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Analysis of Temperature Sensing in *Yersinia pestis*: A Dissertation

Nancy Palme Hoe
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ANALYSIS OF TEMPERATURE SENSING IN YERSINIA PESTIS

A Dissertation Presented

By

Nancy Palme Hoe

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of:

DOCTOR OF PHILOSOPHY IN MOLECULAR GENETICS AND MICROBIOLOGY

JANUARY, 1994

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ANALYSIS OF TEMPERATURE SENSING IN YERSINIA PESTIS

A Dissertation Presented

By

Nancy Palme Hoe

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January 28, 1994
DEDICATION

This work is dedicated to my mother, Hansell Palme and to the memory of my father, Richard Palme.
ACKNOWLEDGEMENTS

I would like to sincerely thank my advisor Jon Goguen for all the time, patience, guidance and considerable computer and automobile expertise he has afforded me during my graduate studies. I would also like to express my gratitude to the members of my thesis committee for their efforts on my behalf, and the faculty in the Department of Microbiology and Immunology at the University of Tennessee Medical School in Memphis for starting me down this road. Thanks also go to past and present members of the lab: Ola Sodiende, Chris Minion, Ellen Nalavaika, and Subramanyam Yerrimili, and to my friends Phyllis Spatrick, Dimitri Blinder, Pat Stow, Carolyn Shaw, and the members of The Blue Room (you know who you are). Thank you all for your encouragement, technical expertise and kind words in times of trouble. Many hugs and kisses go to Hansell Palme (a/k/a Mom) and Pat and Joe Hoe for their love and financial support, and of course this work could not have been undertaken or sustained without the beautiful marvel who is my son Bradley, or the innumerable sacrifices and saintly qualities of my wonderful husband Joe. Thanks guys.
ABSTRACT

The lcrF gene of Yersinia pestis, the etiological agent of plague, encodes a transcription activator responsible for inducing expression of several virulence-related proteins (Yops) in response to temperature. The mechanism of this thermoregulation was investigated. Using a yopE::lacZ reporter fusion, lcrF-mediated thermal regulation was observed in Y. pestis and Escherichia coli. The lcrF gene was sequenced, the 30.8 kDa. LcrF protein identified and purified, and LcrF-dependent yopE-specific DNA binding activity was detected. A sequence similarity search revealed that LcrF exhibits 98% homology to VirF of Yersinia enterocolitica and significant homology to the carboxy termini of other members of the AraC family of transcription activators. During localization studies, a significant proportion of LcrF was found associated with the membrane fraction in E. coli. However, pulse-chase experiments indicated that this result is an artifact of fractionation. lcrF-mediated thermal induction of the yopE::lacZ reporter fusion remains intact in a Shigella flexneri virR mutant. The virR mutation is known to affect thermal induction of Shigella virulence genes, which are also controlled by an activator in the AraC family.

As a first step toward identifying the temperature-sensitive step in the regulation of yop expression, lcrF::lacZ transcriptional fusions were constructed and analyzed in Y. pestis and E. coli. The activity of the fusions was not affected by the native pCD1 virulence plasmid, an intact lcrF gene, or temperature. Thus, induction of lcrF transcription is not essential for temperature-dependent activation of yopE transcription. To confirm these
results, attempts were made to identify both the native \textit{lcrF} message in \textit{Y. pestis}, and a \textit{lcrF-lacZ} hybrid message in \textit{Y. pestis} and \textit{E. coli}. These attempts were unsuccessful. Examination of LcrF protein production revealed temperature-dependent expression in \textit{Y. pestis}. Surprisingly, high-level T7 polymerase-directed transcription of the \textit{lcrF} gene in \textit{Escherichia coli} also resulted in temperature-dependent production of the LcrF protein. Pulse-chase experiments showed that the LcrF protein was stable at both 26 and 37°C, suggesting that translation rate or message degradation is thermally controlled. Comparison of the amount of LcrF protein produced per unit of message at 26 and 37°C in \textit{E. coli} indicated that the efficiency of translation of \textit{lcrF} message increased with temperature. mRNA secondary structure predictions suggest that the \textit{lcrF} Shine-Dalgarno sequence is sequestered in a stem-loop. A model in which decreased stability of this stem-loop with increasing temperature leads to increased efficiency of translation initiation of \textit{lcrF} message is presented.
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CHAPTER I

INTRODUCTION
Yersinia pestis, the causative agent of plague, depends primarily on fleas for transmission between mammalian hosts in nature. Efficient adaptation to this life cycle requires that Y. pestis distinguish between the flea and mammalian environments, and acclimatize to them by expressing the proper combinations of genes. In particular, it is critically important that the machinery required for resistance to mammalian anti-bacterial host defenses be rapidly brought into action following injection of the bacteria into mammalian tissue as an infected flea attempts to take blood. The change in temperature which occurs as the bacteria move from the relatively cool environment of the flea into the warm mammalian environment is one signal which could reasonably be used to trigger the needed response, and there is strong evidence that this signal is exploited for this purpose. In fact, the specific induction of virulence-related genes in response to increased temperature has been observed not only in Y. pestis but also in a variety of other pathogenic bacteria, indicating that temperature-sensing and transduction of the temperature signal to the level of gene control is a common adaptation among pathogens. In several pathogenic species, it has been determined that increased production of specific proteins at elevated temperature is due to increased transcription mediated by the action of one or more transcriptional activators (20, 79, 87). Most of this work has centered on the effect of temperature on expression of these regulatory proteins (see below). Less progress has been made toward the unambiguous identification of the 'thermometer', the bacterial component which serves as the primary temperature sensor, and complete discovery of the pathway by which the temperature signal is transmitted from this sensor to the level of gene expression. These problems have been the focus of my research.
Regulation of virulence gene expression in the Yersiniae

In 1957, Fukui, et al. (37) reported that virulent strains of *Yersinia* (then *Pasteurella*) *pestis* would maintain their virulence during serial passage in liquid media at 26°C but not at 37°C. This loss of virulence at high temperature was caused by overgrowth of the culture with avirulent bacteria as the virulent forms entered stasis several hours after inoculation from solid media (37, 74). It was soon discovered that growth of virulent strains would occur at 37°C only in the presence of calcium (first observed as growth in defined media with skim milk) and that calcium was not required by avirulent strains at high temperature, or by virulent strains at low temperature (57, 74). Under alkaline conditions (pH 7.8), ATP could substitute for Ca++ (159).

Further characterization of the observed growth restriction has revealed that cultures of virulent strains, when switched from 26°C to a higher temperature (34-40°C) in the absence of added CaCl₂, cease DNA replication after two generations of growth, (approximately 3-4 hours after the temperature shift) and subsequently halt stable RNA synthesis (154, 160). The levels of ppGpp do not increase as occurs during metabolic shutdown induced by amino acid starvation (17, 160), and shifting cultures down to 26°C results in restoration of growth. In contrast, CaCl₂ cannot restore growth at 37°C even when it is added immediately after the onset of growth restriction (160). Maximal expression of several virulence-related proteins known as Yops (for *Yersinia* Outer membrane Proteins), also occurs under growth-restrictive conditions (130). The phenotype of growth restriction at 37°C in the absence of added CaCl₂ with concomitant production of Yops has been termed the low-calcium response or LCR (42).
The discovery that all species of *Yersinia* known to cause disease in man (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) contain a plasmid whose presence correlates with the expression of the LCR (5, 103, 130) led to the search for genes controlling the LCR phenotype. Portnoy (103) initially characterized three LCR$^-$ Tn5 insertions in the *Y. pestis* virulence plasmid pYV019. Subsequent Mu d1(Ap$^+$ lac) mutagenesis of the virulence plasmid pCD1 of *Y. pestis* strain KIM produced several LCR$^-$ mutants which clustered in a 17 kb region of the plasmid (42). The mutants were able to grow at 37°C without added CaCl$_2$ and were avirulent for mice. Analysis of β-galactosidase production by the insertion mutants revealed that the expression of affected loci is induced 2-11 fold by temperature, and is insensitive to the addition of Ca$^{2+}$ and ATP. Three distinct transcription units, the *lcrA*, *lcrB*, and *lcrC* loci, were identified.

The significance of the calcium requirement for growth in vitro at 37°C remains a mystery and may not accurately reflect events in vivo, as it is clear the bacteria do not restrict their growth in mammals. Several of the Yops have been identified as being required for virulence (8, 33, 80, 104, 129), and maximal expression of *yop* genes occurs at 37°C in media depleted of Ca$^{2+}$. Although the calcium concentration within a phagolysosome is unknown, it has been proposed that these conditions mimic the mammalian intracellular milieu implying that the presence of bacteria within cells may be essential to the expression of virulence factors. In support of this hypothesis, *Y. pestis* is observed to grow within phagolysosomes of mouse peritoneal macrophages (131). However, the importance of intracellular growth of *Y. pestis* during infection is questionable. Goguen et al. observed that plasmid+ but not plasmid-
Y. pestis pre-incubated at 37°C in the presence of calcium were cytotoxic for a mouse macrophage cell line and resisted phagocytosis (41). In addition, strains of Y. pseudotuberculosis containing the virulence plasmid pIB1 are more resistant to phagocytosis than plasmidless strains, a characteristic dependent on the presence of the yopH gene, whose gene product is also required for virulence (109). Inhibition of phagocytosis is maximal when the bacteria are pre-incubated at 37°C in the absence of calcium, but is also observed when they are pre-incubated in calcium-containing media (109). These results indicate that expression of at least some plasmid-dependent virulence-related activities is not dependent on exposure of the bacteria to the intracellular environment, and that expression of these activities actually inhibits intracellular localization. There are now several new lines of evidence which may help explain these phenomena and their relationship to the observed calcium-dependent growth restriction in vitro. Experiments suggest that YopE is a cytotoxin which may mediate observable alterations in the cytoskeleton upon direct introduction into HeLa cells (110). YopH was initially identified through sequence homology to be a tyrosine phosphatase and this activity has been confirmed biochemically (48). A strain of Y. pseudotuberculosis expressing YopH was found to be responsible for the de-phosphorylation of several cytoplasmic host-cell proteins in a murine cell line (7). These observations suggest the following model: association of the bacteria with the host cell membrane triggers the expression of the Yops which are then exported directly into the host cell, disrupting cell functions required for antibacterial activity. (This notion of direct export of Yops into host-cell cytoplasm was first suggested by H. Wolf-Watz. At present direct evidence for this phenomenon has not been published, but is expected in
the near future.) The region of the bacterial membrane in contact with the mammalian cell membrane may receive the equivalent of a local, rather than global, low calcium-like signal, inducing a response sufficient to poison the mammalian cell, but insufficient to inhibit bacterial growth. If this model proves to be correct, the low calcium response as observed in vitro is an artifact caused by over-stimulation of Yop synthesis and secretion.

Whatever the physiological role of calcium is in inducing the LCR, it is important to note that Ca\(^{++}\) is required for growth only at temperatures above 34°C. Below this temperature, cells grow normally in the absence of Ca\(^{++}\) indicating that temperature has a dominant effect on LCR expression. In the initial analysis by Goguen, et al. (42), the fact that mutations within the lcrA, lcrB, and lcrC loci were LCR\(^{-}\) but still exhibited temperature-dependent increases in transcription indicated that the regulatory loci controlling temperature induction had not been detected. To indentify such loci, Yother et al. first isolated plasmid mutants unable to grow at 37°C even in the presence of Ca\(^{++}\), and then selected second-site suppressors relieving the growth defect (157). The bulk of these suppressors were found to be insertions within a plasmid locus designated lcrF. Genetic evidence suggested that lcrF was a positive regulator of temperature induced yop gene expression (157).

*virF*, the lcrF homologue in *Y. enterocolitica* (98% sequence identity), was subsequently identified by Cornelis, et al. and found to encode a 31 kDa protein with homology to a family of proteins including AraC, the regulator of the *E. coli* arabinose operon, and VirF of *Shigella flexneri* 2a (named for the SalI F fragment containing it and not due to any relation to the *Y. enterocolitica* virF gene-see below) (21). Transcripts of *virF* can be detected by Northern blotting
only in cells grown at 37°C (21), and a 20-fold increase in the expression of chloramphenicol acetyltransferase from a \textit{virF::CAT} operon fusion at 37°C as compared to 25°C has been demonstrated (23). Both of these results suggest that \textit{virF} transcription is thermally regulated. Chromosomal mutations in \textit{Y. enterocolitica} which result in an increase in \textit{virF} and \textit{yop} transcription at low temperature led to the discovery of the \textit{ymoA} gene. The \textit{YmoA} protein resembles histone-like proteins involved in maintaining the bacterial chromosome in a compact structure (23). Although transcription of \textit{virF} increases at 25°C in a \textit{ymoA} mutant as compared with wild type, a further increase is observed when the temperature is elevated to 37°C, indicating that a temperature-sensing mechanism controlling \textit{virF} expression remains intact when \textit{ymoA} is inactivated. Despite the observed thermal regulation of \textit{virF} transcription, IPTG-dependent induction of \textit{virF} transcription at 25°C via use of a Ptac-\textit{virF} fusion does not result in significant expression of Yop proteins (76). This observation indicates that thermal control of \textit{virF} transcription cannot wholly explain the thermal induction of \textit{yop} expression: either additional genes or post-transcriptional thermal control of VirF activity or abundance of VirF must be involved.

Much of the data presented in this dissertation addresses the role of the \textit{Y. pestis lcrF} gene and its product in the thermal control of \textit{yop} expression. Experiments detailed in Chapter II support the hypothesis that post-transcriptional thermal regulation of cell-specific LcrF activity plays a crucial role in this process: an \textit{lcrF::lacZ} fusion construct--residing in \textit{E. coli} to exclude the presence of other \textit{Y. pestis} specific gene products--regulates transcription of a \textit{yop} reporter fusion even though its own transcription does not change
appreciably with temperature. Experiments presented in Chapter IV identify translation of the LcrF protein as the temperature-sensitive step in regulation of yop transcription.

The remainder of this Introduction is devoted to an overview of systems in which the regulation of gene expression by temperature has been extensively investigated. At present, the mechanism of regulation has not been clearly defined in any of these systems. Available data imply that several distinct mechanisms may be involved, but also suggest that a temperature-dependent post-transcriptional event controlling the expression of a central regulatory protein may be a common mechanism of thermal regulation.
Temperature-dependent expression of virulence factors in other pathogens

**The Shigellae.** As in the yersiniae, the major virulence determinants for members of this genus (the ability to invade intestinal epithelium, and the ability to mediate cell-to-cell spread of the bacteria) are expressed preferentially at elevated temperature, and are encoded by a large (230 kb) plasmid (115). Temperature-dependent transcription of the *S. flexneri* genes coding for invasion antigens (*ipa*), and the genes responsible for inter- and intracellular spread (*ics*) is directly mediated by the action of the product of the *virB* gene (1). Transcription of *virB* and possibly *virG* (whose gene product is involved in intercellular spread) is dependent on the product of the *virF* gene (139). The amount of *virF*-specific message has been shown to increase approximately 4-fold upon a temperature shift from 30°C-37°C. However, as with *lcrF* and its homologues in the yersiniae, IPTG-induced transcription of a ptac-*virF* fusion causes increased transcription of a target gene, in this case *virB*, at 37°C but not at 30°C (139). The possibility that VirF synthesis is regulated post-transcriptionally was suggested as a potential explanation for these observations. This hypothesis has yet to be tested.

A search for mutants derepressed for the expression of virulence factors at 30°C identified the chromosomal gene *virR* which is equivalent to the *hns* gene of *E. coli* (64, 88). VirR is thought to act as a repressor of virulence gene transcription at 30°C, and it has been proposed that the synthesis of VirR protein is regulated with temperature (62). Surprisingly, recent attempts to complement *virR* deletion mutations revealed partial complementation by increased dosage of the gene for tRNA{\textsubscript{1}}Tyr. It is unclear at present if this result reflects lowered
translation of virulence gene mRNAs, or modulation of the expression of another, as yet uncharacterized, regulatory locus (62).

*Escherichia coli.* In strains of uropathogenic *E. coli*, expression of the chromosomally-encoded pyelonephritis-associated pili (pap) operon is thermally regulated such that pili are observed on bacteria grown at 37°C, but not on those grown at 25°C (46). The gene for the regulatory protein PapB, and the gene for the major subunit of pilin, PapA, are cotranscribed from a promoter upstream of the *papB* gene (3). Another regulatory gene, *papI*, is upstream of, and divergently transcribed from, *papBA* (3, 44). Transcription of *papI* is induced at 37°C, and IPTG-dependent transcription of *papI* at low temperature increases *pap* operon expression to the 37°C level (44). Thus, *papI* transcription appears to be an important step in the thermal induction pathway. The effect of temperature on *papB* expression is less clear. This gene is required for maximal expression of both the *papI* and *papBA* operons at 37°C (44). It also binds to several sites within the intercistronic region between *papI* and *papBA* and is thought to act by inducing *papI* expression (144, 35). IPTG-dependent overexpression of *papB* does not induce *papBA* transcription at low temperature and, in an early experiment, also failed to increase transcription of *papBA* at 37°C (44). However, in a subsequent experiment, induction of *papBA* was observed at 37°C when lower concentrations of IPTG were used, indicating that *papB* mRNA can be translated and that PapB protein can be an effective inducer at this temperature (35). Unfortunately, a similar experiment was not done at the low temperature, leaving open the possibility that PapB translation or activity is the primary thermally-controlled event. Clearly, the effect of
temperature on translation of papB mRNA and on the specific activity of PapB protein should be examined.

In the search for other genes affecting thermal control of pap pili synthesis, mutations within a locus designated drdX were found to result in constitutive expression of pap operon genes, evoking the hypothesis that the product(s) of the drdX locus might act to 'silence' transcription at low temperatures (45). As is the case for virR of Shigella ssp., drdX is also equivalent to hns, the gene for the H-NS protein (45, 64). An independent search for mutations resulting in constitutive expression of the pap operon identified rimJ which encodes the N-terminal acetylase of the ribosomal protein S5 (153). rimJ mutants have not been characterized sufficiently to permit a mechanistic explanation of this observation.

**Bordetella pertussis.** The etiological agent of whooping cough expresses several virulence factors during the course of infection. These include a filamentous hemagglutinin (encoded by the fhaB gene) mediating adhesion to ciliated epithelium of the upper respiratory tract, and several toxins, two of which, the pertussis toxin (ptx) and adenylate cyclase toxin-hemolysin, have been studied most extensively (150). Expression of these factors has been found to be modulated by temperature such that expression is repressed at 25°C and induced at 37°C. The expression and environmental modulation of these genes is controlled by the bvgAS operon which encodes two proteins, the cytoplasmic membrane protein BvgS and a cytoplasmic protein BvgA. These are homologous to the sensor and receiver modules, respectively, of the two-component regulatory family of signal transducers (111). BvgA controls the
transcription of the 
flaB gene, and is required for the production of pertussis 
toxin and adenylate cyclase (111). However, DNA-binding assays fail to detect 
any BvgA binding to sequences upstream of the 
ptx or adenylate cyclase genes, and 
ptx transcriptional activity is not observed in 
E. coli when the 
bvgAS operon is present in trans (111). Two possibilities have been presented to explain the latter result. The discovery of another protein in 
B. pertussis that binds to 
unique sites upstream of the 
ptx and adenylate cyclase promoters and that is 
distinct from BvgA suggests a regulatory cascade like that seen in Shigella (63). Indeed, the levels of 
flaB message in 
B. pertussis increase immediately after a 
switch from 25°C to 37°C, whereas 
ptx and adenylate cyclase message levels do 
not start increasing until 2 hours after the switch (116). In contrast, Scarlato et al. observe temperature-dependent transcription of the 
ptx gene in 
E. coli when the 
bvgAS operon is present in cis (117). It has been suggested that the absence of 
ptx transcription when the 
bvgAS operon is present in trans is due to 
inhibition of 
ptx promoter activity by local alterations in superhelical density, and that these perturbations are caused by transcription from nearby promoters within the vector sequences. Modulation of 
ptx transcription by 
gyrase and 
topoisomerase in an in vitro transcription assay utilizing 
E. coli RNA polymerase has been offered as evidence in support of this hypothesis (117).

The levels of the BvgA and BvgS proteins have also been examined at various times following a temperature shift from 25°C to 37°C. During the first six hours following this treatment, the amount of BvgA protein increases 60-fold, while the amount of BvgS increases only four-fold (116). Recently, mutant alleles of 
bvgS which confer constitutive expression of the 
bvg-related virulence genes have been described (92). These mutants show that BvgS is a component
of the thermal induction pathway, but do not unambiguously define the thermo-sensing element (92). If BvgS is the thermometer, signal transduction could occur via a conformational change in BvgS either directly effecting the BvgA protein or directly affecting expression of the $bvgA$ gene (92). In light of the membrane location of BvgS, I offer one hypothesis for a temperature-responsive parameter that might control BvgS activity: membrane composition. In order to maintain membrane fluidity within narrow limits, the degree of saturation of membrane lipids is rapidly increased by the bacteria as temperature increases (86). Thus, activation of BvgS by lipids with a high proportion of saturated fatty acid moieties could explain the observed regulation. One proposed mechanism for the alteration of membrane composition is increased solubility of saturated fatty acids in the membrane as temperature increases, giving them greater access to the enzymes involved in membrane lipid biosynthesis (91). If this hypothesis is correct, and if BvgS does respond to lipid composition, then the "programmed selectivity" (91) of the membrane for particular fatty acids at different temperatures would be the primary temperature sensor in this system.

Heat shock

As the name implies, one of the inducers for the heat shock stress response is temperature. The kinetics of gene induction in heat shock differ substantially from the induction observed in thermally regulated virulence genes. For heat shock, a shift from 30°C to 42°C results in a rapid, transient increase in the expression of the affected genes. This is followed by a decline in transcription to a new steady-state level which is modestly higher (2-fold) than the pre-shock
steady state level (11). These events take place within the first 20 minutes after the shift. The kinetics of virulence gene induction is generally much simpler. For example, following a temperature shift transcription of lcrF-regulated genes in Y. pestis increases to a new steady-state level which is maintained as long as the temperature regime is unaltered: there is no transient spike of high transcription, and the induction ratio at the new steady-state level is higher than that observed in the heat-shock system. Nonetheless, the mechanism of temperature sensing in the two systems may, as discussed below, be very similar.

**E. coli.** Transcription of the heat shock genes is controlled by the amount of an alternate sigma factor (σ^{32}) whose levels increase transiently after a heat shock (133). It is now known that the increase in σ^{32} under inducing conditions results from both increased stability and increased synthesis of this protein. The transient increase in the stability of σ^{32}, from a half-life of 1 minute at 30°C to a half-life of 8 minutes following heat shock, occurs within 6 minutes of a temperature shift (133) and is thought to be controlled by the heat shock proteins DnaK, GrpE, and DnaJ (47, 132). Mutants with defects in dnaK, grpE, or dnaJ are unable to reduce production of the heat shock proteins from the high levels achieved at the peak of the transient which immediately follows induction in wild-type cells. These mutants also have elevated levels of heat shock proteins at low temperature. Both phenotypes have been attributed to an increase in the stability of σ^{32} (132, 138). DnaK, GrpE, and DnaJ all selectively bind denatured proteins and are believed to target them for degradation. It has been suggested that DnaK, GrpE, and DnaJ have the same effect on normally folded σ^{32} (24). This has led to the suggestion that increased levels of misfolded
and heat damaged proteins resulting from thermal shock compete with $\sigma^{32}$ as alternative substrates for DnaK, GrpE and DnaJ (81) resulting in an increase in the pool of functional $\sigma^{32}$ and a concomitant increase in the expression of the heat shock proteins. DnaK exhibits weak ATPase and autophosphorylation activities in vitro, activities which increase sharply with temperature (89). Although the physiological role of these enzymatic activities is at present uncertain, there is evidence suggesting that ATP hydrolysis promotes release of bound protein from DnaK (40). These observations have led to the hypothesis that the increased ATPase activity of DnaK at elevated temperature reduces the rate of $\sigma^{32}$ degradation (89). If this mechanism is correct, DnaK may be the thermometer of the heat shock system (24, 89).

The rate of $\sigma^{32}$ protein synthesis is also increased upon heat shock (133). This effect cannot be explained by a concomitant increase in the rate of $\sigma^{32}$ mRNA synthesis (32). This suggests an additional step at the post-transcriptional level regulating the amount of $\sigma^{32}$ protein (32). Recent experiments have identified two regions within the $rpoH$ gene ($rpoH$ encodes $\sigma^{32}$) which are involved in this regulation and which are thought to hybridize in the $rpoH$ mRNA, contributing to its secondary structure (71, 98). It has been suggested that this secondary structure could preclude translation of the $rpoH$ message at low temperature, and that its thermal destabilization during heat shock would allow translation. Indeed, the finding that $\sigma^{32}$-specific message accumulates subsequent to the increase in $\sigma^{32}$ protein synthesis (32) supports the general observation that the rate of message degradation can be reduced by increased translation. However, the two putative hybridizing regions in the $rpoH$ message are widely separated, and due to the coupling between
transcription and translation that occurs in prokaryotes, this proposed secondary structure may not exist in vivo (98).

At sub-lethal temperatures, transcription of the rpoH gene is driven by two promoters recognized by RNA polymerase containing σ^70 (32). When cells are shifted from 30°C to a lethal temperature such as 50°C, transcription occurs solely from a promoter recognized by RNA polymerase containing another alternate sigma factor, σ^E (32a). In addition to rpoH, σ^E is also required for the transcription of the htrA gene at high temperatures (81a). htrA encodes the HtrA protein which had been previously identified as DegP (131a). This protein is required for survival of E. coli at temperatures above 42°C, is an endopeptidase, and may function to process denatured and misfolded proteins which are produced at increased levels at these temperatures (81b). At present, the mechanism controlling σ^E thermal activity is unknown.

**Eukaryotes.** The systems which have been most studied in relation to heat shock are the yeast, *Saccharomyces cerevisiae*, the fruit fly *Drosophila*, and HeLa cells of man. These systems are similar to that of *E. coli* in that transcription of a specific set of proteins (some of which are highly conserved among different phyla) is transiently induced upon heat shock by the action of a heat shock factor, generally designated HSF. HSF binding sites are found in the promoter regions of the induced genes. The sites contain a repeated 5 bp. sequence, nGAAAn, conserved in yeast and HeLa cells (122).

As in *E. coli*, the mechanism of temperature sensing is currently uncertain. In both *Drosophila* and HeLa cells, HSF-mediated binding activity is only seen after a heat shock (123, 161). This phenomenon can be observed in vivo even in the presence of protein-synthesis inhibitors, and can also be seen in vitro,
implying that HSF exists in active and inactive forms in these systems (77, 161). Further work has indicated that the induction of DNA-binding activity is the result of oligimerization of the protein (151). In Drosophila, this involves both a monomer to trimer transition, and a conformational change in the individual protein subunits (152). In contrast, analysis of HSF binding activity in S. cerevisiae indicates that the protein is bound to DNA even under non-inducing conditions and that the oligomeric nature of the protein must be maintained to repress activation of transcription under these conditions (123). In all of these systems it has been observed that the ability of the HSF to activate transcription is dependent upon its state of phosphorylation (77, 124). In summary, it appears there are two distinct steps associated with the induction of heat shock proteins in Drosophila and HeLa cells: 1) the induction of HSF DNA binding activity, and 2) the induction of transcription activating activity by phosphorylation. S. cerevisiae seems to only require the latter.

DNA Topology

Over the past five years, a large body of work has accumulated detailing the effects changes in the topology of DNA have on the expression of virulence genes which are normally induced in response to environmental changes, including temperature (13, 25, 28). Much of this work has grown out of the observation that many searches for regulatory mutations affecting the expression of these virulence genes have yielded alleles of either the E. coli gene hns, or its closely related homologues in other species. The product of this gene, H-NS, is a low molecular weight nucleoid-associated protein capable of condensing chromosomal DNA, and which affects the extent of supercoiling in vivo (64). One hns allele, drdX, and mutants of the hns homologue virR of
Shigella flexneri, have been mentioned above. They result in constitutive expression of virulence genes that are normally induced only at elevated temperature. The ymoA gene of Y. enterocolitica, although not a true hns homologue, does encode a protein that shares the properties of neutral charge, low molecular weight, and probably nucleoid association and DNA compaction with the H-NS-family. Mutations in ymoA affect the expression of both virF and yop genes, causing increased expression at low temperature \( (23, 76) \).

While these results are intriguing, they fail to demonstrate that a temperature-induced change in topology is directly involved in the physiological mechanism for thermal induction of virulence genes. It is now well established that many promoters are affected by changes in DNA topology, some being activated and others repressed by a reduction in superhelical density \( (69) \). Some promoters are also unaffected by such changes \( (69) \). Rather than demonstrating a direct physiological role of supercoiling in gene regulation, these observations may reflect the behavior of promoters forced into physiologically irrelevant regimes of superhelical tension. It is difficult to clearly distinguish between these possibilities, and it is likely that resolution of this issue will only be possible when the induction mechanisms at work in several thermal regulation systems have been established.

Some of the current evidence regarding the role of H-NS-like proteins appears to be contradictory. For example, as pointed out by Hromockyj et al. \( (62) \), null alleles of virR in S. flexneri and hns in E. coli, have opposite effects on supercoiling of plasmids in vivo, even though they give the same phenotype with respect to the induction of virulence genes. Since elevated temperature should have a similar effect on the state of supercoiling in both
systems, this divergent response is not consistent with simple models in which the direct effect of temperature on superhelical density causes changes in promoter activity. Moreover, it is not at all certain that superhelical density in vivo is significantly altered by temperature changes. In fact, the report often cited as evidence for a change in supercoiling with temperature (e.g. see (29, 64)) actually states that plasmid linking numbers change with temperature to maintain a fixed value of superhelical tension (43).

Recognizing the probable constancy of superhelical tension in vivo, Wang and Syvanen have offered a more feasible hypothesis implicating twist (Tw), a parameter determining linking number, as the temperature-sensitive topological parameter (145). (Recall that linking number (L) is partitioned into twist (Tw), the total number of turns of the duplex, and writhe (Wr), the rotation of the duplex about its axis, so that \( \Delta L = \Delta Tw + \Delta Wr \)). Wang and Syvanen propose that many genes, including virulence genes which respond to environmental stimuli, contain 'twist sensitive' promoters (145). The environmental conditions which induce their transcription twist DNA in the promoter region to bring key residues in the -10 and -35 promoter domains into favorable rotational alignment about the axis of the helix (145). For example, the recA promoter of *E. coli* has been shown to be induced by cold shock (a decrease in temperature from 37°C to 10°C), and also by the gyrase inhibitor coumermycin. These two conditions are known to relax negatively supercoiled DNA thus making linking number and twist more positive. This is predicted to bring key bases of -10 and -35 elements of the recA promoter into an alignment that resembles the structure of a consensus *E.coli* promoter. There is at present little experimental support for this interesting hypothesis.
Conclusion

In summary, my review of the literature concerning thermal regulation of gene expression leads me to emphasize the following points:

1. The basis of temperature sensing and the transduction of the temperature signal to effect changes in gene regulation is not clearly understood in any system at present.

2. The extent to which diverse systems share common mechanistic features is unclear, although post-transcriptional regulation of synthesis of a key regulatory protein may be involved in several of the systems described. The finding that genetic manipulations affecting the translation apparatus in both *E. coli* and *Shigella* can alter thermal regulation may also reflect a role for post-transcriptional regulation in these systems.

3. The field of thermo-regulation of gene expression would benefit more from definitive mechanistic descriptions of a few thermo-sensing and regulation systems than from the development of additional theory.

The *Y. pestis lcrF* system is among the most thoroughly investigated thermo-regulation systems in prokaryotes, and offers good prospects for real progress. Significant advances in understanding this system resulting from my research are detailed in the following chapters.
CHAPTER II

TEMPERATURE SENSING IN YERSINIA PESTIS: TRANSCRIPTION OF
THE YOPE GENE BY LCRF
Abstract

In *Escherichia coli*, a *yopE::lacZ* fusion was found to be regulated by temperature in the presence of the cloned *BamHI G* fragment of *Yersinia pestis* plasmid pCD1 which contains the *lcrF* locus. Increasing the copy number of *lcrF* relative to that of the *yopE* reporter had a negligible effect on the induction ratio (26 versus 37°C) but caused large reductions in the absolute levels of *yopE* transcription. We localized the *lcrF* gene by monitoring the induction phenotype of *BamHI G* deletion derivatives. Sequencing revealed an open reading frame capable of encoding a protein of 30.8 kDa. A protein product of this size was detected in a T7 expression system, and LcrF-dependent *yopE*-specific DNA binding activity was observed. As expected, LcrF exhibited 98% homology to VirF of *Yersinia enterocolitica* and significant homology to the carboxy termini of other members of the AraC family of transcriptional regulatory proteins. These proteins could be divided into two classes according to function: those regulating operons involved in catabolism of carbon and energy sources and those involved in regulating virulence genes. *lcrF::lacZ* transcriptional fusions were constructed and analyzed in *Y. pestis* and *E. coli*. The activity of the fusions was not affected by the native pCD1 virulence plasmid, an intact *lcrF* gene, or temperature. Thus, induction of *lcrF* transcription is not essential for temperature-dependent activation of *yopE* transcription. A portion of LcrF was found associated with the membrane fraction in *E. coli*; however, pulse-chase experiments indicated that this result is an artifact of fractionation.
Introduction

Bacteria generally respond to changes in environmental conditions by the action of specific systems which detect these changes and effect adaptive alterations in the pattern of gene expression. While the best known examples of these mechanisms involve responses to availability of nutrients, it has more recently been established that bacterial pathogens adapt to conditions encountered in their hosts in a similar way: they monitor environmental parameters which distinguish host from external environments and alter gene expression accordingly, particularly by induction of virulence genes. Osmolarity and the concentration of specific ions (Ca$^{2+}$, Mg$^{2+}$, and Fe$^{2+}$) are among the environmental parameters demonstrated to regulate such genes (for reviews, see (55), (90), and (93)). Another parameter, temperature, has been implicated in a wide variety of pathogens, including Borrelia burgdorferi (20), Bordetella pertussis, shigellae, salmonellae, and the yersiniae (87).

In the yersiniae (Yersinia pestis, Y. enterocolitica and Y. pseudotuberculosis) at least 12 plasmid-borne genes, many of which have been directly implicated in virulence, are regulated in response to Ca$^{2+}$ and temperature (22, 128). (ATP may also play an important role (105).) Transcription of these genes is induced by increasing the temperature from less than 30°C to greater than 34°C (usually from 26 to 37°C). The induction is substantially greater if the Ca$^{2+}$ concentration is low (μM range). Temperature plays a dominant role in this response in that Ca$^{2+}$ concentration has no effect unless the temperature is elevated. The in vitro phenotype characterized by induction of this gene set (designated yopA-N) at 37°C in the absence of added Ca$^{2+}$ and a coordinated cessation of growth following two rounds of
chromosome replication is known as the low-calcium response (LCR). One favored hypothesis relating these regulatory phenomena to the interaction of bacteria and host is that elevated temperature serves as the primary signal indicating that the bacteria have entered a mammalian host, while reduced concentrations of free Ca\(^{2+}\) indicate entry into the intracellular environment (102). The role of temperature is believed to be similar in other pathogens.

Despite the central role of thermally regulated gene expression in the regulation of virulence genes and in the control of the fundamental stress response known as heat shock, the mechanisms by which temperature changes are translated into changes in gene expression are not well understood. A central question concerning these mechanisms is identification of the "thermometer"-- the bacterial component which functions as the primary temperature sensor. This sensor has not been clearly defined in any system. This is in large measure due to the fact that the regulatory proteins controlling the thermally regulated genes have been found to change in abundance in response to temperature (44, 125, 133), implying that their expression is in turn controlled by a regulatory apparatus that interfaces more directly with the thermometer. For example, the sigma factor RpoH (\(\sigma^{32}\)), which controls expression of many heat shock genes in *Escherichia coli*, is made in increased quantities during the heat shock response and also has a longer half-life (132) (133). Very recent work with this system suggests that the thermometer may be DnaK, which is also a heat shock protein in the Hsp-70 family (24, 89).

Temperature sensing systems used by pathogens do not have the global effect on gene expression that occurs with heat shock; a relatively small and specific set of virulence genes are affected. Another difference between these
systems and heat shock is the character of the induction. In the heat shock response, the initially large increase in transcription is transient and soon decays to a much lower steady state level. In the yersinia system, induction is more directly coupled to temperature, in that the increase in transcription with temperature does not decay unless temperature is lowered. Thus, the systems regulating virulence may be more easily dissected than in heat shock and may also function by fundamentally different mechanisms.

Among pathogens, the thermal regulation system of the yersiniae is the most thoroughly characterized. A central regulatory protein of this system is encoded by a gene designated lcrF (Y. pestis and Y. pseudotuberculosis) or virF (Y. enterocolitica). This gene was initially identified in Y. pestis (157). Cornelis and coworkers have cloned and sequenced the Y. enterocolitica homolog and shown it to be active in trans in E. coli, indicating that it encodes a transcriptional activator (21). They found that transcription of virF was itself induced in response to temperature in Y. enterocolitica and E. coli, leading them to suggest that induction of virF transcription was responsible for induction of virF-regulated genes. They also showed that the VirF protein had significant homology with the DNA-binding carboxy terminus of the E. coli arabinose operon regulatory protein AraC (21). In this report, we present the DNA sequence of the lcrF gene of Y. pestis, extend observations regarding homology with other regulatory genes, and demonstrate that modulation of lcrF transcription is neither required nor sufficient for induction of lcrF target genes.
Materials and Methods

Bacterial strains and plasmids. The strains and plasmids used are described in Table 2.1.

Construction of $yopE::lacZ$ reporter plasmids. A previously characterized (157) $yopE::Mudl\text{ (Ap lac)}$ fusion at 22.8 kb on the pCD1 map (BamH I E fragment) was subcloned for use in many of the experiments described below. On the basis of restriction mapping and the published $yopE$ sequence (34), the fusion point in this plasmid is about 700 bp downstream of the putative $yopE$ initiation codon. The original pCD1 construct (pUT1007::Mudl [22.8 kb]) was digested to completion with BamH I and incompletely with EcoRI and ligated to the vector pMC874 digested to completion with the same enzymes. Among the β-galactosidase positive clones obtained were those in which $yopE::lacZ$ sequences—including approximately 800 bp of pCD1 sequence upstream of $yopE$, and downstream $lacZ$ sequence terminating at the EcoRI site—were substituted for the 5' portion of the $lacZ$ coding sequence of pMC874. One of these clones was designated pUT2016. An EcoRI-SalI fragment (incomplete EcoRI and complete SalI digest) beginning at the EcoRI site 292 bases upstream of the $yopE$ coding sequence and including the $yopE::lacZ$ sequence through the SalI site of $lacZ$ was inserted in EcoRI-SalI digested pBR322, to yield pUM4091, and in EcoRI-SalI digested pLG338, to yield pUM4090. Note that all of these reporters contain operon fusions, producing native β-galactosidase.

Construction of $lcrF::lacZ$ reporter plasmids. Two $lcrF::lacZ$ operon fusions were prepared in vector pNK1915 (120). This vector (a derivative of pBR322) contains a promoterless $lacZ$ and polylinker immediately downstream of
several repeats of the strong T1 transcription terminator. Plasmid pUM4106 was constructed by cloning a 280-bp *HindIII-HaeIII* fragment of the pCD1 *BamHI G* fragment into the polylinker. This fragment contains 206 bp upstream and 80 bp downstream of the start codon of *lcrF*. Plasmid pUM4180 contains a 1.0-kb *HindIII-DraI* fragment of *BamHI G*. The *HindIII* site is the same as that used to construct pUM4106, and the *DraI* site lies 18 bp downstream of the *lcrF* stop codon. There are no sequences suggestive of transcription terminators in the 3' region of the *Y. pestis* fragment included in this construct. Each plasmid was prepared so that vector sequences surrounding the *lcrF* fragments were identical.

Plasmid pUM4101 was created by performing a complete *SalI* and partial *EcoRI* digestion of pUM4100 (pLG338::*BamHI G*) and ligating it to an *EcoRI-SalI* fragment from pNK1915 containing a promoterless *lacZ* gene. This created an *lcrF::lacZ* operon fusion, such that *lacZ* is fused 560 bp downstream of the *lcrF* start codon. This construct also contains approximately 2 kb of *BamHI G* sequences upstream of *lcrF::lacZ*.

**Media and growth conditions.** *Y. pestis* strains were grown in tryptose blood agar base (TBAB) broth (1% tryptose, 0.3% beef extract, 0.5% NaCl) for routine culture or the defined medium (TMH) of Zahorchak and Brubaker (159) as modified by Straley et al. (129) supplemented with 2.5 mM CaCl$_2$ when indicated. TBAB (Difco) supplemented with 2.5 mM CaCl$_2$ was used for growth on solid medium. *E. coli* strains were grown in Luria broth (LB) or on Luria broth agar (94). Ampicillin, kanamycin, and tetracycline, were used at concentrations of 50, 50, and 25 μg/ml, respectively, unless otherwise noted.
For determination of LCR, cultures were grown overnight at 26°C in TBAB broth supplemented with 2.5 mM CaCl₂. Equal numbers of cells were then resuspended in 0.033 M NaPO₄ buffer, pH 7.0. Serial dilutions were spotted onto duplicate TBAB plates containing 2.5 mM CaCl₂ or TBAB rendered calcium deficient by the addition of sodium oxalate (58). Each set of plates (with or without Ca²⁺) was incubated for 48 h at 26 or 37°C.

**Determination of β-galactosidase levels.** Overnight cultures of *Y. pestis* strains grown at 26°C in TMH were used to inoculate duplicate 5-ml TMH cultures (with and without added CaCl₂) to an optical density at 600 nm of 0.1. The cultures were then incubated in a shaking water bath at 26°C until they reached an optical density at 600 nm of 0.25 (approximately 2.5 h). At this time, half of the cultures were switched to 37°C, and all of the cultures were allowed to grow for an additional 4 h. β-Galactosidase levels were determined as described by Miller (94). *E. coli* strains were grown in LB following the protocol described above, except that cultures were allowed to grow for 2 h after the temperature switch.

**DNA sequencing.** The BamHI G fragment of pCD1 was cloned into the Bluescript M13-based sequencing vectors. Unidirectional deletions used in sequencing and the functional analysis of *lcrF* were prepared by using the exonuclease III-mung bean nuclease system of Stratagene (San Diego, Calif.). Sequencing was carried out on double-stranded templates by the chain termination method of Sangar et al. (114) with the Sequenase kit of United States Biochemical.

**DNA methodology.** Large-scale plasmid preparations were by the method of Birnboim and Doly (6) as described by Maniatis et al. (85), except that a phenol
extraction of the supernatant was performed after the first spin. The boiling method of Holmes and Quigley (61) was used to prepare some DNA for transformations and restriction analysis. To screen large numbers of clones for their plasmid content, we utilized the method of Kado and Liu (70) in a 96-well microtiter dish format. Restriction digests, ligations, production of blunt ends by use of the Klenow fragment, and gel electrophoresis were carried out essentially as described by Maniatis et al. (85). Restriction enzymes, Klenow fragment, and T4 DNA ligase were obtained from New England Biolabs, Boehringer Mannheim, and GIBCO/BRL. Transformation of E. coli was performed using the cold CaCl$_2$ method of Dagert and Ehrlich (26). Y. pestis cells were prepared for electroporation according to the manufacturer's instructions for the electroporation apparatus (Gene Pulser, Bio-Rad) except that cells were incubated at 30°C for 2 h before being plated. Cells were electroporated at 12,500 V/cm$^2$, with a pulse time length of approximately 4 ms.

**Analysis of proteins.** The protein products of the BamHI G fragment and its deletions present in the Bluescript M13 plasmids were visualized by utilizing the T7 overexpression system of Tabor and Richardson (137). Growth and induction of cultures, labeling of proteins with $[^{35}$S]methionine (Amersham Corp., Arlington Heights, Ill.), and preparation of samples for electrophoresis followed a protocol of Tabor. Samples were analyzed on 16.5% polyacrylamide gels as described by Schägger and von Jagow (118) to facilitate visualization of low-molecular-weight proteins.

**Pulse-chase.** Cells were prepared for labeling with $[^{35}$S]methionine following a protocol of Tabor, except that the cells were resuspended in 100 ml of M9 medium supplemented with 20μg of thiamine per ml and a 0.01% solution
of all amino acids except methionine and cysteine (18 amino acids). After rifampin addition and incubation at 42°C, the culture was filtered through a 0.2-
μm-pore-size nitrocellulose filter (Nalgene) to reduce the culture size to 1 to 3
ml. Filtering was performed at 37°C. The culture was then incubated at 30°C for 30 min, at which time cells were pulsed with 2.5 mCi of [35S]methionine. The cells were chased by adding the culture to medium containing a 500-fold excess of unlabeled methionine. Samples were withdrawn at various times and added to tubes chilled in an ice water bath, a method which has been shown to effectively block export of β-lactamase (72).

**Cell fractionation.** Cells were fractionated by a modification of the method of Straley and Brubaker (130). Briefly, frozen cells were resuspended in 0.01 M HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.5) and passed twice through a French pressure cell (American Instrument Co., Silver Springs, Maryland) at 20,000 lb/in². The extract was spun briefly in a microcentrifuge to spin down whole cells. MgCl₂ was added to the cell extract to a final concentration of 1 mM, lysozyme was added (1 mg/ml), and the mixture was incubated on ice for 90 min. The extract was then loaded onto a discontinuous sucrose gradient of 1.5 ml of 55% and 3.0 ml of 20% sucrose (all sucrose solutions were prepared [wt/wt] in 0.01 M HEPES). The gradient was spun at 40,000 rpm in a Beckman SW50.1 rotor for 1 h (150,000 × g). Approximately 1 ml was taken off the top of the gradient as the soluble fraction. Total membranes were harvested, diluted 1:1 with 0.01 M HEPES, and loaded on top of a second discontinuous sucrose gradient (1.0 ml of 55%, 1.9 ml of 45%, and 2.0 ml of 40% sucrose). This was centrifuged under the same conditions as before for 5 h. The gradients were fractionated from the top down with a peristaltic
pump (approximately 0.5 ml per fraction). Each fraction was then diluted to 5.4 ml with 0.01M HEPES and spun at 50,000 rpm in the SW50.1 rotor for 1h (234,000 x g). Pellets were resuspended in a small volume of 0.01 M HEPES. The relative positions of inner and outer membranes were checked by determining the density of each fraction with a refractometer and by visualizing fraction proteins on Coomassie blue-stained acrylamide gels.

**Gel Mobility Shift Assay.** The method used is that of Byerly et al. (12) with the following modifications. Plasmid pUM4200 was digested with EcoRI and PstI and 5' end labeled with [$\gamma$-32P]ATP and T4 polynucleotide kinase (Boehringer Mannheim). This resulted in the production of two labeled fragments: one of 679 bp which contains 292 bp of sequence upstream of *yopE* and 387 bp of *yopE* coding region and a 2.9-kb fragment containing only vector sequences. The reaction mixture, consisting of 1 ng of labeled DNA, 1 µg of unlabeled supercoiled Bluescript plasmid DNA (-KS), 10 µl of 2X DNA binding buffer (2X DNA-binding buffer is 20 mM Tris-hydrochloride [pH 7.5], 100 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, and 10% glycerol), and dH2O to 20 µl, was preincubated at 37°C for 5 min at which time crude cell extracts or partially purified LcrF was added, and incubation was continued at 37°C for an additional 15 min. After the addition of 1 µl of loading dye (0.1% xylene cyanol and 50% glycerol), the samples were loaded onto 5% polyacrylamide gels (1:30 bisacrylamide-acrylamide buffered with 10 mM Tris [pH 7.4], 0.38 M glycine, and 1 mM EDTA) which had been prerun at 9 V/cm for 1 h. Gels were run at 12 V/cm for approximately 1 h, dried, and exposed to Kodak X-Omat AR film overnight at -70°C, with one intensifying screen.
To prepare cells for use in the DNA-binding assays, *E. coli* BL21 (DE3) containing pNF409 was used (135). DE3 is a lambda lysogen containing the T7 polymerase gene under control of the *lacUV5* promoter, conferring isopropyl-b-D-thiogalactopyranoside (IPTG) inducibility upon polymerase expression. The cells were grown at 37°C in LB broth, and IPTG was added as described elsewhere (135). Crude cell extracts were prepared according to Webster et al. (148). To prepare partially purified LcrF, crude cell extracts were passed through a Sephadex G-25M column equilibrated with the following phosphate buffer based on that of Tobin and Schleif (140): 10 mM potassium phosphate (pH 7.2), 2 mM EDTA, 10% glycerol, and 0.1 mM dithiothreitol. The final pH was 7.0. The extract was then applied to a phosphocellulose column (Whatman P11; 1 ml of resin volume) equilibrated in the phosphate buffer. Proteins were eluted with a linear NaCl gradient (to 1 M) in phosphate buffer, and selected fractions were used in the gel mobility shift assay.

**Nucleotide sequence accession number.** The nucleotide sequence data reported in this paper have been submitted to GenBank and assigned the accession number M86690.
Results

Regulation of a cloned \textit{yopE}:\textit{lacZ} fusion in \textit{Y. pestis}. The \textit{lcrF} locus on the native \textit{Y. pestis} virulence plasmid pCD1 was originally identified by selecting Tn5 insertion mutants which permitted growth of a specially constructed \textit{Y. pestis} strain at 37°C (157). This strain carries a mutant allele of the \textit{lcrE} gene designated \textit{lcrE1} (158), as well as a \textit{yopE}:Mud1 (Ap lac) fusion. The \textit{lcrE1} mutation confers a calcium-blind phenotype, i.e. the bacteria behave as if calcium is absent, even when it is available at a high concentration. This behavior includes inability to grow at 37°C. The \textit{yopE} fusion serves as a reporter of transcription of the \textit{lcrF}-regulated \textit{yopE} gene. In order to characterize \textit{yopE} regulation by the \textit{lcrF} locus in a simplified system in \textit{E. coli} (see below), we needed to determine whether a cloned \textit{yopE}:\textit{lacZ} fusion would be regulated in the same manner as the Mud1 fusion in pCD1. Also, we wanted to be sure that this fusion behaved as expected in trans with native pCD1 in \textit{Y. pestis}, responding to both temperature and Ca\textsuperscript{2+} concentration.

The \textit{yopE}:\textit{lacZ} fusion plasmid pUM4090 was introduced into KIM5, KIM6, and UTP1007 by electroporation, and β-galactosidase assays were performed in TMH-grown cultures. The low copy number vector pLG338 was used to construct this reporter because its copy number (estimated to be 7.5 [126]) is probably similar to that of pCD1. (Although no direct measurements of the pCD1 copy number have been made, we estimate its copy number to be in the range of 3 to 5. This estimate is based on the relative amounts of pBR322 and pCD1 DNAs present in cells containing both plasmids [estimated from ethidium stained gels], the known sizes of the plasmids, and using 40 to 60 as the copy number of pBR322.) The results of β-galactosidase assays on these strains are
FIG. 2.1. Effect of temperature and Ca$^{2+}$ concentration on the expression of a cloned yopE::lacZ fusion. (A) Results of β-galactosidase assays on Y. pestis KIM5 (pCD1+ Lcr$^+$), KIM6 (pCD1- Lcr$^-$), and UTP1007 (lcrEl Lcr$^c$) containing pUM4090. (B) Effect of increasing the copy number of plasmids bearing the BamHI G fragment of pCD1 on temperature induction of a cloned yopE::lacZ fusion in E. coli. Reporter, plasmids bearing the yopE::lacZ fusion; activator, plasmids bearing the BamHI G fragment. (C) Map of the yopE::lacZ fusion construct contained in the reporter plasmids used in these experiments. The break indicates truncation for scaling of the drawing; the lacZYA genes are intact through the SalI site downstream of lacA. E, EcoRI; P, PstI; H, HindIII; S, SalI.
given in Fig. 2.1A. Our results clearly indicate that a cloned yopE::lacZ fusion can be used as a reporter gene to study lcrF-mediated regulation. The fusion is regulated by calcium and temperature in the same manner as on the native plasmid: in KIM5, maximal induction occurs at 37°C in the absence of calcium. In the lcrE1 strain UTP1007, maximal induction occurs at 37°C regardless of the calcium concentration. In the pCD1- strain KIM6, there is no induction, as expected. Moreover, the basal level of synthesis is much lower in KIM6 than in KIM5 at 26°C, suggesting that the presence of lcrF has a substantial effect even at low temperature.

**Induction of a yopE::lacZ fusion in E. coli.** To determine whether this reporter would also function in E. coli, a strain (UME4079) containing pUM4090 and a pBR322 derivative carrying the lcrF-containing BamHI G fragment of pCD1 (pUT2027) was constructed. Thermal induction of β-galactosidase activity similar to that displayed by Y. pestis constructs in Ca²⁺-deficient medium was observed, although the induced level in the E. coli strain was somewhat lower (Fig. 2.1B, middle). One difference between the E. coli and Y. pestis strains compared in these experiments was that the lcrF gene was present in E. coli in a much higher dosage relative to the reporter. To determine the importance of this dosage effect, a second E. coli strain was tested. In this strain (UME4081), the relative copy numbers of lcrF and the reporter were reversed via a reciprocal exchange of vectors to form the plasmids pUM4091 (reporter in pBR322) and pUM4100 (BamHI G in pLG338). As shown in the left of Fig. 2.1B, the results obtained with this strain are somewhat more similar to those obtained by using pUM4090 in Y. pestis, in that the induced β-galactosidase levels are higher. Because the differences observed were relatively minor, despite the large
changes in relative copy numbers of reporter and lcrF, we concluded that the induction mechanism, at least in E. coli, was fairly insensitive to relative dosage and that both constructs were adequate for further analysis of induction.

We also examined the effect of increasing lcrF dosage even more by cloning the BamHI G fragment on a very high-copy-number Bluescript vector to form plasmid pNF001. An E. coli strain (UME4085) with this plasmid and the low-copy-number reporter (pUM4090) (Fig. 2.1B, right) had an eightfold induction ratio, but absolute β-galactosidase levels at both 26 and 37°C were markedly reduced. Taken with the results reported above, this observation suggests that increased dosage of lcrF or some other Y. pestis gene in the BamHI G fragment reduces yopE expression at both 26 and 37°C. Because deletions in pNF001 could be made easily and induction activity was readily observable, this plasmid and its derivatives were used in the experiments described below to delimit the sequences required for induction.

The sequence of a 1.5-kb region of plasmid pYV019 of Y. pestis EV76, which includes yopE and upstream sequences, has been reported elsewhere (34). pYV019 is virtually identical to pCD1. An open reading frame in this upstream region, termed yerA, was identified and, when deleted, reduced the production of YopE protein. All of the reporter plasmids used in our E. coli experiments contain only the 3' third of the yerA coding region and are presumably yerA negative. Thus, yerA is not required for the induction observed in E. coli.

**Mapping lcrF.** Nested deletions were prepared from plasmids pNF001 and pNF002. Each deletion was then tested for LcrF activity by transforming it into strain UME4077 (CSH50 with the low-copy-number pUM4090 reporter) and assaying for β-galactosidase at 26 and 37°C. A 1.1-kb region of BamHI-G
FIG. 2.2. Proteins exhibiting homology to LcrF. See Table 2.2 for full descriptions and sequence references for each protein. (Top) Multiple alignment. Each amino acid sequence was maximally aligned to the LcrF sequence by utilizing the algorithms of Lipmann and Pearson (82) and Needleman and Wunsch (99), as implemented by the DNA* AALIGN program, with a gap penalty of 4 and a deletion penalty of 12. The region of each protein determined to align with amino acids 171 to 271 of LcrF is listed. Numbers next to the protein names indicate the first amino acids in each alignment. (Bottom) Similarity histogram. Values were calculated by the following equation: \[ \text{Similarity} = \frac{\text{number of identical residues} + (0.5 \times \text{number of conserved residues})}{\text{number of family members}} \]. Letters, identical amino acid; filled circles, conserved amino acids. The relevance of boxed regions in the alignment is discussed in the text. A deletion starting from the 3' end of lcrF and extending to the arrow in region C abolishes LcrF activity.

Abbreviations: LCRF, LcrF of Y. pestis; VIRF, VirF of Y. enterocolitica; ARACEWC, AraC of Erwinia carotovara; RNS, Rns of enterotoxigenic E. coli; VIRFS, VirF of S. flexneri; ARACEC, AraC of E. coli; ARACST, AraC of Salmonella typhimurium; RHAR, RhaR of E. coli; RHAS, RhaS of E. coli; MELR, MelR of E. coli; XYLSP, XylS of Pseudomonas putida.

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(coordinates, 55.7 to 56.8kb of pCD1, on the basis of the map of Goguen et al. [42])
required for yopE induction was clearly defined in these experiments (data not shown).

**Identification of the LcrF protein.** Selected deletion constructs from the
mapping experiments described above were transformed into K38(pGP1-2). The
proteins expressed from the T7 promoter in these plasmids were specifically
labeled with [35S]methionine as described. The results (data not shown) clearly
indicate that yopE induction in *E. coli* is linked to the presence of a protein
species with a molecular mass of approximately 30 kDa.

**Sequencing lcrF.** Sequencing the region of the *Bam*HII G fragment
identified by the deletion analysis revealed an open reading frame consisting of
271 amino acids which has the potential to code for a protein with a molecular
weiths of 30,836. A potential Shine-Dalgarno sequence occurs 13 nucleotides
upstream of the first methionine. While this spacing is different from the
optimal 6 to 7 nucleotides found in *E. coli* (127), this has also been seen for the
VirF protein of *Y. enterocolitica* (21). Of the six amino acid residues altered
between VirF and LcrF, only one replacement (Gly-95_{LcrF} for Arg-95_{VirF}) is not a
conservative one. A consensus sequence DnaA binding site (5' TTATCCAAA
3') is centered 153 bp upstream from the presumptive lcrF translation start. We
find that this site is also present upstream of virF. Curiously, a similar site
upstream of rpoH, the gene encoding the heat shock sigma factor σ^{32}, has been
noted elsewhere (147).

**Proteins homologous to LcrF.** Like VirF of *Y. enterocolitica*, LcrF is a
member of the AraC family of transcription activators (14, 21, 54, 107, 140). In
addition to LcrF, we add another previously unrecognized member to this
family, the virF gene of *Shigella flexneri* (112). Note that there is no special relationship between the *Y. enterocolitica* and *S. flexneri* proteins: the identical gene designations are merely coincidental. The current members of the AraC family are listed in Table 2.2, which also illustrates that the family can be readily divided into two subsets: those which regulate catabolic operons in response to substrate availability and those which regulate virulence genes in response to temperature. A multiple alignment of carboxy-terminal residues of the current AraC family is shown schematically in Fig. 2.2. This region displays the strongest sequence similarity among these proteins. Domain A was identified by Cornelis et al. (21) as containing a helix-turn-helix region similar to a domain in lambda repressor known to be involved in the DNA binding (101). Similarity in this region among the AraC family (43%) is no higher than the average for the carboxy-terminal region shown (44%). In contrast, region B, identified by Caron and colleagues as a potential helix-turn-helix motif (domain II in reference 5), shows a very high similarity (55%), with scores of greater than 80% at three positions. The behavior of one of our deletion mutants is consistent with an important function for region B: a deletion from the 3' end of *lcrF* up to and including the codon for Arg-253 (plasmid pNF15; deletion endpoint indicated by arrow in Fig. 2.2) destroys LcrF function.

**Transcription of lcrF reporter fusions is not induced in *Y. pestis*.** One potential mechanism for *lcrF*-mediated induction is that transcription of the *lcrF* gene itself is modulated, providing more of the activator protein at elevated temperature. To investigate this possibility, three *lcrF:*lacZ transcriptional fusions were constructed (Fig. 2.3A). Two of these were designed as an isogenic pair which differed with respect to production of active LcrF protein. In one
FIG. 2.3. Expression of lcrF::lacZ fusions. (A) Structure of lcrF::lacZ fusion constructs. pUM4106 and pUM4180 were constructed in pNK1915. T1 denotes the 4 to 5 repeats of the T1 terminator sequence present in this plasmid. pUM4101 is constructed in pLG338, which lacks a terminator sequence. Arrows show directions of transcription. Note that the intact lcrF gene is present only in pUM4180. H, HindIII; Ha, HaeIII; D, DraI; E, EcoRI; Bc, BclI; S, SalI. (B) Results of β-galactosidase assays performed on Y. pestis KIM5 (pCD1+) and KIM6 (pCD1-) containing pUM4106, pUM4180, and pUM4101 grown in TMH and on E. coli CSH50 containing pUM4180 grown in LB. β-galactosidase values for pNK1915 (data not shown) were never above 15 U for any condition in any strain. Note that significant differences in β-galactosidase values at 26 and 37°C are observed only with pUM4101, in which case induction is only twofold or less.
member of this pair (pUM4106), the fusion to a promoterless lacZ cassette occurs near the upstream end of the lcrF reading frame, while in the other (pUM4180), it occurs 18 bp downstream from the lcrF termination codon. Each of these constructs includes 206 bp of sequence upstream of the lcrF start codon, immediately preceded by several copies of the strong T1 transcription terminator. Each plasmid was introduced by electroporation into KIM5 and KIM6, and β-galactosidase assays were performed. The results are shown in Fig. 2.3B. Note that activity was not affected by temperature, an intact lcrF gene, or the presence of the native pCD1 plasmid.

The third lcrF reporter, pUM4101, was designed to reflect lcrF regulation in Y. pestis. It was constructed with the low-copy-number pLG338 vector and includes approximately 2 kb of native pCD1 sequence upstream of lcrF. The point of fusion in this clone is well within the lcrF coding region. The results of β-galactosidase assays on Y. pestis strains containing this plasmid are shown in Fig. 2.3B. This plasmid exhibits weak (approximately twofold) temperature induction in both KIM5 (pCD1+) and KIM6 (pCD1−) strains.

Transcription of lcrF reporter fusions is not induced in E. coli. Plasmid pUM4180 was transformed into CSH50 and β-galactosidase activity was assayed at 26 and 37°C (Fig. 2.3B, right). No increase in transcription was observed in response to the temperature increase, although the actual β-galactosidase values are lower than those observed in Y. pestis.

A constitutively transcribed clone of lcrF mediates thermoregulation of yopE. If modulation of lcrF transcription with temperature is the important step in induction of target genes, then induction should not be observed with a constitutively transcribed lcrF construct like pUM4180. To determine the
FIG. 2.4. Thermoregulation mediated by constitutively transcribed lcrF. (A) Map of the lcrF::lacZ' construct in pUM4186. Note that this construct is identical to the constitutively expressed pUM4180 construct, except for deletion of lac sequences to inactivate lacZ. H, HindIII; D, DraI; Bc, BclI. (B) Results of β-galactosidase assays showing the effect of the presence of pUM4186 on induction of the yopE::lacZ reporter plasmid pUM4090 in Y. pestis KIM6 (pCD1-) (left) and E. coli CSH50 (right). Arrows reflect probable underestimation of β-galactosidase values at 37°C (see text). Note that pUM4186 in CSH50(pUM4090) causes an eightfold induction (26 versus 37°C), even though the absolute β-galactosidase values are lower than those observed in Y. pestis.
pattern of \textit{yopE::lacZ} expression in the presence of a constitutively transcribed \textit{lcrF} gene, we first deleted the \textit{lacZYA} sequences from pUM4180 to permit use of a \textit{lacZ} reporter fusion with this clone. The deleted version, pUM4186 (Fig. 2.4A), was inactive in a β-galactosidase assay. When placed in trans with the reporter pUM4090 in \textit{Y. pestis} KIM6 (Fig. 2.4B, left), pUM4186 caused a substantial increase in β-galactosidase levels at 26°C and also mediated induction in response to temperature. Although this induction appears to be modest (twofold), the strain grows very poorly at 37°C and segregates plasmid pUM4090 at a high rate. Thus, values obtained for β-galactosidase production at 37°C probably underestimate induction substantially. When placed in trans with the reporter pUM4090 in \textit{E. coli} CSH50 (Fig. 2.4B, right), the presence of pUM4186 resulted in a sixfold increase in β-galactosidase levels in response to temperature, demonstrating that this construct retains inducing activity.

\textbf{A constitutively transcribed clone of \textit{lcrF} does not induce LCR-mediated growth restriction at 26°C.} To determine whether pUM4186 in combination with the native pCD1 plasmid could prevent growth at 26°C by inducing expression of the LCR, dilutions of either KIM5 or KIM6 with or without pUM4186 were plated and grown as described in Materials and Methods. All strains grew well at 26°C, regardless of Ca²⁺ concentration. Thus, despite overproduction of \textit{lcrF}, pUM4186 does not fully induce LCR at 26°C. Both KIM5 and KIM5 (pUM4186) showed the expected LCR-mediated growth restriction at 37°C in the absence of Ca²⁺. However, at 37°C in the presence of Ca²⁺, KIM5 (pUM4186) formed abnormally small colonies and plated with an efficiency of 10⁻², further illustrating the instability induced by this plasmid at elevated temperature.
FIG. 2.5. Results of gel mobility shift assay. EcoRI-PstI-digested pUM4200 was used to detect LcrF-specific binding. Lanes: 1, no extract added; 2, crude extract prepared from BL21(DE3, Bluescript KS M13\(^{-}\)); 3, crude extract prepared from BL21(DE3, pNF409). The second shifted band in the region labeled \textit{yopE} specific is observed only at high LcrF concentrations. Lane 4, LcrF-containing fraction from phosphocellulose column.
**Gel mobility shift assay.** Cellular extracts containing LcrF were prepared to detect \( yopE \)-specific DNA-binding activity as described in Materials and Methods. As can be seen in Fig. 2.5, lane 3, an LcrF-containing extract is sufficient to retard the \( yopE \)-specific 679-bp lower band. While there is some nonspecific binding activity in the crude extract, as evidenced by retardation of the vector-specific 2.9-kb upper band, partial purification of the extract over a phosphocellulose column (Fig. 2.5, lane 4) eliminated this activity.

**Localization of LcrF in *E. coli.*** To determine the cellular location of LcrF, cell fractionation and pulse-chase experiments were performed as described in Materials and Methods. *E. coli* K38 (pGP1-2) containing pNF409 was used in these experiments. This clone contains approximately 2 kb of BamHI-G sequences upstream of lcrF. Initial fractionation studies indicated that LcrF was also found predominantly in the outer membrane. However, pulse-chase experiments in which the distribution of LcrF among cell fractions was monitored show that this result is an artifact (Fig. 2.6). The distribution of labeled LcrF did not change significantly as a function of chase time, even when lengths of time as short as 15 s and as long as 30 min were compared (data not shown). This suggested that the observed distribution is not the result of sequential progress of the protein through a secretion pathway but results instead from association of the protein with membranes after lysis of the cells.
FIG. 2.6 Localization of LcrF in *E. coli*. Pulse-chase experiments were performed as described in Materials and Methods with K38(pGP1-2) containing pNF409. Cells were pulsed for 20 s with 2.5 mCi of $[^{35}\text{S}]$methionine and chased, and samples were withdrawn at 15 and 30 s and 1, 2, 5, and 10 min. The samples were then loaded onto discontinuous sucrose gradients, centrifuged, and fractionated. Samples of each fraction are displayed. Top and bottom refer to the positions of the fractions in the gradient. The two samples shown are representative of results observed at all time points. Arrows denote LcrF. the origin of the two lower bands, whose molecular masses are less than 14.4 kDa, is unknown.
Discussion

Many of the results reported here for the lcrF gene of Y. pestis are similar to those of Cornelis et al. for the virF gene of Y. enterocolitica (21). The two genes, as expected, are highly homologous, and both can activate transcription of a reporter gene in E. coli in the absence of additional yersinia genes. We have also shown that LcrF, as predicted by its sequence, is a DNA-binding protein with specificity for a fragment containing yopE sequences. Our most important new finding is that modulation of lcrF transcription is not required for induction of a reporter gene, indicating that posttranscriptional mechanisms affecting lcrF activity or abundance may be important events in thermoregulation. The fact that overexpression of lcrF in E. coli does not affect the thermal induction ratio for yopE but does reduce the absolute levels of yopE expression also has implications for models of LcrF function. As discussed below, these results imply a rather different mechanism than do available data concerning the role of VirF of Y. enterocolitica.

There are two lines of evidence demonstrating that transcription of Y. enterocolitica virF is itself regulated by temperature: an impressive increase in the abundance of virF message with increasing temperature in a nearly wild-type Y. enterocolitica strain has been demonstrated by Northern (RNA) blotting, and the chloramphenicol acetyltransferase activity of a vir::cat fusion has been shown to increase substantially in both Y. enterocolitica and E. coli (21, 23). There are no data from this system suggesting the operation of posttranslational mechanisms modulating VirF activity. Thus, solely on the basis of available virF data, the simplest model for thermal induction of VirF-regulated genes is one in which the primary regulatory event is induction of virF transcription.
This results in the production of increased VirF activator protein which binds to sites upstream of the target genes and activates transcription. In this model, the mechanism responsible for temperature sensing is independent of virF and is present in both Y. enterocolitica and E. coli. Modulation of VirF activity by some posttranscriptional mechanism is not ruled out but need not be invoked to explain the current data.

Our results with lcrF suggest a rather different model for two reasons: a reporter regulated by lcrF responds to temperature even though lcrF transcription remains essentially constant, and abundant transcription of lcrF does not result in the expression of the LCR at low temperature in Y. pestis. The simplest model consistent with these data is one in which the activity, stability, or rate of translation of LcrF protein is controlled by temperature to provide increased active LcrF as temperature increases. Recently, results similar to those reported here for LcrF have been reported for another member of the AraC family involved in thermal regulation of transcription, the virF gene of S. flexneri; i.e., the virF message increased only modestly (fourfold) with temperature, as evidenced by quantitative Northern blotting, and overexpression of virF at 30°C via the use of a Ptac-virF fusion did not result in significant expression of virB, as observed by S1 nuclease protection assays (139). In S. flexneri, virB is dependent on virF and is normally induced approximately 20-fold in response to temperature.

Given the high degree of homology of LcrF and Y. enterocolitica VirF, we think it unlikely that their function is as different as these models suggest. One possibility for reconciliation of the models would require evidence that lcrF transcription in Y. pestis is thermoregulated and evidence that VirF activity is
modulated posttranscriptionally in Y. enterocolitica. Such data would support a model in which both increased transcription of lcrF and virF and posttranscriptional mechanisms provide some increment of induction of the target genes. Although we have no evidence suggesting significant thermal regulation of lcrF transcription, all of the relevant observations in Y. pestis were made with reporter constructs; despite repeated attempts, we were unable to measure the lcrF message in Y. pestis by Northern blotting. Because all of the constructs so far examined in the virF system show significant virF induction, no measurements of the effect of temperature on the expression of VirF target genes in the absence of virF induction have been made. Thus, there is no evidence either for or against posttranslational changes in VirF activity. Clearly, additional data addressing these points are required.

The fact that three distantly related thermoregulatory proteins controlling virulence-related genes in different genera of enterobacteria all fall within the AraC family also has interesting implications. Among the members of this family which regulate catabolic operons, functional homologies are greater than would be predicted solely from sequence homologies. Except in the case of the three AraC proteins, nearly all sequence homology is restricted to the carboxy terminal region involved in DNA binding. Nonetheless, all of the members of the catabolic family apparently not only bind DNA but also interact with small inducer molecules which activate them. Moreover, sequence comparisons show that LcrF and the other thermoregulatory proteins are about as closely related to AraC as are the catabolite operon regulators in the family (again excluding AraC proteins from different species). Thus, it is likely that functional homology of the thermoregulators and AraC is also more extensive
than indicated by sequence alone. This leads to the hypothesis that LcrF activity is also modulated by interaction of the protein with an inducer molecule that serves as a temperature messenger. The availability of the messenger would in turn be controlled by a temperature-responsive process (e.g., synthesis by a thermosensitive enzyme) serving as the central cellular thermometer. Although there is little evidence to support this model, it could explain one unusual feature of our data: the decline in absolute levels of yopE transcription with increases in levels of lcrF. Consider a model in which availability of the inducer is limited and both LcrF and the LcrF-inducer complex compete for binding sites upstream of the yopE gene. Under these conditions, the fraction of LcrF complexed with inducer would decline with overexpression of LcrF, decreasing absolute induction levels. Preliminary results with LcrF-containing extracts indicate no changes in in vitro binding activity with either growth temperature or reaction temperature, suggesting that competition for binding sites independent of activator activity may well occur. In this inducer model, the ability of LcrF and VirF to function in E. coli would be explained by the use of common temperature messengers in the different species. We are initiating a search for such molecules.

Alternative possibilities that have been suggested for a central E. coli thermometer include the DnaK protein, which stabilizes σ^{32} at elevated temperatures and could have a similar effect on other proteins (24), and the state of DNA supercoiling (56). The differences in dynamics between LcrF-mediated and heat-shock mediated regulation suggest that they may not share a common mechanism. Although mutations in a protein with histonelike properties do affect expression of genes regulated by VirF (23), and presumably
LcrF, we are unsympathetic to the supercoiling hypothesis, both because it is
difficult to understand the basis for regulation of specific genes by global changes
in DNA topology and because there is little evidence that the state of
supercoiling varies with temperature in a stable manner. Indeed, the major
result of the report most often cited as evidence for modulation of supercoiling
by temperature (43) is that compensating changes in linking number maintain a
fixed degree of supercoiling over a broad temperature range!

In addition to its role as a transcriptional activator, AraC has other
properties that could be sensibly incorporated into models of LcrF and VirF
function. For example, AraC represses its own transcription in the absence of
inducer by a mechanism that involves formation of a DNA loop by binding of
the protein to two operators in the *ara* regulatory region (30). A similar
mechanism controlling transcription of thermal regulatory proteins in the AraC
family could account for a variety of observations, including the sensitivity of
thermal regulation to supercoiling and the effect of mutations in genes
encoding putative DNA-bending proteins (55); DNA loop formation can be
either negatively or positively affected by bending proteins (83, 96) and is also
known to be dependent on supercoiling (50). AraC activator activity is also
modulated by interaction with another protein (cyclic AMP receptor protein
[83]). Given the complexity of the LCR regulation, the potential of such
interactions modulating LcrF activity should not be overlooked.

Unfortunately, there is little evidence to suggest autoregulation of *lcrF* and
*virF* transcription and fairly strong evidence against it. In *Y. pestis*, we observe
very weak thermal induction of an *lcrF* reporter (pUM4101). This induction is
not altered by the presence of an intact *lcrF*. In *Y. enterocolitica*, regulation of
virF transcription in response to temperature in a strain containing no other source of virF is observed with a virF::cat fusion (23). Although the virF gene in this fusion is disrupted close to the downstream end of the coding region, a highly conserved sequence is affected and a deletion downstream of this point is known to yield inactive LcrF. This result suggests that the observed regulation is not dependent on VirF protein.

Finally, we comment on two additional results: discovery of a putative DnaA-binding site immediately upstream of lcrF and virF and the apparent affinity of LcrF for membranes. Although its major role is in the initiation of chromosomal DNA synthesis, DnaA has also been implicated in the repression of transcription of several genes, including rpoH (encoding σ32) (147) and the dnaA gene itself (39). This repression may not be significant at physiological DnaA levels, however (10). Curiously, DnaA undergoes at least one reaction that is strongly temperature dependent, between 24 and 38°C: dissociation of ADP by binding of membrane lipid (119). This may be an important mechanism for the reactivation of DnaA initiation activity, but its effect on repressor activity has not been determined. Given these properties, involvement of DnaA in control of lcrF and virF transcription is at least conceivable.

The results of our attempts to determine the subcellular location of LcrF suggest that it and similar proteins may have an affinity for outer membrane that interferes with localization by fractionation methods. Given their lack of a signal sequence, their inclusion in the AraC family, and the demonstrated DNA-binding properties of LcrF, we think it very likely that these proteins are cytoplasmic. Obviously, techniques other than standard cell fraction methodology will be required to demonstrate this.
Acknowledgments

We thank Ellen Nalavika, Zhanzhi Guan, and Steve Goguen for their assistance and Stuart Austin, Stanley Tabor, and Martin Marinus for their generous gifts of strains.

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Addendum

During review of this report, two other relevant articles have been published. Lambert de Rouvroit et al. (76) report the purification of Y. enterocolitica VirF, along with gel shift assays and footprinting. They also present additional genetic evidence in support of the supercoiling hypothesis. Skurnik and Toivanen (121) report on the role of Y. pseudotuberculosis LcrF in regulation of the yadA gene.
TABLE 2.1. Bacterial strains and plasmids

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<tr>
<td>JM101</td>
<td>K12 supE thi Δ(lac-proAB) F' traD36 proAB lacIqZM15</td>
<td>(155)</td>
</tr>
<tr>
<td>K38</td>
<td>HfrC λ</td>
<td>(137)</td>
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<tr>
<td>BL21(DE3)</td>
<td>B F⁻ ompT rB⁻mB⁻ (λ DE3)</td>
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<tr>
<td>UME4077</td>
<td>CSH50 (pUM4090)</td>
<td>This study</td>
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<td>UME4079</td>
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<td>CSH50 (pUM4090, pNF001)</td>
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<td>UME4144</td>
<td>CSH50 (pUM4086)</td>
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### Plasmids

<table>
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<th>Plasmid</th>
<th>Description</th>
<th>Reference</th>
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<tbody>
<tr>
<td>pUT1007</td>
<td>lcrE1 mutant of pCD1; Lcr&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(158)</td>
</tr>
<tr>
<td>pGW600</td>
<td>encodes high levels of Mu repressor; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(73)</td>
</tr>
<tr>
<td>pMC874</td>
<td>Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(15)</td>
</tr>
<tr>
<td>pGP1-2</td>
<td>Expression of T7 polymerase under control of λ promoter P&lt;sub&gt;L&lt;/sub&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>S. Tabor</td>
</tr>
<tr>
<td>pLG338</td>
<td>Derivative of pSC101; Km&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>S. Austin</td>
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<tr>
<td>PBR322</td>
<td>ColE1 replicon; Ap&lt;sup&gt;r&lt;/sup&gt; Tc&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(9)</td>
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<tr>
<td>pNK1915</td>
<td>Derivative of pBR322 containing T1 terminator upstream of promoterless lacZ; Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>N. Kleckner</td>
</tr>
<tr>
<td>pUT2016</td>
<td>8.3-kb BamHI-EcoRI fragment containing yopE-lacZ sequences from pCD1 of UTP1422 inserted into pMC874</td>
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<td>pUT2017</td>
<td>4.8-kb BamHI G fragment of pCD1 inserted into pACYC184</td>
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<td>pUT2027</td>
<td>4.8-kb BamHI G fragment of pCD1 inserted into pBR322</td>
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<td>pUM4087</td>
<td>1.0-kb HindIII-DraI fragment of pUT2017 containing lcrF sequences inserted into SmaI-digested Bluescript KS M13&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>This study</td>
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<td>8.3-kb EcoRI-SalI fragment containing yopE-lacZ sequences from pUT2016 inserted into pBR322</td>
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<td>pUM4101</td>
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<td>Vector</td>
<td>Description</td>
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</tr>
<tr>
<td>--------</td>
<td>-------------</td>
<td></td>
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<td>pNF001</td>
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<td>pNF002</td>
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<td>pNF409</td>
<td>1.6-kb deletion of pNF001</td>
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<tr>
<td>pNF15</td>
<td>1.7-kb deletion of pNF001</td>
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<td>pUM4106</td>
<td>280-bp HindIII-HaeIII fragment of pNF409 containing sequences around start codon of lcrF inserted into SmaI-digested pNK1915</td>
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<td>pUM4186</td>
<td>pUM4180 deleted for lacZYA sequences (to BclI site)</td>
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<td>pUM4200</td>
<td>680-bp EcoRI-PstI fragment containing yopE sequences inserted into EcoRI-PstI-digested Bluescript KS M13&lt;sup&gt;+&lt;/sup&gt;</td>
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### TABLE 2.2 Proteins with significant homology to LcrF

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<tr>
<th>Protein</th>
<th>Species</th>
<th>Operon or gene regulated</th>
<th>Inducer</th>
<th>% Identity with LcrF</th>
<th>Ref.</th>
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<tr>
<td><strong>Catabolism</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>AraC</td>
<td><em>E. carotovara</em></td>
<td>Arabinose operon</td>
<td>Arabinose</td>
<td>28.0</td>
<td>(78)</td>
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<td>AraC</td>
<td><em>E. coli</em></td>
<td>Arabinose operon</td>
<td>Arabinose</td>
<td>23.2</td>
<td>(95)</td>
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<td>AraC</td>
<td><em>S. typhimurium</em></td>
<td>Arabinose operon</td>
<td>Arabinose</td>
<td>22.9</td>
<td>(19)</td>
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<td>RhaR</td>
<td><em>E. coli</em></td>
<td>Rhamnose operon</td>
<td>Rhamnose</td>
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<td>(141)</td>
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<td>RhaS</td>
<td><em>E. coli</em></td>
<td>Rhamnose operon</td>
<td>Rhamnose</td>
<td>21.4</td>
<td>(141)</td>
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<td>XyIS</td>
<td><em>P. putida</em></td>
<td>Plasmid-borne xylene operon</td>
<td>Benzoate</td>
<td>20.9</td>
<td>(65)</td>
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<td>MelR</td>
<td><em>E. coli</em></td>
<td>Melibiose operon</td>
<td>Melibiose</td>
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<td><strong>Virulence</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td>VirF</td>
<td><em>Y. enterocolitica</em></td>
<td>Plasmid-borne</td>
<td>?&lt;sup&gt;c&lt;/sup&gt;</td>
<td>97.8</td>
<td>(21)</td>
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<tr>
<td>Rns</td>
<td>Enterotoxigenic</td>
<td>Colonization factors</td>
<td>?</td>
<td>24.2</td>
<td>(14)</td>
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<td></td>
<td><em>E. coli</em></td>
<td>CS1 and CS2</td>
<td></td>
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<tr>
<td>VirF</td>
<td><em>S. flexneri</em></td>
<td>Plasmid-borne virulence genes</td>
<td>?</td>
<td>23.5</td>
<td>(112)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Regulatory proteins controlling operons involved in catabolism of carbon and energy sources

<sup>b</sup> Regulatory loci controlling expression of virulence genes

<sup>c</sup> Denotes inducer is unknown
Appendix

The following figures were removed from the original manuscript comprising Chapter II in accordance with the recommendations of the reviewers. The data in the figures were considered redundant as sequencing of the \textit{virF} gene in \textit{Y. enterocolitica} and identification of the VirF protein had been demonstrated previously by Cornelis et al. (21), however the experiments are completely detailed in Chapter II. The figures are included here as a record of the work accomplished.
FIG. 2A.1. Nucleotide sequence of lcrF. The sequence starts at the HindIII site in the BamHI G fragment of pCD1. The putative ribosome binding site is outlined. Sequences underlined once have been noted to exhibit limited homology to sequences found upstream of yopE and yopH of Y. pseudotuberculosis, and yop51 of Y. enterocolitica (21). A consensus DnaA binding site is underlined twice. The deduced amino acid sequence is given above the nucleotide sequence. The six amino acids which differ between LcrF and VirF of Y. enterocolitica are shown in bold type.
FIG. 2A.2. Deletion mapping of the lcrF gene. Plasmids containing deletions of the BamHI G fragment of pCD1 were transformed into CSH50 (pUM4090) and β-galactosidase assays performed on cultures incubated at 26°C and 37°C. Only clones showing a 6-fold increase in β-galactosidase at 37°C are scored as positive for induction. Lane numbers correspond to fluorograph at right showing proteins produced by the indicated deletion plasmids. Plasmids proteins were radiolabeled as described in Materials and Methods. Lane 1: Bluescript KS M13+.
CHAPTER III

INTRODUCTION

The results presented in this chapter were obtained during the course of my thesis work and relate to the main objective of identifying the temperature sensor in Y. pestis. However, they do not constitute complete bodies of work appropriate for publication and are presented here as a record of work accomplished. Results presented in section A demonstrate that lcrF-mediated transcription of a yopE::lacZ reporter fusion remains thermally regulated in a S. flexneri virR mutant, and section B details my attempts to identify lcrF-specific message in Y. pestis and E. coli.
SECTION A

LCRF-MEDIATED TRANSCRIPTION OF THE YOPE GENE IS STILL TEMPERATURE-DEPENDENT IN A SHIGELLA FLEXNERI VIRR MUTANT

Abstract

The effect of a virR mutation on the LcrF-mediated thermal induction of a yopE::lacZ operon fusion was investigated. β-galactosidase assays were performed on selected S. flexneri strains containing the lcrF gene and a yopE::lacZ reporter fusion incubated at 26 and 37°C. The absolute levels of β-galactosidase produced from the yopE reporter fusion at both temperatures increase 2-fold in the virR mutant independently of the presence of lcrF, however thermal induction remains intact.
Introduction

Mutations in the chromosomal gene virR of *S. flexneri* result in the constitutive expression of plasmid-borne virulence genes normally induced in response to temperature (88). virR is equivalent to hns, the *E. coli* gene for H-NS, a histone-like protein capable of condensing DNA (64). Because mutations in hns (then designated *drdX*), cause constitutive transcription of thermally-regulated fimbrial genes in *E. coli* (45), it has been suggested that temperature-induced changes in DNA topology are a specific signal used by pathogens to respond to their environment and that mutation of hns and virR affect expression of thermally regulated genes because they mimic the topological effects of increased temperature. However, Maurelli and colleagues have proposed an alternative model in which post-transcriptional steps in the synthesis of VirR protein, rather than supercoiling, are controlled by temperature (62). The fact that the presence of the gene for tRNA$_{1}$Tyr (tyrosyl transfer RNA) on a high-copy-number plasmid can partially complement a virR deletion mutation indicates this control may be exerted at the level of translation (62). In the Maurelli model, VirR protein would be produced in significant quantities only at low temperature, and would act as a repressor of transcription by binding to sites within vir-specific promoters (88, 62).

At the time virR was discovered, it was known that hns and virR mutations gave similar phenotypes in *E. coli* and *S. flexneri* in that they caused constitutive expression of genes normally repressed at 30°C. Given the likelihood that close virR homologues are present in all members of the Enterobacteriaceae, we reasoned that lcrF-mediated transcription of a *yop* gene might also be affected by VirR-like proteins. At this time, we had established
that the simple system consisting of lcrF and a yop reporter work well in E. coli (see Chapter II), and was thus very likely to function in Shigella. We had also established that transcription of the lcrF gene does not change with temperature, however, the posttranscriptional step(s) regulating lcrF expression were still unknown. Finally, we had also shown that VirF, another regulatory protein controlling the expression of vir genes with temperature in S. flexneri, is a member of the AraC family of transcriptional activators, and that this family also includes LcrF. Given these observations, I thought it worthwhile to determine if a yopE::lacZ fusion would be induced by lcrF in a temperature-dependent manner in an S. flexneri virR mutant.
Materials and Methods

Bacterial strains and plasmids. The strains used are described in Table 3.1. Plasmid pUM4100 consists of the 4.8-kb BamHI G fragment of pCD1, the native Yersinia pestis virulence plasmid, inserted into pLG338, a low-copy-number plasmid derived from pSC101 (126). The BamHI G fragment contains the entire lcrF gene. Plasmid pUM4091 consists of an 8.3-kb fragment containing yopE-lacZ sequences inserted into pBR322.

Media and growth conditions. Shigella flexneri strains were grown in Luria broth (LB) or on Luria broth agar (94). Ampicillin and kanamycin were used at concentrations of 500 and 50 μg/ml, respectively.

Determination of β-galactosidase levels. Overnight cultures of S. flexneri strains grown at 26°C in LB were used to inoculate duplicate 5-ml LB cultures to an optical density at 600 nm of 0.05. The cultures were then incubated in a shaking water bath at 26°C for one hour. At this time, half of the cultures were switched to 37°C, and all of the cultures were allowed to grow for an additional 2 h. β-Galactosidase levels were determined as described by Miller (94).

DNA methodology. To screen clones for their plasmid content, we utilized the method of Kado and Liu (70). Gel electrophoresis was carried out essentially as described by Maniatis et al. (85). Transformation of S. flexneri was performed using the cold CaCl₂ method of Dagert and Ehrlich (26).
Results

Induction of a yopE::lacZ fusion in an S. flexneri virR mutant. To determine if lcrF function would be affected by the virR mutation, β-galactosidase assays were performed on the strains described in Table 3.1 following incubation at 26°C or 37°C. The results are presented in Fig. 3.1 and show that the 26°C vs 37°C induction ratios for yopE expression in the presence of lcrF remain essentially constant whether or not the functional virR gene is present. The absolute β-galactosidase values increase approximately two-fold in the virR mutant as compared to wild type but this is independent of the presence of lcrF. Parallel control experiments with S. flexneri strain BS217, which is identical to the virR::Tn10 mutant except that virR is functional and Tn10 is inserted elsewhere in the chromosome, gave similar results (data not shown), indicating that lcrF-mediated induction of transcription of the yopE::lacZ fusion is not affected by the presence of Tn10.
Fig. 3.1. lcrF-mediated transcription of a yopE::lacZ fusion in S. flexneri. β-galactosidase assays were performed on strains 2457T (left) or BS189 (right) containing the yopE reporter plasmid pUM4091. A "+" indicates the presence of plasmid pUM4100, the source of the lcrF gene. Cells were incubated at either 26 or 37°C and β-galactosidase levels determined according to Miller (94).
Discussion

The impetus for these experiments was the discovery of the virR gene in S. flexneri, and the observation that the expression of a vir::lac transcriptional fusion, normally induced by temperature, was now derepressed at low temperature (88). We had thought the virR mutation might effect lcrF-mediated transcription of a yopE::lacZ fusion due to the fact that virR is an analog of the hns gene in E. coli, and mutations in these genes have the same effect on virulence gene induction. Therefore, temperature-induced changes in the topological state of the DNA might represent a general mechanism used by pathogenic bacteria to sense their environment, and lcrF-mediated thermal regulation might be affected by an alteration in virR even though it is expressed in an S. flexneri background. In addition, LcrF and VirF, the transcription activator responsible for the expression of virulence genes in S. flexneri, are members of a family of regulatory proteins and might be affected similarly by VirR. However, our results clearly indicate that lcrF-mediated transcription of yopE remains thermally regulated even in the virR mutant.

It is possible that lcrF-mediated control of yopE transcription may not respond to the changes elicited by the virR mutation. The assortment of histone-like proteins in S. flexneri and the Yersinia may be dissimilar and unable to complement one another. Mutations in the Y. enterocolitica ymoA gene, whose product also has H-NS-like properties, increase the level of β-galactosidase produced from a yop: lacZ operon fusion at 26°C in a VirF+ background, and are proposed to effect DNA 'compaction' (23). Therefore, it appears that yop promoters are not insensitive to mutations in a gene affecting the topological state of the DNA. It is interesting to note, however, that
transcription of the \textit{yop} fusion still increases with temperature in the \textit{ymoA} mutant (23). It appears that even in the \textit{Yersinia}, these mutations do not seem to significantly interfere with VirF/LcrF-mediated thermal induction and it is unlikely they represent a specific mechanism of gene regulation.
<table>
<thead>
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<th>Strain</th>
<th>Relevant genotype and/or description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2457T</td>
<td>mal xyl lac man⁺ rha fuc nic</td>
<td>T. Maurelli</td>
</tr>
<tr>
<td>BS217</td>
<td>Tc⁺ transductant of 2457T using P1vir lysate grown on E. coli strain NK6051 (purE::Tn10)</td>
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<tr>
<td>BS189</td>
<td>Tc⁺ transductant of 2457T using p1L4 grown on S. flexneri strain BS185 (virR1::Tn10)</td>
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SECTION B

ATTEMPTS TO OBSERVE LCRF-SPECIFIC RNA
Introduction

Following the identification of lcrF as the transcription activator responsible for the thermal induction of the yop regulon, we focused our attention on identifying the thermally sensitive step controlling lcrF expression. As a first step toward this goal, we constructed several lcrF::lacZ operon fusions in order to determine if transcription of lcrF was itself thermally regulated. The construction of these fusions and the results of β-galactosidase assays performed on Y. pestis and E. coli strains containing them is presented in Chapter II. Our results indicated that the level of transcription of the lcrF gene does not change with temperature, implying a posttranscriptional step in the control of lcrF expression.

Two of these fusion constructs, pUM4106 and pUM4180 (Chapter II, Fig. 2.4), were prepared as a pair, identical except for the point of fusion of the lcrF gene and the promoterless lacZYA cassette. For pUM4106, the point of fusion is only 74 bp downstream of the start site of translation of the lcrF gene, retaining only 24 N-terminal codons of lcrF, while for pUM4180, the point of fusion is 18 bp downstream of the lcrF stop codon. Both plasmids contain 208 bp of pCD1 sequence upstream of the lcrF coding region. Similar levels of β-galactosidase are produced by the two fusion constructs, making it unlikely that lacZ transcription is being driven by promoter sequences within the lcrF coding region. However, we wanted to determine the start site of lcrF transcription in these fusion constructs to confirm the results of the β-galactosidase assays. Northern blotting was performed to detect the lcrF message and primer extension assays were carried out on RNA isolated from E. coli strains.
containing either of the two fusion plasmids to identify the 5' end(s) of the $lcrF$ message.
Materials and Methods

Bacterial strains and plasmids. Descriptions of plasmids pUM4106 and pUM4180 can be found in Chapter II, and complete maps are presented in Fig. 2.4.

Construction of lcrF::lacZ reporter plasmids. See Chapter 2.

Media and growth conditions. Y. pestis strains were grown in tryptose blood agar base (TBAB) broth (1% tryptose, 0.3% beef extract, 0.5% NaCl) for routine culture or the defined medium (TMH) of Zahorchak and Brubaker (159) as modified by Straley et al. (129) supplemented with 2.5 mM CaCl$_2$ when indicated. TBAB (Difco) supplemented with 2.5 mM CaCl$_2$ was used for growth on solid medium. E. coli strains were grown in Luria broth (LB) or M9 liquid medium, or on Luria broth agar (94). Ampicillin was used at a concentration of 500 µg/ml unless otherwise noted.

Overnight cultures of Y. pestis strains grown at 26°C in TMH were used to inoculate duplicate 5-ml TMH cultures (with and without added CaCl$_2$) to an optical density at 600 nm of 0.1. The cultures were then incubated in a shaking water bath at 26°C until they reached an optical density at 600 nm of 0.25 (approximately 2.5 h). At this time, half of the cultures were switched to 37°C, and all of the cultures were allowed to grow for an additional 4 h. E. coli strains containing plasmids were grown in LB following the protocol described above, except that cultures were allowed to grow for 2 h after the temperature switch.

Preparation, electrophoresis, and Northern blotting of RNA. RNA was isolated from selected Y. pestis or E. coli strains grown as described for the determination of β-galactosidase. Several methods were used to extract the
RNA. The hot-phenol method is described in the Material and Methods section of Chapter 4. The protoplasting method of Summers (136) was carried out as described (2). The guanidinium/cesium chloride centrifugation method of MacDonald was carried out as described (84), except that the guanidinium thiocyanate solution was added to cell pellets which had been obtained after centrifugation. Electrophoresis of RNA samples through duplicate formaldehyde-agarose gels and transfer of RNA to Zeta Bind nylon membranes (Bio-Rad Laboratories) in 10XSSC (1XSSC is 0.15M NaCl, 0.02M citrate) was as described in (85).

Northern blotting was carried out at 46°C using a pre-hybridization solution of 6XSSPE (1XSSPE is 0.15M NaCl, 0.01M NaH2PO4·H2O, 0.0013M EDTA), 5X Denhardt's, 0.5% SDS, 50% deionized formamide, and 100 μg/ml of denatured, fragmented salmon sperm DNA. The hybridization solution was identical except the Denhardt's was omitted. When oligonucleotide probes were used for hybridization, the formamide concentration was calculated for each RNA:DNA hybrid (16) such that the hybridization temperature of 46°C was equal to Tm minus 12 (85). Blots were washed at room temperature in 6XSSC, 0.5% SDS, dried and exposed to Kodak X-Omat AR film at -70°C with one intensifying screen.

Preparation of probes. A 363 bp fragment corresponding to bps 246-609 of the lcrF coding region was isolated from bisacrylylcystamine (BAC)-acrylamide gels as described (51), labeled by the random primer extension technique (59), and purified by passage over a Sephadex G-50 column (85). An lcrF-specific probe of the sequence 5'-CCCATTCTAATTTAATAATCTCTAG-3' complementary to nucleotides 10-34 of the lcrF coding region was prepared by
Genosys (The Woodlands, Texas). Oligonucleotide probes were labeled at the 5' end by phosphorylation with polynucleotide kinase and [\gamma\textsuperscript{32P}]ATP (5,000 Ci/mmol; Amersham) as described (85). The radioactive probe was purified by electrophoresis through 19% acrylamide/7M urea gels and subsequent elution from a C\textsubscript{18} Sep-Pak cartridge (Waters, Milford, MA).

**Primer extension.** Labeled oligonucleotide primers were hybridized to total RNA isolated from selected *E. coli* strains, extended with reverse transcriptase and the extension products analysed on 8% acrylamide/7M urea gels as described (85).
Results and Discussion

Initially, a large amount of time was spent trying several methods of RNA isolation. A method in which hot, acidic phenol is added directly to cells in liquid media followed by several rounds of extraction was found to be the simplest method giving good reproducibility and sharp ribosomal RNA bands upon ethidium bromide staining of formaldehyde/agarose gels. Subsequently, using specific restriction fragments or oligonucleotides as probes, Northern blotting was performed with RNA isolated from pCD1+ and pCD1- Y. pestis with or without pUM4180 grown at 26 and 37°C. No lcrF-specific RNA could be detected in these experiments (data not shown). To minimize possible loss of the lcrF RNA during transfer from the gel to the nylon membrane, attempts were made to detect the RNA by hybridization directly in formaldehyde/agarose gels which had been dried. This method was not successful primarily due to the inability to reduce a high background due to nonspecific binding of the probes to the dried gel.

As we wished to identify the 5' ends of the lcrF message, primer extension assays were performed on RNA isolated from E. coli strain CSH50 (Lac-) containing pUM4106 or pUM4180 grown at 26°C or 37°C. These experiments were unsuccessful in detecting any lcrF-specific extension products.

Our inability to detect lcrF-specific message using two standard techniques is in agreement with a result presented in Chapter IV. We describe an experiment in which we observe that the presence of lcrF sequences substantially decrease the levels of a plasmid-length RNA produced by T7 RNA polymerase. We note the fact that the lcrF gene contains a large number of
putative sites for RNase E, an endoribonuclease involved in general RNA stability, and suggest the presence of these sites may contribute to the unusual instability of $lcrF$ message. At present, the available technology does not appear to be sufficient to enable us to detect $lcrF$ message in $Y. \text{pestis}$.

Acknowledgements

I would like to thank Danuta Klimuszo for completing an experiment during my maternity leave.
CHAPTER IV

TEMPERATURE SENSING IN YERSINIA PESTIS: TRANSLATION OF THE LCRF PROTEIN IS THERMALLY REGULATED
Abstract

The lcrF gene of Yersinia pestis encodes a transcription activator responsible for inducing expression of several virulence-related proteins in response to temperature. The mechanism of this thermoregulation was investigated. An lcrF clone was found to produce much lower levels of LcrF protein at 26 than at 37°C in Y. pestis, although it was transcribed at similar levels at both temperatures. High level T7 polymerase-directed transcription of the lcrF gene in Escherichia coli also resulted in temperature-dependent production of the LcrF protein. Pulse-chase experiments showed that the LcrF protein was stable at 26 and 37°C, suggesting that translation rate or message degradation is thermally controlled. The lcrF mRNA appears to be highly unstable and could not be reliably detected in Y. pestis. Insertion of the lcrF gene into plasmid pET4a, which produces high levels of plasmid-length RNA, aided detection of lcrF-specific message in E. coli. Comparison of the amount of LcrF protein produced per unit of message at 26 and 37°C indicated that the efficiency of translation of lcrF message increased with temperature. mRNA secondary structure predictions suggest that the lcrF Shine-Dalgarno sequence is sequestered in a stem-loop. A model in which decreased stability of this stem-loop with increasing temperature leads to increased efficiency of translation initiation of lcrF message is presented.
Introduction

There are now many examples of pathogenic bacteria which modulate the expression of virulence-related genes in response to temperature. By distinguishing between environmental temperatures and the body temperatures of warm-blooded hosts, this regulation presumably promotes adaptation to these distinct environments, especially via induction of genes required for protection from antibacterial host defenses. The lcrF genes of *Yersinia pestis* and *Yersinia pseudotuberculosis*, and the virF gene of *Yersinia enterocolitica*, are closely related homologs (98% sequence identity) each involved in thermal control of a suite of plasmid encoded virulence genes, collectively termed *yops*. In each of the *Yersinia* species, the regulatory genes and the *yops* reside on a plasmid of about 75 kb. In *Y. pestis* KIM, used in our laboratory, this plasmid is designated pCD1. The *yop* genes encode secreted proteins, including a tyrosine phosphatase (48) and serine threonine kinase (38) thought to be exported to the cytoplasm of mammalian cells with which the bacteria make contact. Transcription of the *yop* genes requires lcrF or virF, and is strongly induced at 37 compared with 26°C (21, 60). The level of *yop* expression at 37°C can also be modulated by other environmental factors, particularly Ca²⁺ concentration (4, 105).

The thermally sensitive step(s) in the pathway of *yop* gene expression has not yet been clearly identified. lcrF and virF are known to encode transcription activators that bind to specific sequences upstream of the *yop* genes and are required for *yop* expression (21, 60, 76). Thus, one potential mechanism for thermal induction of *yop* gene transcription is increased transcription of lcrF and virF at elevated temperature. Both experiments with virF::cat operon
fusions and measurements of virF mRNA on Northern (RNA) blots indicate that such induction does occur in Y. enterocolitica (21, 23) In contrast, lcrF::lacZ operon fusions in Y. pestis show that lcrF transcription is insensitive to temperature between 26 and 37°C, implying that a posttranscription mechanism may modulate LcrF abundance or specific activity in response to temperature (60). There is evidence that a posttranscriptional mechanism also operates with VirF in Y. enterolitica, despite the apparent modulation of virF transcription: forced transcription of virF driven by a heterologous promoter causes only weak induction of yops at 26°C, but causes strong induction at 37°C (76). There is one observation that argues against thermally induced changes in specific activity of the VirF and LcrF proteins as the major mechanism of yop induction: temperature has little if any effect on the ability of these proteins to bind at their normal sites upstream of yop genes. However, this does not completely rule out models in which these proteins are thermally activated: activator proteins which bind their normal target sequences in both active and inactive forms have been described previously (100, 146) and binding constants for the LcrF and VirF proteins at both temperatures have not been carefully measured.

Attempts to identify genes involved in the regulation of virF transcription by temperature failed to discover a classical activator or repressor protein. However, they did lead to the discovery of an interesting gene, ymoA, in Y. enterocolitica. Mutations in this gene affect the expression of both virF and yop genes presumably by altering DNA topology (23). Because ymoA mutations apparently have a global effect on chromatin structure (23), these observations must be interpreted cautiously.
In summary, current evidence indicates that thermal regulation of activator gene transcription may have a role in regulation of yop induction in *Y. enterocolitica* but is probably not important in *Y. pestis*; some thermally sensitive step occurs subsequent to activator transcription in both species; ability of the activator proteins to bind DNA appears to be independent of temperature in the relevant range for both species; and changes in chromatin structure affect the induction pathway.

Because we have not observed thermal induction of *lcrF* transcription in *Y. pestis*, our recent efforts have been focused on identification of some other thermally sensitive process affecting LcrF abundance or specific activity. In this report, we present evidence that the synthesis of LcrF in both *Y. pestis* and *Escherichia coli* is strongly regulated by a thermally sensitive posttranscriptional mechanism, most likely controlling translation of the *lcrF* mRNA. We also present a model in which destabilization of an mRNA secondary structure sequestering the *lcrF* Shine-Dalgarno (SD) sequence is the thermosensitive element regulating LcrF synthesis.
Materials and Methods

Bacterial strains and plasmids. The plasmids used are shown in Fig. 4.1. *E. coli* BL21(DE3) (135) was utilized to direct overexpression of *lcrF*. DE3 is a lambda lysogen containing the T7 polymerase gene under control of the *lacUV5* promoter, conferring isopropyl-β-D-thiogalactopyranoside (IPTG) inducibility upon polymerase expression. The *Y. pestis* strains employed were KIM5 (pCD1+, *Lcr*+) and KIM6 (pCD1-, *Lcr*−). The *Lcr* designation refers to the low-calcium response, a *Y. pestis* phenotype characterized by the induction of the *yop* gene set and the eventual cessation of growth following incubation at 37°C in media low in Ca²⁺. pCD1 is the native *Y. pestis* 75-kb virulence plasmid which contains the structural genes for the Yops and genes involved in *yop* expression, including *lcrF*.

DNA methodology. Large-scale plasmid preparation and analysis, DNA techniques, and transformation of *E. coli* were as described previously (60).

Plasmid construction. Plasmid pUM4087 is a 1.0-kb *HindIII*-DraI fragment of pCD1 containing *lcrF* sequences inserted into *SmaI*-digested Bluescript KS M13⁺ (60). Plasmid pUM4087B was made by digesting plasmid pUM4087 to completion with *HindIII*, followed by ligation to a *HindIII*-BamHI adaptor (New England Biolabs, Beverly, Mass.). This destroyed the *HindIII* site contained within the multiple cloning site downstream of the *lcrF* sequences and added an additional BamHI site. The subsequent BamHI fragment from pUM4087B containing *lcrF* sequences was inserted into BamHI-digested pET4a (135) to make pET4a-F1 (Fig. 4.1).
Media and growth conditions. *E. coli* strains were grown in M9 liquid medium, or on Luria broth agar (94). The enriched medium of Tabor (137) was used for the growth of cells during the purification of LcrF. *Y. pestis* strains were grown in the defined medium (TMH) of Zahorchak and Brubaker (159), as modified by Straley (129). When temperature-shift experiments are conducted in Ca²⁺-deficient TMH, growth rates of *Y. pestis* at 26 and 37°C are similar, until growth restriction begins 2.5 to 3 h after the temperature shift. Tryptose blood agar base (Difco) supplemented with 2.5 mM CaCl₂ was used for growth on solid medium. Ampicillin was used at a concentration of 200 μg/ml.

Induction of LcrF. Overnight cultures of *E. coli* strains grown at 26°C in M9 were used to inoculate duplicate 5-ml M9 cultures to an optical density at 600 nm (OD₆₀₀) of 0.2. Cultures were incubated in a shaking water bath at either 26 or 37°C to an OD₆₀₀ of 0.5 at which time IPTG was added to a final concentration of 1 mM. The cultures at 37°C were placed on ice 1 h after IPTG addition. To normalize protein production to bacterial growth rate rather than the absolute time of postinduction incubation, the 26°C cultures were incubated until their OD₆₀₀ equaled that of the 37°C cultures (approximately 2 h after IPTG addition), at which time they were placed on ice. Cells were pelleted rapidly in a microcentrifuge and immediately resuspended in lysis buffer (100 mM potassium phosphate [pH 7.4], 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol) (148) to a concentration of approximately 1 mg of protein per ml.

Cultures of *Y. pestis* strains were grown at 26°C overnight in TMH without added CaCl₂ and used to inoculate duplicate 10-ml cultures of the same medium to an OD₆₀₀ of 0.1. Incubation was continued in a shaking water bath at 26°C until the OD₆₀₀ of the cultures reached 0.25. At this time, half of the cultures
were switched to 37°C, and all of the cultures were incubated for an additional 3 h. Under these conditions, the growth rate of *Y. pestis* at 26 and 37°C is similar until growth of the 37°C cultures begins to slow near the end of the incubation period. Cultures were then harvested as described above for *E. coli* except that cell pellets were resuspended in lysis buffer to a concentration of approximately 10 mg of protein per ml.

**Preparation of LcrF antiserum.** Two rabbits were injected subcutaneously with 250 µg of purified LcrF (see Results) in the presence of Freund's complete adjuvant. Animals were subsequently boosted at 2 and 4 weeks with 100 µg of LcrF in the presence of incomplete Freund's adjuvant. Antibody production was monitored by detection of LcrF on immunoblots of whole cell lysates of BL21(DE3) containing pNF28.

**Electrophoresis of proteins.** Analysis of proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed the procedure of Laemmli (75).

**Immunoblotting.** Proteins separated by SDS-polyacrylamide gel electrophoresis were electrophoretically transferred to Immobilon P nylon membranes (Millipore, Bedford, Mass.). LcrF was detected by incubating membranes with dilutions of anti-LcrF antiserum preadsorbed against a whole-cell suspension of BL21(DE3, Bluescript KS M13+). To detect LcrF in *Y. pestis* lysates, the antiserum was affinity purified in small quantities with membrane-bound antigen (106). β-Lactamase was detected with anti-β-lactamase antibodies (5 Prime-3 Prime, Inc., Boulder, Colo.). Affinity-purified goat anti-rabbit immunoglobulin G conjugated with alkaline-phosphatase (Bio-Rad Laboratories, Richmond, Calif.) was used as the second antibody. Protein-
antibody complexes were detected by either staining with 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (Bio-Rad), or by chemiluminescence, with the substrate Lumi-Phos 530 (Boehringer Mannheim) in conjunction with exposure of the blot to X-ray film (Kodak X-Omat AR). Relative amounts of LcrF in blots visualized by chemiluminescence were determined by densitometry of the resulting films, with a Zeineh soft laser scanning densitometer (Biomed Instruments, Inc., Fullerton, Calif.). Only samples processed within the same blot were used in making these comparisons.

**Pulse-chase.** Five-milliliter cultures of selected *E. coli* strains were grown in M9 supplemented with a 0.01% solution of 18 amino acids (minus cysteine and methionine) and treated with IPTG as described under "Induction of lcrF" except that when the cultures had reached an OD₆₀₀ of 1.0, rifampin was added to a final concentration of 20 µg/ml. Fifteen minutes after the addition of rifampin, cells were pulsed with 15 µCi of [³⁵S]methionine per ml (1,000 Ci/mmol). The cells were chased by adding a 500-fold excess of unlabeled methionine. Samples were withdrawn at the indicated times, added to tubes chilled in an ice-water slurry, and spun at 4°C to pellet the cells. Cell pellets were resuspended in electrophoresis sample buffer and stored at -20°C until use.

**Preparation, electrophoresis, and Northern blotting of RNA.** Ten-milliliter M9 cultures of selected *E. coli* strains were treated as described under "Induction of lcrF". Total cellular RNA was prepared by adding 1 ml of culture to a tube containing a mixture of 1 ml of TES-SDS (50 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA, 50 mM NaCl, 1% SDS) and 2 ml of phenol (equilibrated with acetate buffer to pH 5.0) which had been incubated at 95°C. The entire contents of the
tube were vortexed for 1 min, incubated at 65°C for 5 min, vortexed again briefly, and spun at 10,000 rpm in an SS-34 rotor for 10 min (11,950 × g). A total of 1.8 ml of the upper phase was removed, mixed with 4 ml of cold ethanol, and incubated at -20°C to precipitate the RNA. Samples were spun as before. The RNA samples were electrophoresed through formaldehyde-agarose gels as described in reference (113).

RNA was transferred to Zeta Bind nylon membranes (Bio-Rad Laboratories) in 101SSC (1SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 75 mm Hg for 1 h with a pressure blotting apparatus (PosiBlot, Stratagene, La Jolla, Calif.). After transfer, blots were allowed to air dry and were stored at room temperature.

A 1.0 kb BamHI fragment was isolated from digests of pUM4087B and radiolabeled with [α-32P]dCTP (3,000 Ci/mmol) by the random primer extension technique (59). This fragment contains the entire lcrF gene including 210 bp upstream of the lcrF start codon (206 bp of pCD1 sequence plus 4 bp of vector sequence) and 50 bp downstream of the lcrF termination codon (18 bp of pCD1 sequence and 32 bp of vector sequence). Northern blotting of RNA was carried out as described in reference (113). Blots were dried and exposed to Kodak X-Omat AR film overnight at -70°C, with one intensifying screen. For relative quantitation of message levels, autoradiographs were scanned with the Zeineh laser densitometer. To allow normalization of message levels to the amount of 16S rRNA in the crude RNA preparations used in these experiments, relative amounts of the rRNA were estimated by comparing intensities of the corresponding bands in dilution series on ethidium bromide-stained gels.
**Computer analyses.** RNA secondary structure predictions were determined with the MFOLD and SQUIGGLES programs as implemented in the Genetics Computer Group sequence analysis application package (version 7.2; University of Wisconsin, Madison,). These programs use the general algorithm of Zuker (162) and Zuker and Jaeger (68, 67) with the energy rules developed by Freier (36) and Turner (142, 143) to determine optimal and suboptimal secondary structures of RNA.

**Image processing.** Images of blots, gels, and X-ray films exposed to radioactivity or chemiluminescence were acquired by a Microtek II XE desktop scanner (Microtek Lab, Inc., Torrance, Calif.) equipped with a custom-made transilluminator and Adobe Photoshop software (Adobe Systems Inc., Mountain View, Calif.). Brightness and contrast of the entire image were adjusted to reflect the appearance of the original as closely as possible, as would be done by traditional photographic methods. Where the order of gel lanes has been changed to improve clarity of the figure, a gap has been left between adjacent lanes.
Results

Purification of LcrF. The initial steps in the purification of LcrF from BL21(DE3) containing plasmid pNF28 (Fig. 4.1) were based on those developed by Lambert de Rouvroit for the purification of VirF (76). Cells (7 liters) in enriched media were incubated at 37°C to an OD600 of 0.5, at which time IPTG was added to a final concentration of 1 mM and incubation was continued for an additional 2 h. Cells were pelleted and resuspended in sodium phosphate buffer (pH 7.4) containing 1 M NaCl, and DNase I was added to a final concentration of 0.1 mg/ml. Cells were broken by passage through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 20,000 lb/in². Dialysis of the lysate and elution of the protein from a phosphocellulose (Whatman P11) column (2 by 20 cm) were as described previously (76). To obtain additional purification of LcrF, we introduced the following steps: the pooled fractions containing LcrF (40 ml) from the phosphocellulose column were dialyzed overnight against a buffer (P buffer) containing 50 mM sodium phosphate (pH 7.0), 2mM EDTA, 10% glycerol, and 100 mM NaCl and applied to a Mono S HR 5/5 column (5 by 50 cm) (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer. The column was washed with 5 ml of P buffer and eluted with a linear gradient (9 ml) from 100 mM to 1 M NaCl. LcrF eluted between 400 and 500 mM NaCl. The LcrF-containing fractions (3.5 ml) were pooled, passed over a Sephadex G-25M column (1.5 by 12 cm) equilibrated with P buffer (100 mM NaCl), and reloaded onto the Mono S column. The column was washed as before and eluted with the same linear gradient in a volume of 16 ml. The LcrF protein recovered
FIG. 4.1 Plasmid constructions used in lcrF analysis. (a) Structure of the BamHI G fragment of Y. pestis plasmid pCD1 and deletion derivatives contained in Bluescript KS M13-. The lcrF gene is oriented such that its transcription can be directed by T7 polymerase. (b) Structure of lcrF::lacZ operon fusion constructs. pUM4180 and pUM4186 were constructed in pNK1415 (120). This plasmid was erroneously identified as pNK1915 in reference (60). T1 ter denotes the four to five repeats of the T1 transcription terminator sequence present in this plasmid. The HindIII and DraI sites are shown to indicate the limits of the pCD1-specific DNA inserted into pNK1415 and are not reconstituted in pUM4180 and pUM4186. (c) Structure of the lcrF gene in plasmid pET4a-F1. This plasmid was constructed from plasmid pET4a (135). Pφ10 denotes the promoter sequences of the T7 10 gene. R1.1 denotes the RNase III cleavage site present in this plasmid. Arrows show directions of transcription. Heavy black line, sequence from the BamHI G fragment of pCD1 external to lcrF and other indicated features. Heavy gray line, vector sequence external to indicated features. All maps are drawn to a scale of 1 kb = 1.22 cm.
FIG. 4.2 Purification of LcrF. SDS-polyacrylamide gel analysis of a fraction from the final Mono S column. One microgram of protein was mixed with sample buffer and incubated at 95°C for 3 min prior to being loaded on the gel, which was subsequently stained with Coomassie Brilliant Blue. Lane M, protein size standards (in kilodaltons) as indicated. The image was acquired electronically as described in Materials and Methods.
from this column was judged to be greater than 95% pure from Coomassie blue-stained polyacrylamide gels (Fig. 4.2). The purified material was used to produce LcrF-specific polyclonal rabbit antisera as described above.

**Expression of LcrF in Y. pestis is temperature dependent.** We have previously shown that lcrF::lacZ operon fusions produce nearly equivalent cell-specific levels of β-galactosidase at both 26 and 37°C, independently of Ca²⁺ concentration (60). This result is independent of the point of fusion within the lcrF transcription unit: constructs which retain only 24 N-terminal codons of lcrF sequence behave similarly to constructs in which the point of fusion is further within, or slightly downstream of, the lcrF coding region. Thus, it is unlikely that lacZ transcription is driven by a promoter within the lcrF coding region. We were not able to determine whether this equivalent level of transcription led to equivalent levels of LcrF protein synthesis at both temperatures in our earlier studies. Using the specific anti-LcrF antibody produced for this study, we measured LcrF levels in Y. pestis, with both the native pCD1 plasmid and a plasmid construct used in the earlier fusion experiments as carriers of the lcrF gene.

Plasmid pUM4186 (Fig. 4.1) is derived from the lcrF::lacZ fusion plasmid pUM4180 and in addition to the lcrF coding region contains 206 bp upstream of the lcrF start codon, and 18 bp downstream of the termination codon. It differs from pUM4180 only by deletion of most of the lac sequences. Cultures of pCD1⁺ and pCD1⁻ Y. pestis with and without plasmid pUM4186 were grown in TMH without added CaCl₂ at 26 and 37°C, and LcrF levels were assayed in immunoblots (Fig. 4.3). Production of LcrF protein is temperature dependent
FIG. 4.3 Production of LcrF mediated by constitutively transcribed lcrF in Y. pestis. Immunoblot of cell lysates of selected Y. pestis strains. Cells were incubated at either 26 or 37°C in Ca²⁺-free TMH and processed as described in Materials and Methods. One hundred fifty micrograms of cell protein from each sample was loaded onto an SDS-polyacrylamide gel. The immunoblot was incubated with a 1:100 dilution of affinity purified anti-LcrF antibody for 2 h and developed by the chemiluminescence technique described in Materials and Methods. F: 50 ng of purified LcrF protein. The image was acquired electronically as described in Materials and Methods.
in strain KIM5, which contains only the native lcrF gene in pCD1, and remains so in the presence of pUM4186. As expected, LcrF is not detected regardless of the growth temperature in strain KIM6, which lacks the native pCD1 plasmid. Most importantly, LcrF is not detected in 26°C-grown KIM6 containing pUM4186 but is present in the same strain grown at 37°C. Several conclusions can be drawn from these experiments: (i) the amount of LcrF protein in native Y. pestis is strongly temperature dependent; (ii) this result holds even in a construct in which transcription of IcrF is known to be unaffected by temperature, and (iii) no genes of the native pCD1 virulence plasmid, with the possible exception of lcrF itself, are required for thermal regulation of LcrF levels.

**Expression of LcrF in E. coli is temperature dependent.** The latter conclusion and the observation that lcrF properly regulates the transcription of yop fusions in E. coli imply that LcrF expression in E. coli should also be temperature dependent. Moreover, this temperature effect should be independent of the promoter driving transcription. As a first approach to testing these predictions, we examined the effect of temperature on production of LcrF by the IPTG-induced T7 polymerase-driven overexpression construct used in production of LcrF for purification. lcrF transcription was induced in cultures of BL21(DE3) containing pNF28 at 26 and 37°C as described above. Induction caused a decrease in growth rate of about 20% at both temperatures (from 0.93/h to 0.78/h at 37°C; from 0.60/h to 0.45/h at 26°C), although the effect was slightly greater at the lower temperature. When extracts of these cultures were examined on Coomassie blue-stained polyacrylamide gels, we were unable to discern the LcrF protein in extracts of cultures induced at 26°C or in
uninduced cultures at either temperature. In 37°C-induced cultures, the LcrF protein formed a prominent band (Fig. 4.4A). This thermal sensitivity is not characteristic of the T7 polymerase transcription: a control protein (the Saccharomyces cerevisiae protein CP1) in a similar construct was synthesized as a roughly equivalent fraction of total protein at both temperatures (data not shown). To allow detection of low levels of LcrF in cultures induced at 26°C, immunoblots of cell lysates were prepared. As shown in Fig. 4.4B, small but detectable amounts of the protein are made at this temperature. Comparison of a dilution series made from these samples indicates that the amount of LcrF present in the 37°C-induced samples is about 16-fold greater than that in the 26°C-induced samples.

**Thermal control of LcrF expression does not require functional LcrF.** Data presented in Fig. 4.4C show that thermal regulation of LcrF levels in *E. coli* does not require a functional lcrF gene. Plasmid pNF409 (Fig. 4.1) is a deletion derivative of pNF001 and contains the entire lcrF gene in addition to approximately 2 kb of native pCD1 sequence upstream of lcrF. Plasmids pNF15, pNF14, and pNF13 are deletion derivatives of pNF409 containing varying amounts of the lcrF coding region in addition to the upstream pCD1 sequence, and, on the basis of sequence predictions, should produce proteins with molecular masses of 28.9, 22.7, and 15.9 kDa, respectively. None of these deletion alleles are capable of inducing the transcription of a *yopE::lacZ* reporter fusion in response to temperature, and all delete at least part of the region required for LcrF DNA binding activity (60). Each of these plasmids was introduced into BL21(DE3), and the expression of lcrF was induced at 26 and 37°C. As expected, the amount of full-length protein produced from
Fig. 4.4. T7 polymerase driven expression of LcrF in *E. coli*. All plasmids were contained in strain BL21(DE3) and all cultures were induced with IPTG at the indicated temperatures. Post-induction incubation was continued for an equal number of cell generations at both temperatures. Equal amounts of cell protein from each sample were applied to SDS-polyacrylamide gels. The images were acquired electronically as described in Materials and Methods.

**Panel A:** Coomassie blue stained whole-cell lysates carrying the LcrF over-producing plasmid pNF28. Note that LcrF (arrow) forms a major band at 37°C, but is not detectable at 26°C. Lane M shows protein size standards (kDa) as indicated.

**Panel B:** Immunoblot prepared from cultures containing the vector Bluescript KS M13+ or pNF28 as indicated. Note that a small amount of LcrF is visible in the 26°C pNF28 sample. The blot was stained using anti-LcrF antiserum pre-adsorbed against BL21 (DE3, Bluescript KS M13+).

**Panel C:** Immunoblot prepared from cultures containing plasmid pNF409, which produces full-length LcrF, and the series of lcrF deletion derivatives as indicated. Note that much less LcrF is produced at 26°C for all constructs, showing that the regulation does not require intact LcrF. Affinity purified anti-LcrF antibody was used for detection, and yields reduced background as compared with Panel B. The blots in both panels were developed by the alkaline phosphatase-tetrazolium reduction technique described in Materials and Methods.
plasmid pNF409 is significantly increased at 37°C. The truncated proteins produced from plasmids pNF15, pNF14, and pNF13 are detected only in extracts from cultures induced at 37°C and are clearly thermally induced. They may be made at somewhat lower levels than is the wild-type protein, although the reduced staining intensity may also be due to the deletion of major epitopes detected by the antiserum.

The LcrF protein is stable. A possible explanation for the low levels of LcrF observed at the lower temperature is that stability of the protein—rather than its rate of synthesis—is affected by temperature, resulting in rapid degradation at 26°C. To determine whether the LcrF protein produced at 26°C is unstable, a pulse-chase experiment was performed with E. coli BL21(DE3) containing plasmid pNF28 (Fig. 4.5). In addition to LcrF, the segment of Y. pestis DNA contained in this plasmid also includes the yscB locus, which lies downstream of lcrF and is transcribed independently (49). The locus encodes a 14 kDa protein. Labeling of LcrF is much more temperature sensitive than is labeling of YscB, again confirming the operation of specific thermoregulation. There is no significant change in the amount of labeled LcrF or YscB during the 30-min chase period at either temperature. Moreover, we see no hint of LcrF degradation products at the low temperature either in this experiment or in LcrF immunoblots from other experiments described here. We conclude that LcrF is stable through the temperature range relevant to these experiments.

Comparison of mRNA and protein levels. Taken together, the results presented above indicate that the cell-specific rate of LcrF synthesis is thermally modulated at the level of translation. Since there is no reason to believe that
FIG. 4.5 Effect of temperature on the stability of LcrF protein. Pulse-chase experiments were performed as described in Materials and Methods with BL21(DE3) containing pNF28. Cells were pulsed for 5 s with $[^{35}\text{S}]$methionine, and samples were withdrawn at the indicated times (in minutes). The arrow denotes LcrF. The protein with a molecular mass of approximately 14 kDa is the product of the $yseB$ gene (49) present downstream of $lcrF$ in pNF28. The band visible immediately below LcrF in the 37°C samples often appears when LcrF is expressed at high levels and is reactive with LcrF-specific antibodies, suggesting that it is the result of aberrant translation or proteolysis. The image was acquired electronically as described in Materials and Methods.
the properties of ribosomes are affected, this most likely involves changes in the effective concentration of the lcrF mRNA, or in the efficiency with which this mRNA can be translated. One simple experiment to distinguish between these possibilities is comparison of the concentrations of lcrF message and protein at 26 and 37°C. Interpretation of such an experiment is complicated by the fact that in prokaryotic systems the rate of message degradation, like the rate of premature transcription termination, is often coupled to the frequency of translation initiation (156). This coupling results in rapid degradation of poorly translated mRNA species. However, it has recently been shown that these processes can be substantially uncoupled when an operon is transcribed by T7 polymerase (18). Polarity is lost because the T7 enzyme is insensitive to the terminator-like structures normally inducing premature termination, and mRNA degradation is reduced because the long regions of naked mRNA resulting from the rapid T7 elongation rate (200 nucleotides per s versus 25 nucleotides per s for E. coli RNA polymerase [66] ) may allow the mRNA to fold into a nuclease-resistant form (18). Thus, an increase in translation efficiency should be more directly reflected by an increase in the ratio of protein to mRNA for T7-driven genes compared with genes driven by bacterial RNA polymerase.

Figure 4.6 shows the results of experiments intended to measure the efficiency of translation initiation for lcrF message produced by T7 polymerase. Because we have been unable to detect lcrF mRNA in Y. pestis despite numerous attempts and suspect it to be highly unstable, we took advantage of several features available in plasmid pET4a, one in a series of plasmid vectors developed by Studier (135). This plasmid contains the promoter for gene 10 of phage T7 upstream, and the R1.1 RNase III cleavage site downstream, of the
BamHI cloning site. It also contains a β-lactamase gene which provides selection and is in the proper orientation for message production by transcription initiated at φ10. Due to the processivity of T7 polymerase, transcription initiating at the φ10 promoter can continue completely around the plasmid several times, producing greater-than-plasmid-length RNAs (134). The presence of the RNase III cleavage site results in the conversion of these species to linear, plasmid length RNAs. A second discrete species corresponding to the distance between the φ10 transcription initiation site and the RNAse III site (157 nucleotides for pET4a) should also be produced for each round of transcription initiation. As has been demonstrated by Studier et al. (135), the plasmid-length species is produced at such high levels that it can be directly visualized on ethidium-bromide-stained agarose gels prepared from small quantities of crude cell extracts. We assumed that even a very unstable message would be detectable by hybridization if produced from this vector, and constructed plasmid pET4a-F1 by inserting a BamHI fragment containing the entire lcrF gene and its translation initiation signals into BamHI-digested pET4a (Fig. 4.1). As seen in panel A of Fig. 4.6, the vector pET4a does produce large amounts of plasmid-length RNA when induced with IPTG. However, pET4a-F1 produces no RNA species visible by ethidium bromide staining.

Panel B of Fig. 4.6 shows a Northern blot prepared from the gel of panel A and hybridized with a probe specific for lcrF. At both 26 and 37°C, two sizes of lcrF-specific message can be detected. These correspond to the major species expected from this construct: the larger is the length of the plasmid, and the
**Fig. 4.6.** Detection of lcrF-specific message and determination of relative translational efficiency.

**Panel A:** Total cellular RNA from BL21 (DE3) containing pET4a or pET4aF1. RNA was isolated from cultures incubated at 26°C or 37°C with or without IPTG addition as indicated. RNA was electrophoresed through a 1% formaldehyde-agarose gel and stained with ethidium bromide. The V (vector) indicates plasmid-length message produced by T7-directed transcription of pET4a.

**Panel B:** Northern blot of the pET4a-F1 lanes of Panel A. The [32P]-labeled probe was a 1.0-kb fragment containing the entire lcrF gene. (A) denotes the band corresponding to plasmid-length message. The identity of the smaller band (B) is discussed in the text. Relative amounts of each species were determined by densitometry.

**Panel C:** Histogram comparing the protein/mRNA ratios of β-lactamase and LcrF at 26 and 37°C. Relative amounts of β-lactamase and LcrF protein in aliquots of the same samples used for RNA analysis in Panels A and B were determined by densitometry of film exposed to chemiluminescence-developed immunoblots.

The images were acquired electronically as described in Materials and Methods.
smaller is the distance between the φ10 promoter and the RNase III cleavage site in pET4a-F1. The larger species encodes both LcrF and β-lactamase, while the smaller species encodes only LcrF. When the amount of lcrF-specific RNA in the two bands is quantitated by densitometry and normalized to the amount of 16S rRNA (see panel A), essentially equal amounts are present at both temperatures. This is consistent with the expectation that transcription from the φ10 promoter in this construct is not temperature sensitive. Panel C shows a comparison between the ratio of β-lactamase to large mRNA and that of LcrF protein to lcrF-specific mRNA from this experiment. Although the five fold temperature-specific induction of LcrF protein is somewhat less than that observed in experiments with other T7 constructs reported above, it is clear that the ratio of LcrF protein to mRNA is substantially increased at 37°C, indicating increased translation efficiency at this temperature.
Discussion

We have previously reported that *lcrF*-regulated genes respond normally to thermal induction under conditions in which transcription of *lcrF* does not change (60), and Lambert de Rouvroit et al. (76) have shown that forced transcription of the *lcrF* homolog *virF* at low temperatures does not cause induction of *virF*-regulated genes. These results implied that either the synthesis or specific activity of the LcrF and VirF proteins was modulated by temperature, independently of transcription. The primary and, to us, surprising observation reported here is that the amount of LcrF protein—rather than the activity of LcrF molecules—is strongly thermally regulated. This regulation is observed not only in *Y. pestis*, but even when an *lcrF* clone containing only 208 bp of 5' sequence is transcribed by T7 polymerase in *E. coli*. Thermal regulation in the T7 polymerase-driven constructs is unaffected by gross mutation of the *lcrF* gene, including truncation of half of the coding region from the 3' end. The region deleted includes the *lcrF* DNA binding sequences and completely eliminates the ability of LcrF to induce its normal target genes. Pulse-chase experiments show no indication of LcrF degradation, suggesting that protein stability is not thermally sensitive. Taken together, these results indicate that either some aspect of the translation process per se, or some posttranscription process that controls the level of message (e.g., mRNA degradation), must be modulated by temperature, and that a feature of RNA structure or sequence essential for this modulation is contained within the 5' half of the *lcrF* mRNA sequence.

Despite repeated attempts, we have been unable to measure *lcrF* message directly in *Y. pestis*. However, we do have some direct evidence that the
efficiency of lcrF mRNA translation increases with temperature in E. coli. In constructs in which lcrF is transcribed at high levels and the message is cut to discrete lengths by RNAse III to improve detection, the protein/mRNA ratio was increased by a factor of 5 at 37°C compared with 26°C. The magnitude of this effect is less than the difference in LcrF expression observed in Y. pestis at these temperatures. Because these experiments were performed under conditions in which translation and mRNA degradation are substantially uncoupled (i.e., there may be reduced degradation of untranslated message), the full effect of inefficient translation on protein levels may not be achieved. It should also be noted that measurements of the LcrF protein/mRNA ratio were made under conditions in which LcrF is produced at roughly 1,000 times its normal level in Y. pestis, although it remained a reasonable fraction of total cell protein (~5%). Because we do not know the details of the regulatory mechanism involved, the potential for aberrant behavior due to overexpression is difficult to evaluate.

In these experiments, we also observed that addition of the lcrF gene to the pET4a vector causes a dramatic decrease in mRNA levels independently of temperature. This effect could result from either decreased transcription or increased degradation when the lcrF sequences are added to pET4a. We do not have sufficient data to distinguish between these hypotheses, but offer the following observations. No changes were introduced into the φ10 promoter region during construction of plasmid pET4a-F1, and a search of the lcrF gene does not reveal any T7 transcription terminator-like structures (135). Although this search cannot rule out the presence of weak terminators such as those found downstream of the RNAse III cleavage site of pET4a, the fact that these
terminators are highly inefficient (134) makes it unlikely that their presence
could cause a large decrease in transcription. T7 polymerase is also not subject
to the premature termination mechanisms responsible for polarity effects in
poorly translated mRNAs, and so polarity effects due to lcrF sequences can also
be discounted. On the other hand, the presence of some element within the lcrF
mRNA causing rapid degradation of the lcrF mRNA is consistent with the
difficulty we have encountered in detecting this message. By causing
degradation to begin quickly, the presence of such an instability element early in
the lcrF sequence could also account for the low level of degradation products
seen in the autoradiographs. One class of known instability determinants is
cleavage sites for RNase E, the only endoribonuclease found to have a general
role in message stability in E. coli (97). The RNase E consensus sequence is
(G/A)AUU(A/U) (31). Not all occurrences of this site are cleaved, probably
because of as yet poorly defined requirements for appropriate secondary
structure. Sequence analysis shows that the Y. pestis sequence included in the
pET4a-F1 construct contains 26 occurrences of the RNase E consensus sequence,
20 of which occur within the lcrF coding region. In contrast, the β-lactamase
gene and tetracycline resistance genes of the vector contain only four sites each.
Thus, lcrF message may be especially sensitive to RNase E, resulting in extreme
instability.

There is a simple model for thermal regulation of lcrF translation which
could account for many features of our data. This model is based on the
presence of a predicted stem loop structure which sequesters the lcrF SD
sequence (Fig. 4.7A). We are not the first to suggest that the decreased stability of
such a structure with increasing temperature could lead to increased translation,
although the theoretical plausibility of such a mechanism has not been discussed in detail (71, 98). At a minimum, operation of such a mechanism would require the following conditions. (i) The sequestration of the SD sequence by secondary structure is sufficient to have a large effect on the efficiency of translation initiation. There is very strong experimental support for this effect, especially from the work of de Smit and van Duin (27), who have shown that mutations altering the stability of SD-containing secondary structures strongly affect translation. These authors have developed a simple equilibrium model for the effect of secondary structure on translation initiation. This model fits available data very well. (ii) The effect of temperature on liberating the SD sequence by melting of secondary structure must be much greater than its effect on association of the mRNA with appropriate sequences on the ribosome. If this condition is not met, the effect of increased temperature on SD availability is offset by a reduction in affinity for the 30S ribosome subunit. There is substantial evidence that this requirement is also satisfied. As pointed out by Ringquist et al. (108), several studies have shown that mutational alteration of SD sequences affects ribosome binding and/or translation initiation much less than predicted by the effect of the mutation on stability of the duplex formed between the mRNA and the 3′ end of the 16S rRNA. To account for this observation, it has been suggested that factors other than simple duplex formation are the primary determinants of stability in the ribosome mRNA interaction. The major function of the SD sequence appears to be proper positioning of the initiation codon on the ribosome, rather than association of the mRNA with the 30S subunit. In any case, if simple hybridization of mRNA and 16S rRNA sequences were the major determinant
FIG. 4.7 Predicted secondary structure of the translation initiation region of the lcrF mRNA and its potential role in thermoregulation.

(A) Schematic representation of the predicted secondary structure within the 5' region of the lcrF message, computed as described in Materials and Methods. This structure was predicted in full-length lcrF message, as well as in all members of a series containing progressively larger 3' deletions. Thus, the prediction is quite robust, and the predicted structure could well form during folding of nascent mRNA in vivo. Boldface letters indicate the putative lcrF SD sequence.

(B) Computed values of the free energy of helix formation ($\Delta G^\circ_t$ kilocalories[kilojoules] per mol) for the structure in panel A as a function of temperature.

(C) Relative expression ($E$) of lcrF as a function of temperature predicted by the model of de Smit and van Duin (27). Each filled circle indicates the calculated value of $E$ at the corresponding temperature. (The solid line was fitted to these points by eye.) In the context of the model, maximal expression corresponds to $E = 1.0$. The equation used was

$$E = \frac{\exp \left[ - \frac{\Delta G^\circ_t}{RT} [30S] \right]}{1 + \exp \left[ - \frac{\Delta G^\circ_t}{RT} \right] + \exp \left[ - \frac{\Delta G^\circ_{30S}}{RT} \right]} [30S]$$

with parameter values as follows: $\Delta G^\circ_{30S} = -13.5$ kcal[ca. 56.5 kJ] per mol at all temperatures, $\Delta G^\circ_t$ as per panel B, [30S] (the concentration of 30S ribosome subunits) = 8.5 mM, $R$ is the gas constant (1.99 cal[ca. 8.33 J] mol$^{-1}$ K$^{-1}$), and $T$ is the absolute temperature (K). de Smit and van Duin developed this equation on the basis of changes in equilibrium protein levels caused by mutations altering the stability of mRNA secondary structure. Data supporting their model were drawn from experiments conducted at a single temperature. Our treatment differs only in that we explicitly consider the destabilizing effect of temperature. Note the large increase in predicted expression between 26 and 37°C (dashed lines).
of translation initiation, translation would, contrary to general observation, be a highly temperature-sensitive process. (iii) Thermal destabilization of secondary structure over the temperature range in which translation increases must be sufficient to improve formation of productive mRNA-ribosome complexes. There is good reason to believe that this requirement will be met by the SD-sequestering structure of the lcrF message, even though the predicted melting temperature (T_m) of this structure (52°C) is far above the physiologically relevant temperature range. In their work with mutationally altered SD-sequestering structures, de Smit and van Duin showed that maximum translation rates were achieved at temperatures far below T_m, and that this could be accounted for by the negative free energy (ΔG°) of association of mRNA and the 30S subunit (27). In other words, ribosomes serve as a sink for the unfolded mRNA species, greatly reducing the proportion of total mRNA that remains in the folded state at equilibrium.

Using the model of de Smit and van Duin (27), assuming that the value they used for the mRNA-30S ΔG° (-13.5 kcal [ca. 56.5 kJ]/mol) can be reasonably applied at both 26 and 37°C, and, using computed ΔG° values for the lcrF stem-loop (Fig. 4.7B), we calculate that the change in stability of the lcrF stem-loop between these temperatures could account for a 40-fold increase in expression (Fig. 4.7C). We conclude that control of translation by thermal destabilization of the stem-loop is a viable hypothesis for thermal control of lcrF translation and should be rigorously tested.
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CHAPTER V

DISCUSSION
The major finding of the research presented is that translation appears to be the temperature-sensitive step controlling production of LcrF, a critical component of the pathway mediating the response of *Y. pestis* to elevated temperature. We have proposed the existence of a stem-loop structure in the *lcrF* RNA as an explanation for the observed increase in LcrF translation with temperature, and present a model in which it can serve as the primary temperature-sensing apparatus. This hypothesis must now be rigorously tested. Four strategies will be used to confirm the existence of the stem-loop and its importance in the regulation of LcrF translation. These include:

1. Replacement of *lcrF* upstream sequences including the stem-loop region with a heterologous promoter and translation initiation region to determine if thermal regulation of LcrF protein production can be abolished.

2. A genetic approach in which stabilizing, de-stabilizing, and compensatory mutations are introduced into the putative stem-loop sequences, and their effect compared with predictions of the model.

3. Confirmation that the putative stem-loop structure does form under simulated physiological conditions by chemical and enzymatic analysis of RNA secondary structure.

4. Confirmation of sharp temperature dependence of 30S ribosomal subunit-mRNA binding reaction in vitro. The "toeprinting" technique of Hartz et al. (53) will be used in these experiments. In this method, RNA is hybridized to a primer complementary to sequences upstream of the initiation region, followed by incubation of the duplex with purified
30S ribosomal subunits. The extent of subunit binding to the RNA is demonstrated by its ability to inhibit extension of the primer by reverse transcriptase. The degree of primer extension inhibition observed in vitro by this method has been shown to correlate well with translation efficiency in vivo (52).

Our working model posits that destabilization of the stem-loop with temperature is sufficient to regulate LcrF synthesis. Although this model is attractive in its simplicity and may play a major role in the thermal control of lcrF-regulated genes, the regulatory system functioning in vivo is probably more complicated. There are two important observations which are not readily explained by our simple model. The first of these, reported in Chapter 11, is the inhibitory effect of increasing lcrF gene dosage on the absolute levels of yopE transcription. It is possible that gene dosage does not accurately reflect LcrF protein levels in these experiments, and direct measures of LcrF levels are required before this observation is pursued further. If high LcrF levels do indeed reduce yop transcription without affecting induction ratios as suggested by the present data, models in which active and inactive forms of LcrF compete for binding sites upstream of the yop promoters must be considered. Such models could explain the observed results if the capacity of the cell to "activate" LcrF is limited, resulting in low ratios of the active to inactive forms when large amounts of LcrF are synthesized. Note that this effect could result from a mechanism for LcrF activation that is not directly related to thermoregulation: for example, the Ca++ signal may act in part through regulation of LcrF specific activity. The second line of evidence that suggests more complicated regulatory machinery than is encompassed by our simple model is data indicating
transcription regulation of the lcrF homologue in Y. enterocolitica. Our data do not definitively exclude such regulation in Y. pestis. Given our findings that lcrF message is poorly translated at low temperature, and the observation that forced transcription of either lcrF or Y. enterocolitica virF fails to induce yop transcription at low temperature, we think it likely that increased transcription of these activator genes is secondary to the major thermal induction event. Further investigation of these phenomena with be required for a complete description of thermoregulation of gene expression in the yersiniae.
Bibliography


