA (2.3 μM monomer) was found to unfold cooperatively as temperature was increased from 25°C to 55°C as monitored by the loss in α-helicity measured by CD at 222 nm (Figure 5A). The presence of a well-defined isodichroic point (203.4 nm, $-4.57\ M^{-1}cm^{-1}$) in spectra recorded at different temperatures is strong evidence of a two-state equilibrium as described in Equation 1. The position and magnitude of this isodichroic point agree well with the mean values observed in a survey of 46 peptides and tropomyosins known to undergo a two-state helix-to-random-coil transition (203 nm, $-4.58\ M^{-1}\ cm^{-1}$; Holtzer and Holtzer, 1992). Reversibility of the thermal unfolding transitions was demonstrated by the recovery of 94% of the initial $\Delta\epsilon_{222}$ following cooling of the thermally unfolded protein to 25°C (Figure 5A). Subsequent thermal unfolding–folding cycles (Figure 5A, inset) displayed essentially 100% recovery. Using the α-helical content ($\Delta\epsilon_{222}$) as a probe for the native conformation, TlpA was found to have a marked concentration dependence (Figure 5B) as predicted by the proposed two-state equilibrium between unfolded monomers and highly helical, folded coiled coils (Equation 1). Due to instrument limitations, concentrations lower than 0.1 μM could not be analyzed precluding an accurate determination of the dissociation constant; however, the value must be below 0.1 μM, assuming the oligomer is a dimer (Figure 5B). The midpoint, T_m, of the thermal unfolding transition was also shown to be concentration dependent, with values of 42.4°C and 39°C at concentrations of 3.6 and 0.1 μM, respectively (Figure 5C), covering the range of concentrations used in GMSA (Figure 4). The relatively small change in T_m as a function of concentration is characteristic of long coiled-coil-containing proteins such as tropomyosin (Lehrer and Stafford, 1991) and is in stark contrast to that seen with

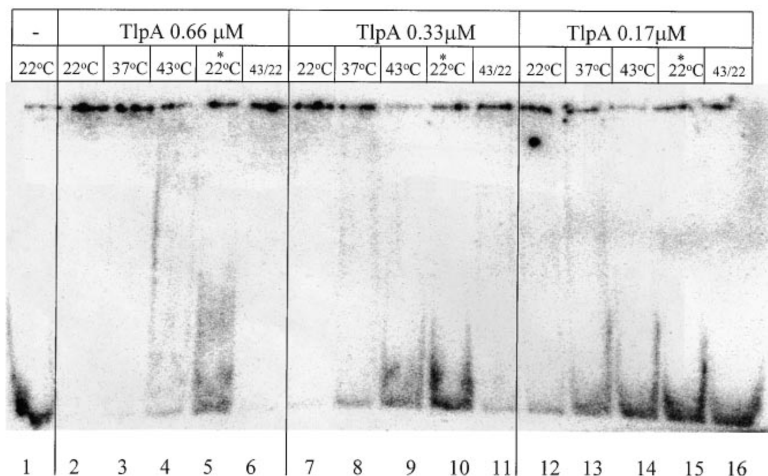

Figure 4. Gel Mobility Shift Analysis of TlpA Interaction with Target DNA as a Function of Temperature and Protein Concentration

Dash indicates no addition of protein and shows the mobility of the free probe. Temperatures at which each reaction was carried out are indicated. Indicated protein concentrations are for monomer TlpA. An asterisk marks the lanes where an excess of the cold target probe was added; all lanes included a poly dI-dC as a nonspecific competitor at 0.24 μg/μl. 43/22 denotes the sample which was kept at 43°C for 20 min and then let cool to 22°C before loading.

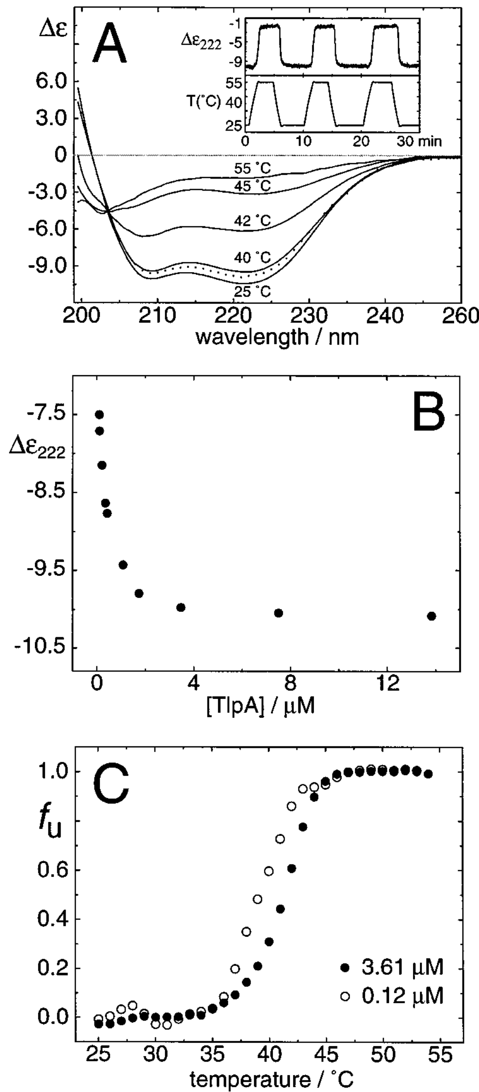

Figure 5. Conformational Analysis of TlpA Using CD Spectroscopy

(A) CD spectra, $\Delta\epsilon$ ($M^{-1}cm^{-1}$) versus wavelength, of TlpA (2.3 μM monomer) as a function of temperature (solid lines). The spectrum of the sample following cooling to 25°C is overlayed using a dotted line. Reversibility of the thermal unfolding transition of TlpA as monitored at a single wavelength ($\Delta\epsilon_{222}$) is demonstrated (inset) as temperature is varied in a stepwise linear fashion between 25°C and 55°C.

(B) Concentration dependence of the folded conformation of TlpA (0.1–13.8 μM) at 25°C.

(C) Concentration dependence of the thermal unfolding of TlpA. Values of $\Delta\epsilon_{222}$ were converted to f_u for graphical comparison; see Experimental Procedures for details. Every fourth point is displayed for clarity.

short coiled coils such as Fos and Jun (O'Shea et al., 1989b). Decreased DNA binding by TlpA is observed in GMSA at temperatures of 37°C and above (Figure 4), which is entirely consistent with the thermal unfolding behavior of TlpA, which indicated the protein is increasingly unfolded at these temperatures (Figure 5C).

Intracellular Concentration of TlpA

The intracellular concentration of TlpA in wild-type strain 1275 was determined by Western blotting of cells

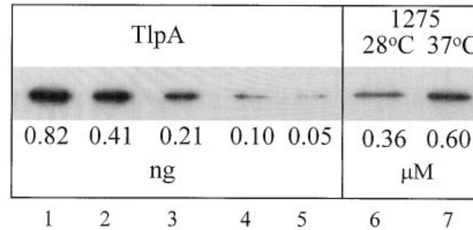

Figure 6. Intracellular Concentration of TlpA

A dilution series with the indicated amount of purified TlpA (lanes 1–5) was run in an SDS-PAGE along with total cell lysates of S. typhimurium 1275 grown at 28°C and 37°C (lanes 6 and 7). The gel was analyzed by Western blotting using TlpA antibodies and chemiluminescence detection. From the standard curve of pure TlpA, we derive the indicated intracellular concentration of TlpA as micromolar monomer (lanes 6 and 7). In lanes 6 and 7, 3.2×10^6 cells were loaded.

grown to the exponential phase at 28°C and 37°C (Figure 6). Dilution series of purified TlpA run in the same gel were used to construct the standard curve from which the intracellular concentration could be determined. From the blot, we estimate that in cells grown at 28°C, the intracellular monomer TlpA concentration is 0.36 μM and at 37°C, 0.6 μM, corresponding to 396 and 684 dimers per cell, respectively.

Discussion

Here we have shown that TlpA is a novel gene regulator with built-in thermosensing capacity. According to our model, the sensor–regulator activity of TlpA is based on a monomer-to-coiled-coil equilibrium, which represents a unique adaptation of the coiled-coil structure for the regulation of function.

The concentration dependence of TlpA conformation measured by CD spectroscopy was indicative of a cooperative system and is the expected behavior of a monomer-to-coiled-coil equilibrium. Moreover, CD spectra recorded as a function of temperature contained a well-defined isodichroic point, strongly supporting the reversible two-state model of unfolded monomer interconverting with the highly α-helical coiled coil. As expected in such a system, the midpoint of the thermal unfolding transition in TlpA is also concentration dependent. Based on this model system, the unfolded TlpA monomers are incapable of binding DNA, which explains the dramatically reduced activity seen in GMSA at higher temperatures. The temperatures where we see unfolding in CD and reduced DNA binding in GMSA are in the same range (37°C–43°C) and show that more unfolding leads to less repressor activity. Importantly, both GMSA and CD demonstrate that TlpA is not irreversibly denatured by high temperatures, and upon cooling both function and full α-helicity are regained. Collectively, the in vitro data draw a convincing picture of TlpA as a molecule dependent on both temperature and subunit concentration for its fully folded conformation, which in turn dictates the DNA-binding activity of this protein.

The in vitro behavior of TlpA was reproducible in its genetic background S. typhimurium, underscoring the temperature adaptability of this naturally occurring system. Transcription data showed that the *tlpA* promoter

did not respond to temperature, since *tlpA* is constitutively active as long as TlpA repressor is not available. Utilizing the deletion mutant of TlpA, dTlp5, which is unable to bind DNA, we saw constitutive *lacZ* activity and protein accumulation. This is direct evidence that the TlpA protein is the key mediator of this system. Furthermore, the control experiments on supercoiling or heat shock involvement lead us to believe that the mechanism of thermoregulation of *tlpA* need not involve any accessory functions or conditions other than temperature.

We suggest here that the molecular basis of TlpA action that determines the degree of promoter repression is the temperature- and concentration-dependent equilibrium between unfolded (monomer) and folded (coiled-coil) TlpA. The outcome of TlpA–promoter interaction can lead to one of the following three events: repression, partial repression/derepression, or derepression. The repressed state is maintained at maximal efficiency if the ambient temperature is below the T_m of the TlpA concentration present in the cell. TlpA is stable to about 35°C as measured by CD, which correlates well with in vivo transcription data. At 28°C, we find an intracellular concentration of 0.36 μM TlpA in the wild-type cell, which probably represents the lowest concentration in Salmonella required for efficient repression. A handful of TlpA molecules would not suffice to bring about the repression, since then the monomer-to-coiled-coil equilibrium would favor the nonfunctional monomer. Thus, finding a relatively large quantity of ∼400 molecules of TlpA at 28°C and close to 700 at 37°C in Salmonella is explained by the need to have a sufficient quantity of folded repressor required by the concentration-dependent oligomerization. Subjecting Salmonella transformed with *tlpA::lacZ* fusion, pRHTF01, to elevated temperatures by shifting the culture from 28°C to 37°C, 39°C, or 42°C, showed a gradient of increasing transcription derepression indicative of partial repression/derepression. Indeed, from 35°C–44°C, TlpA is increasingly unfolded, which explains the increased transcription. In this temperature range, as more TlpA is produced, the increased monomer concentration shifts the equilibrium toward folded coiled coils, thus increasing repression. The shift toward folded oligomers raises the T_m of the system, thereby increasing the number of folded and functional molecules. At and above 45°C, regardless of TlpA concentration, a total derepression ensues, owing to the lack of functional repressors. At such temperatures, TlpA is overproduced "in vain," since as long as such a high temperature is maintained, the protein is unable to fold into a functional repressor. A temperature downshift will then establish repression quite efficiently, owing to the excess of protein.

Virulence plasmids of Salmonella are quite heterologous in size and content but carry at least one conserved region, the *spv* gene cluster, which is needed to confer a growth advantage while deep in host tissue (Gulig et al., 1993). All plasmid-carrying serovariants of Salmonella analyzed by us to date contain the *tlpA* gene and express its protein product, indicating that *tlpA* is also conserved. An intriguing finding is the sequence homology between *tlpA* promoter and various Salmonella promoters (data not shown). Qi et al. (1995) have recognized a motif ($TGTTN_{8–12}TGTT$) in promoter regions of genes activated in response to bactericidal proteins. We have extended that homology (Qi et al., 1995) search and found this motif in *tlpA* promoter region and in several de facto virulence genes of Salmonella. We considered as significant only those entries that had the completely matching sequence localized to regions known to, or predictably involved in, regulation of the adjacent coding gene. This strategy produced nine matches. Of these nine genes, four (*pagC*, *prgH*, *orgA*, and *spvA*) are involved in virulence functions (Foster and Spector, 1995; Galán and Bliska, 1996; Jones and Falkow, 1996), and two (*bipA* and *envM*) are induced in response to bactericidal agents (Qi et al., 1995; Groisman, 1996). Interestingly, thus far no reports have emerged that would implicate temperature as an inducer signal in Salmonella virulence. It is possible that the above genes have a global, as-yet-unidentified regulatory factor in common that recognizes the TGTT motif in various contexts. It is equally plausible that TlpA could be involved in direct regulation of these genes, e.g., as an accessory repressor part of the complex and multifaceted regulation of Salmonella virulence. Indeed, the intracellular concentration of TlpA places the equilibrium in a range rendering it most sensitive to regulation by temperature. This suggests that TlpA has evolved to work at temperatures characteristic to target hosts of salmonellosis and is evidence that upon entry into a host organism, TlpA will undergo some degree of derepression. In this context, we speculate that TlpA could sense the entry into the warm host organism and relieve its accessory repressor control over *orgA* and *prgH*, which are critical in the initial stages of infection, whereas genes involved in later stages, such as *spvA* and *pagC*, could be relieved from TlpA action at the onset of host entry or upon a further temperature cue when the infected organism experiences fever. Also, it is important to note that certain natural hosts for virulent Salmonella have body temperatures higher than, e.g., a human host organism (37°C), which could cause increasing levels of derepression (analogous to effects depicted in Table 5); for example, cows (38.5°C), sheep (39.1°C), pigs (39.2°C), or chickens (41°C). Current efforts are aimed at elucidating the putative link between *tlpA* and the above genes.

The DNA-binding activity of an elongated coiled coil, like TlpA, is not unheard of in prokaryotes or eukaryotes (Jagura-Burdzy and Thomas, 1992; Niki et al., 1992; Wang et al., 1996). The uniqueness of TlpA stems from the capacity to modify its gene regulatory activity by responding to temperature changes. The only protein that can be compared to TlpA in function is the eukaryotic heat shock transcription factor (HSF). In vitro studies with the purified protein show that trimerization into a DNA-binding competent form can be induced by temperature (Goodson and Sarge, 1995; Larson et al., 1995). Both TlpA and HSF contain coiled-coil domains, although their function is quite different: in the latter, the intramolecular form is transformed via heating into a coiled-coil trimer (Peteranderl and Nelson, 1992; Zuo et al., 1994), whereas TlpA is constitutively DNA binding at physiological conditions and regulates its activity through the effect of temperature on the monomer-to-coiled-coil equilibrium. Also unlike TlpA, the HSF system is complex and requires the interplay of different domains and association with hsp70, which is likely to be necessary for exerting the full in vivo function (Wu, 1995).

Table 6. Strains Used in this Study

1275	wild-type isolate of S. typhimurium[b]
1275-1	*tlpA*$^{-}$, manufactured by allelic replacement, Km^{r} [a]
1275-2	recipient 1275; P22 donor lysate TT15266 pyrE2419::MudQ[c]
1275-3	recipient 1275; P22 donor lysate TT1709 zgf-1716::MudQ[c]
1275-4	recipient 1275; P22 donor lysate TT15240 putA1019::MudP[c]

[a] This study.
[b] Baird et al., 1985.
[c] Benson and Goldman, 1992.

Very diverse roles have been attributed to the coiled-coil motif, which is present in numerous proteins. These roles range from homo- and heterodimerization and positioning of DNA-binding heads to serving an architectural yet dynamic role in the eukaryotic cytoskeleton or acting in virus-eukaryote membrane fusion (Cohen and Parry, 1994; Oas and Endow, 1994; Lupas, 1996). We believe TlpA represents a novel adaptation of the coiled-coil motif to function, namely the coupling of activity with the protein-folding equilibrium in response to temperature cues to achieve dynamic regulation of transcription repression.

Experimental Procedures

Plasmid Constructs, Allelic Replacement

Methods for DNA manipulation and transformation have been previously described (Sambrook et al., 1989). All enzymes were used as suggested by the manufacturers (Boehringer Mannheim; New England BioLabs). *tlpA-lacZ* transcription fusion plasmids pRHTF01–03 were based on a previously described vector pOF (Hurme et al., 1996), which was modified by cloning into ClaI-HindIII sites the ribosomal RNA terminators, which were PCR amplified from pKK223–2 (Pharmacia) to generate vector pRHTFV. HindIII-BamHI fragment containing *tlpA* from pMR11 or pMR12 was cloned into Bluescript SK+ (Stratagene). From the resultant plasmids, the inserts were cut out as HindIII-BamHI fragments before ligation into the corresponding cloning sites of vector pRHTFV to generate pRHTF01 and pRHTF03, respectively. Insert for pRHTF02 was cloned from pMR11 as a SmaI-PstI fragment into SK+, from where it was recovered as a HindIII-BamHI into pRHTFV. pOF70 has been described previously as pHUB70 (Rhen et al., 1993); pOF11 is pRHTFV without the transcription terminators. Allelic replacement in strain 1275 to generate 1275-1, where *tlpA* genes from 5′ SmaI to XhoI are replaced by a kanamycin-resistance block from pUK4 (Pharmacia), was achieved using the pMAK700 plasmid with a temperature-sensitive replicon according to published protocols (Hamilton et al., 1989). All plasmids were transformed into Salmonella by electroporation. Strains 1275-2, -3, and -4 were generated by P22 HT *int* generalized phage transduction (Schmieger, 1972) from donor strains that are MudP and MudQ insertion mutants in S. typhimurium (Benson and Goldman, 1992), described in Table 6, into the recipient 1275.

Western Blotting

Equivalent number of cells, as measured by OD_{600}, were suspended in SDS-PAGE sample buffer. Gels were loaded with 1.45×10^5 cells per lane of the 12% SDS-gel in figure 2A, whereas in figure 2B, the gel was loaded with 2.22×10^6 cells per lane. Proteins were transferred onto Immobilon-P PVDF membranes (Millipore) as suggested by the manufacturer. Probing was done with TlpA affinity-pure antibodies at 0.3 μg/ml (antiserum was purified on cyanogen bromide-activated sepharose-coupled TlpA [Pharmacia]) and 1:8000 diluted CAT antiserum (5′-3′ Prime Inc). Western blotting was carried out using the chemiluminescence kit according to the manufacturer's instructions (Boehringer Mannheim). Hyperfilm ECL (Amersham) was used for the blots with various exposure times, ranging from 15 s to 2 min. Films were scanned with a Molecular Dynamics, Inc., densitometer and were quantitated by Image Quant Program. Several dilutions of the antigens and antibodies were tested in order to ensure the linearity of the signal.

GMSA

A 223 bp fragment containing the target sequence was available from our previous work (Hurme et al., 1996). DNA fragments for the mobility-shift assays were end-labeled with ATP (Amersham) using polynucleotide kinase (Boehringer Mannheim), following published protocols (Sambrook et al., 1989). GMSA proteins stored in 50 mM phosphate, 0.15 M NaCl (pH 7.0) were mixed with target DNA in a binding buffer (2 mM $MgCl_2$, 5 mM NaCl, 6.5% glycerol, 2.4 μg poly dI-dC, 10 mM DTT in 4 mM Tris [pH 8.0]) in a final volume of 10 μl. NaCl was adjusted to 150 mM. Purified TlpA protein was available from previous work (Hurme et al., 1996) and used as indicated. DNA was at 3.0 ng per reaction, while competitor DNA containing the target sequence was used at 300 ng per reaction. Protein–DNA mixes were preincubated for 20 min at a given temperature, and then the labeled target was added for an additional 20 min before the gel was loaded. Samples were run in a 4% polyacrylamide (29:1 acrylamide:bis) gel cast in 1 × TBE (50 mM Tris, 50 mM borate, 1 mM NaEDTA [pH 8.3]). Gels were prerun in 1 × TBE for at least 3 hr until the current remained constant at 10–12 mA. Gels were dried and analyzed using phosphorimaging technology (Molecular Dynamics, Inc.).

Transcriptional Fusions

Strains carrying the appropriate fusion were grown in Luria broth supplemented with 30 μg/ml chloramphenicol. Single colonies were inoculated in growth media and then split into two or more separate vessels and grown to the exponential phase, then aliquoted and placed at different temperatures for 1 or 2 hr on a heating block or a water bath. Fusion activity was assayed according to Miller (1972) and expressed as Miller Units. Each experiment was repeated at least four times.

RNA Isolation and Northern Blotting

Messenger RNA was isolated from S. typhimurium 1275 grown to an OD_{600} of 1.2–1.5 according to a previously published protocol (von Gabain et al., 1983). Northern blotting was carried out according to published protocols (Ausubel et al., 1997). Probes were PCR amplified from a total DNA preparation from 1275; *tlpA* fragment was amplified with primers to residues 15–40 and 540–560 in the *tlpA* gene (accession #M88208), whereas *ompA* (accession #X02006) was amplified with primers to nucleotides 51–70 and 881–861 in the *ompA* sequence. Both were purified from agarose gels with Gene Clean II kit (BIO101) and labeled with [α^{32}P]dCTP by random priming, using the Prime-It-II Kit (Stratagene). 1275 was grown to the logarithmic state at 28°C and divided into two tubes, each containing 2.5 ml of culture. The (+) tube included 5 μl of chloramphenicol (10 mg/ml in methanol), whereas the (−) tube included only 5 μl of the solvent. The "zero" sample was taken prior to the addition. Samples were incubated for 30 min in a 43°C water bath, and 1.2 ml samples were withdrawn for immediate RNA isolation. After an additional 30 min at 28°C, a second set of samples was withdrawn. Gels were loaded with 10–13 μg of total RNA. Transfer was onto Hybond+ (Amersham) filters. Filters were incubated in phosphorimager cassettes for 2–48 hr, and the signal was quantitated with Image Quant program (Molecular Dynamics, Inc.). Probed filters were stripped in boiling 0.1% SDS and reprobed with either *tlpA* or *ompA* probes as a measure of reproducibility between gels.

Circular Dichroism Spectroscopy

CD spectra were recorded using an Aviv 62 DS spectropolarimeter. CD spectra of wild-type TlpA were recorded in 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl and 10 mM DTT. Protein concentrations were determined by amino acid analysis (Mora et al., 1988) of known aliquots of protein solutions. Thermal denaturation of *tlpA* was monitored by CD at 222 nm ($\Delta\epsilon_{222}$) as the temperature was increased in 0.2° steps, allowing a 5 min equilibration at each step before recording the data. For purposes of presentation, $\Delta\epsilon_{222}$ was converted into fraction of unfolded protein (f_u) using the relationship $f_u = [(\Delta\epsilon_F + m_F T) - \Delta\epsilon_{Exp}]/[(\Delta\epsilon_F + m_F T) - (\Delta\epsilon_U + m_U T)]$, assuming a

standard two-state equilibrium between unfolded monomeric TlpA and helical, folded oligomeric protein (Pace et al., 1989). Here $\Delta\epsilon_F$, $\Delta\epsilon_U$, and $\Delta\epsilon_{Exp}$ represent the values of the fully folded, the fully unfolded, and the experimentally determined CD. The values m_F and m_U represent the slopes of the pre- and posttransition regions. Data for two concentrations was fit independently for these variables. The midpoints of thermal denaturation curves T_m were obtained by the maximum of the first derivative of a plot of $\Delta\epsilon_{222}$ as a function of temperature.

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