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Temperature Sensing in *Yersinia pestis*: Regulation of *yopE* Transcription by *lcrF*

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In *Escherichia coli*, a *yopE::lacZ* fusion was found to be regulated by temperature in the presence of the cloned *Bam*HI 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 *Bam*HI 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.

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 references 22, 36, and 37). Another parameter, temperature, has been implicated in a wide variety of pathogens, including *Borrelia burgdorferi* (8), *Bordetella pertussis*, shigellae, salmonellae, and the yersiniae (34).

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 (9, 54). (ATP may also play an important role [44].) 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 (micromolar 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 (43). 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 (19, 51, 58), 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 (σ^{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 (57, 58). Very recent work with this system suggests that the thermometer may be DnaK, which is also a heat shock protein in the Hsp70 family (12, 35).

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-

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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* (68). 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 (11). 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 (11). 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 1.

Construction of *yopE::lacZ* reporter plasmids. A previously characterized (68) *yopE*::Mud1 (Ap *lac*) fusion at 22.8 kb on the pCD1 map (*Bam*HI E fragment) was subcloned for use in many of the experiments described below. On the basis of restriction mapping and the published *yopE* sequence (15), the fusion point in this plasmid is about 700 bp downstream of the putative *yopE* initiation codon. The original pCD1 construct (pUT1007::Mud1 [22.8 kb]) was digested to completion with *Bam*HI and incompletely with *Eco*RI 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 *Eco*RI site—were substituted for the 5′ portion of the *lacZ* coding sequence of pMC874. One of these clones was designated pUT2016. An *Eco*RI-*Sal*I fragment (incomplete *Eco*RI and complete *Sal*I digest) beginning at the *Eco*RI site 292 bases upstream of the *yopE* coding sequence and including the *yopE::lacZ* sequence through the *Sal*I site of *lacZ* was inserted in *Eco*RI-*Sal*I-digested pBR322, to yield pUM4091, and in *Eco*RI-*Sal*I-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 (50). 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 *Hin*dIII-*Hae*III fragment of the pCD1 *Bam*HI 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 *Hin*dIII-*Dra*I fragment of *Bam*HI-G. The *Hin*dIII site is the same as that used to construct pUM4106, and the *Dra*I 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 *Sal*I and partial *Eco*RI digestion of pUM4100 (pLG338::*Bam*HI G) and ligating it to an *Eco*RI-*Sal*I 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 *Bam*HI-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 (70) as modified by Straley and Bowmer (55), 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 (38). 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 $CaCl_2$. Equal numbers of cells were then resuspended in 0.033 M $NaPO_4$ buffer, pH 7.0. Serial dilutions were spotted onto duplicate TBAB plates containing 2.5 mM $CaCl_2$ or TBAB rendered calcium deficient by the addition of sodium oxalate (24). Each set of plates (with or without Ca^{2+}) 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 $CaCl_2$) 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. β-Galactosidase levels were determined as described by Miller (38). *E. coli* strains were grown in LB by the protocol described above, except that cultures were allowed to grow for 2 h after the temperature switch.

DNA sequencing. The *Bam*HI 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 Sanger et al. (47) with the Sequenase kit of United States Biochemical.

DNA methodology. Large-scale plasmid preparations were by the method of Birnboim and Doly (1) as described by Maniatis et al. (33), except that a phenol extraction of the supernatant was performed after the first spin. The boiling method of Holmes and Quigley (25) 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 (27) 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. (33). Restriction enzymes, Klenow fragment,

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype and/or description	Source or reference
Y. pestis		
KIM5	pCD1$^+$ Lcr$^+$	R. Brubaker
KIM6	pCD1$^-$ Lcr$^-$	Spontaneous pCD1 segregant of KIM5
UTP1007	*lcrE1* Lcr^c	69
UTP1422	pUT1007::Mu dl 22.8, pGW600, Ap^r Tc^r	68
E. coli		
CSH50	K-12 F$^-$ *araD(lac-pro) strA thi*	CGSC
JM101	K-12 *supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI^{q}ZM15*)	67
K38	HfrC λ	60
BL21(DE3)	B F$^-$ *ompT* r_B^- m_B^- (λ DE3)	59
UME4077	CSH50(pUM4090)	This study
UME4079	CSH50(pUM4090, pUT2027)	This study
UME4080	CSH50(pUM4091)	This study
UME4081	CSH50(pUM4091, pUM4100)	This study
UME4085	CSH50(pUM4090, pNF001)	This study
UME4144	CSH50(pUM4086)	This study
UME4147	CSH50(pUM4086, pUM4090)	This study
Plasmids		
pUT1007	*lcrE1* mutant of pCD1; Lcr^c	69
pGW600	Encodes high levels of Mu repressor; Tc^r	29
pMC874	Km^r	6
pGP1-2	Expression of T7 polymerase under control of λ promoter p_L; Km^r	S. Tabor
pLG338	Derivative of pSC101; Km^r Tc^r	S. Austin
pBR322	ColE1 replicon; Ap^r Tc^r	2
pNK1915	Derivative of pBR322 containing T1 terminator upstream of promoterless *lacZ*; Ap^r	N. Kleckner
pUT2016	8.3-kb *Bam*HI-*Eco*RI fragment containing *yopE-lacZ* sequences from pCD1 of UTP1422 inserted into pMC874	This study
pUT2017	4.8-kb *Bam*HI G fragment of pCD1 inserted into pACYC184	This study
pUT2027	4.8-kb *Bam*HI G fragment of pCD1 inserted into pBR322	This study
pUM4087	1.0-kb *Hin*dIII-*Dra*I fragment of pUT2017 containing *lcrF* sequences inserted into *Sma*I-digested Bluescript KS M13$^+$	This study
pUM4090	8.3-kb *Eco*RI-*Sal*I fragment containing *yopE-lacZ* sequences from pUT2016 inserted into pLG338	This study
pUM4091	8.3-kb *Eco*RI-*Sal*I fragment containing *yopE-lacZ* sequences from pUT2016 inserted into pBR322	This study
pUM4100	4.8-kb *Bam*HI G fragment of pUT2017 inserted into pLG338	This study
pUM4101	6.2-kb *Eco*RI-*Sal*I fragment of pNK1915 containing promoterless *lacZ* inserted into partial *Eco*RI- complete *Sal*I-digested pUM4100	This study
pNF001	4.8-kb *Bam*HI G fragment of pUT2017 inserted into Bluescript KS M13$^-$	This study
pNF002	4.8-kb *Bam*HI G fragment of pUT2017 inserted into Bluescript KS M13$^+$	This study
pNF409	1.6-kb deletion of pNF001	This study
pNF15	1.7-kb deletion of pNF001	This study
pUM4106	280-bp *Hin*dIII-*Hae*III fragment of pNF409 containing sequences around start codon of *lcrF* inserted into *Sma*I-digested pNK1915	This study
pUM4180	0.9-kb *Cla*I-*Bam*HI fragment of pUM4087 inserted into partial *Cla*I-*Bam*HI-digested pUM4106	This study
pUM4186	pUM4180 deleted for *lacZYA* sequences (to *Bcl*I site)	This study
pUM4200	680-bp *Eco*RI-*Pst*I fragment containing *yopE* sequences inserted into *Eco*RI-*Pst*I-digested Bluescript KS M13$^-$	This study

and T4 DNA ligase were obtained from New England Biolabs, Boehringer Mannheim, and GIBCO/BRL. Transformation of *E. coli* was performed by the cold $CaCl_2$ method of Dagert and Ehrlich (13). *Y. pestis* cells were prepared for electroporation according to the manufacturer's instructions for the electroporation apparatus (Gene Pulser; Bio-Rad), except that the 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 of approximately 4 ms.

Analysis of proteins. The protein products of the *Bam*HI G fragment and its deletions present in the Bluescript M13 plasmids were visualized by utilizing the T7 overexpression system of Tabor and Richardson (60). 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 (48) to facilitate visualization of low-molecular-weight proteins.

Pulse-chase. Cells were prepared for labeling with [^{35}S]methionine by a protocol of Tabor, except that the cells were resuspended in 100 ml of M9 medium supplemented with 20 μg 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 [^{35}S]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 (28).

Cell fractionation. Cells were fractionated by a modification of the method of Straley and Brubaker (56). 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 Spring, Md.) at 20,000 lb/in^2. The extract was spun briefly in a microcentrifuge to spin down whole cells. $MgCl_2$ 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 0.01 M HEPES and spun at 50,000 rpm in the SW50.1 rotor for 1 h (234,000 × *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 for the gel mobility shift assay was that of Byerly et al. (4), with the following modifications. Plasmid pUM4200 was digested with *Eco*RI and *Pst*I and 5′ end labeled with [γ-^{32}P]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 2× DNA-binding buffer (2× DNA-binding buffer is 20 mM Tris-hydrochloride [pH 7.5], 100 mM KCl, 2 mM EDTA, 2 mM dithiothreitol, and 10% glycerol), and H_2O 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 (59). 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 cells were grown at 37°C in LB broth, and IPTG was added as described elsewhere (59). Crude cell extracts were prepared according to Webster et al. (65). 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 (63): 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 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 *yopE::lacZ* fusion in *Y. pestis*. The *lcrF* locus on the native *Y. pestis* virulence plasmid pCD1 was originally identified by selecting Tn*5* insertion mutants which permitted growth of a specially constructed *Y. pestis* strain at 37°C (68). This strain carries a mutant allele of the *lcrE* gene designated *lcrE1* (69), as well as a *yopE*::Mud1 (Ap *lac*) fusion. The *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 *yopE* fusion serves as a reporter of transcription of the *lcrF*-regulated *yopE* gene. In order to characterize *yopE* regulation by the *lcrF* locus in a simplified system in *E. coli* (see below), we needed to determine whether a cloned *yopE::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 *Y. pestis*, responding to both temperature and Ca^{2+} concentration.

The *yopE::lacZ* fusion plasmid pUM4090 was introduced into KIM5, KIM6, and UTP1007 by electroporation, and β-galactosidase assays were performed on TMH-grown cultures. The low-copy-number vector pLG338 was used to construct this reporter because its copy number (estimated to be 7.5 [52]) 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 given in Fig. 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 *Bam*HI G fragment of pCD1 (pUT2027) was constructed. Thermal induction of β-galactosidase activity similar to that displayed by *Y. pestis*

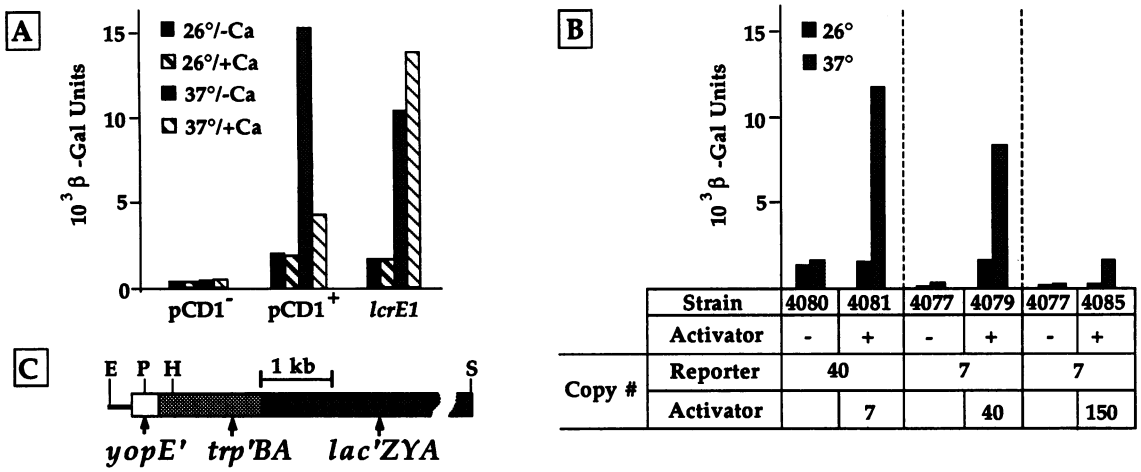

FIG. 1. Effect of temperature and Ca^{2+} concentration on the expression of a cloned *yopE::lacZ* fusion. (A) Results of β-galactosidase assays on *Y. pestis* KIM5(pCD1$^+$ Lcr$^+$), KIM6(pCD1$^-$ Lcr$^-$), and UTP1007 (*lcrE1* Lcr^c) containing pUM4090. (B) Effect of increasing the copy number of plasmids bearing the *Bam*HI 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 *Bam*HI 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 *Sal*I site downstream of *lacA*. E, *Eco*RI; P, *Pst*I; H, *Hin*dIII; S, *Sal*I.

constructs in Ca^{2+}-deficient medium was observed, although the induced level in the *E. coli* strain was somewhat lower (Fig. 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 dose 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 (*Bam*HI-G in pLG338). As shown in the left of Fig. 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 *Bam*HI 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. 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 *Bam*HI 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 (15). 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 *Bam*HI-G (coordinates 55.7 and 56.8 kb of pCD1, on the basis of the map of Goguen et al. [17]) 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 [^{35}S]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*HI 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 weight 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* (53), this has also been seen for the VirF protein of *Y. enterocolitica* (11). Of the six amino acid residues altered between VirF and LcrF, only one replacement (Gly-95_{LcrF} for Ala-95_{VirF}) is not a conserved 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 (64).

Proteins homologous to LcrF. Like VirF of *Y. enterocolitica*, LcrF is a member of the AraC family of transcription

TABLE 2. Proteins with significant homology to LcrF

Protein	Species	Operon or gene regulated	Inducer	% Identity with LcrF	Reference
Catabolism[a]					
AraC	*E. carotovara*	Arabinose operon	Arabinose	28.0	30
AraC	*E. coli*	Arabinose operon	Arabinose	23.2	39
AraC	*S. typhimurium*	Arabinose operon	Arabinose	22.9	7
RhaR	*E. coli*	Rhamnose operon	Rhamnose	22.6	62
RhaS	*E. coli*	Rhamnose operon	Rhamnose	21.4	62
XylS	*P. putida*	Plasmid-borne xylene operon	Benzoate	20.9	26
MelR	*E. coli*	Melibiose operon	Melibiose	20.8	66
Virulence[b]					
VirF	*Y. enterocolitica*	Plasmid-borne virulence genes	?[c]	97.8	11
Rns	Enterotoxigenic *E. coli*	Colonization factors CS1 and CS2	?	24.2	5
VirF	*S. flexneri*	Plasmid-borne virulence genes	?	23.5	46

[a] Regulatory proteins controlling operons involved in catabolism of carbon and energy sources.
[b] Regulatory loci controlling expression of virulence genes.
[c] ?, inducer unknown.

activators (5, 11, 21, 45, 63). In addition to LcrF, we add another previously unrecognized member to this family, the *virF* gene of *Shigella flexneri* (43). 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, 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. This

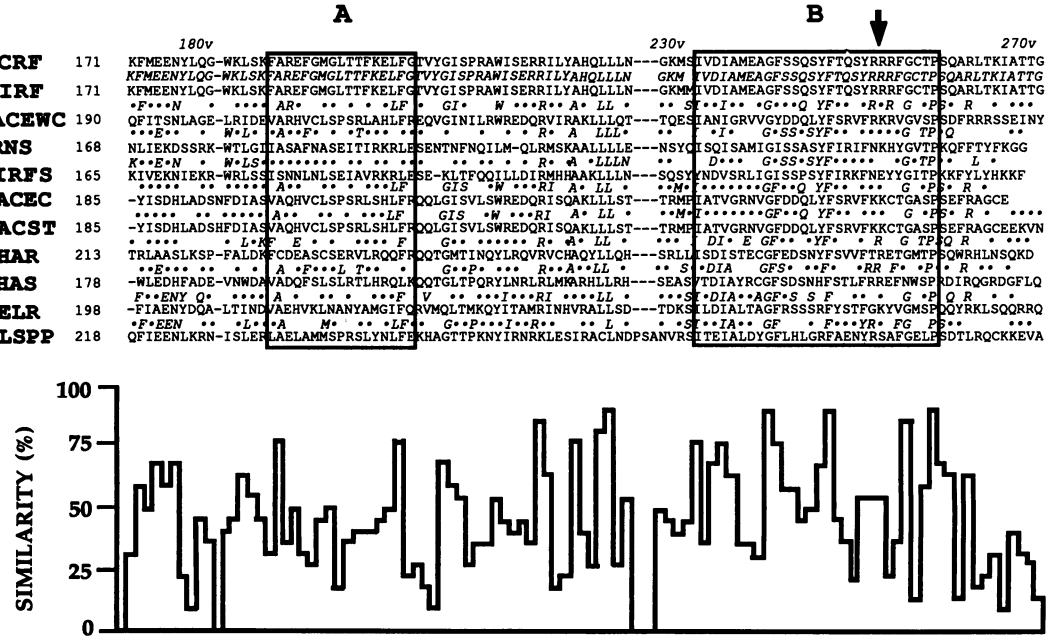

FIG. 2. Proteins exhibiting homology to LcrF. See Table 2 for full descriptions and sequence references for each protein. (Top) Multiple alignment. Each amino acid sequence was maximally aligned to the LcrF sequence by utilizing the algorithms of Lipmann and Pearson (31) and Needleman and Wunsch (41), 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: [number of identical residues + (0.5 × number of conserved residues)]/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*; XYLSPP, XylS of *Pseudomonas putida*.

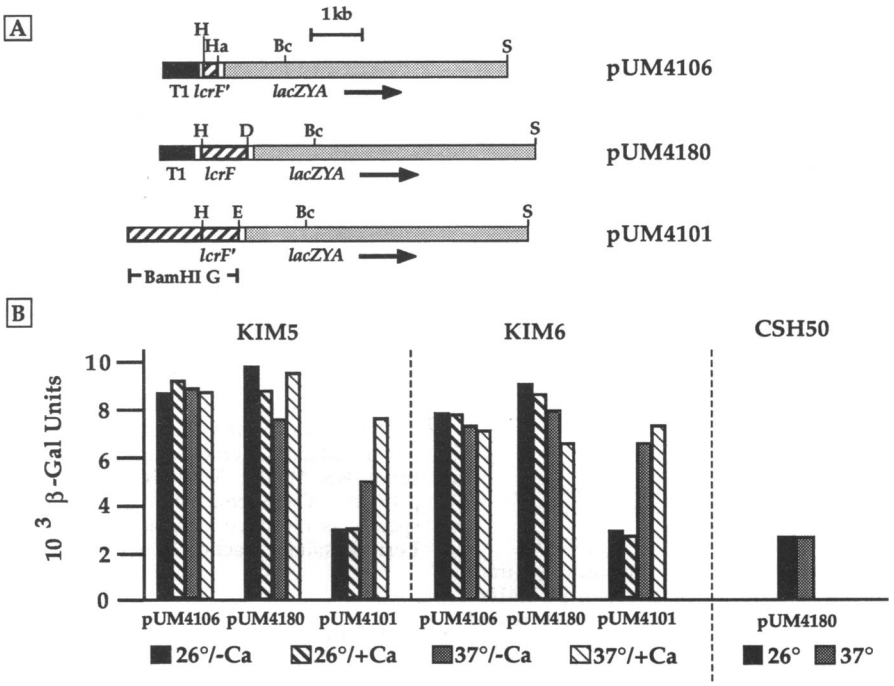

FIG. 3. Expression of *lcrF*::*lacZ* fusions. (A) Structure of *lcrF*::*lacZ* fusion constructs. pUM4106 and pUM4180 were constructed in pNK1915. T1 denotes the 4 to 5 repeats of the T1 terminator sequence present in this plasmid. pUM4101 is constructed in pLG338, which lacks a terminator sequence. Arrows show directions of transcription. The hatched region represents sequence specific to the *Bam*HI G fragment of pCD1. Note that the intact *lcrF* gene is present only in pUM4180. H, *Hin*dIII; Ha, *Hae*III; D, *Dra*I; E, *Eco*RI; Bc, *Bcl*I; S, *Sal*I. (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.

region displays the strongest sequence similarity among these proteins. Domain A was identified by Cornelis et al. (11) as containing a helix-turn-helix region similar to a domain in lambda repressor known to be involved in DNA binding (42). 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) 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. 3A). Two of these were designed as an isogenic pair which differed with respect to production of active LcrF protein. In one 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. 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. 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. 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 pattern of *yopE*::*lacZ* expression in the presence of a constitutively transcribed *lcrF* gene, we first deleted the *lacZYA* sequences from pUM4180 to permit use of a *lacZ* reporter fusion with this clone. The deleted version, pUM4186 (Fig. 4A), was inactive in a β-galactosidase assay.

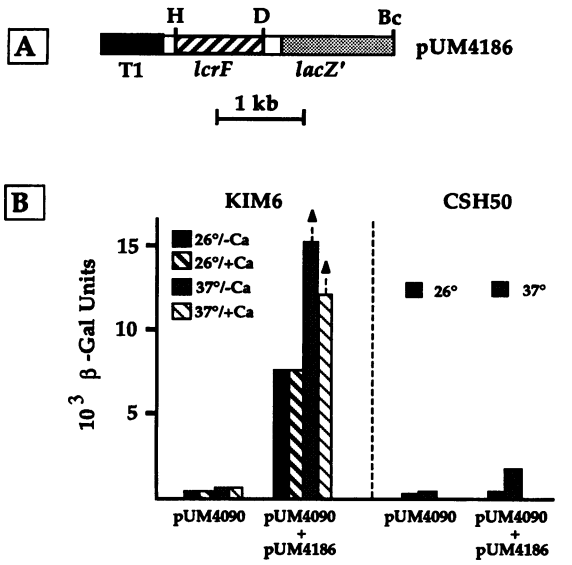

FIG. 4. Thermoregulation mediated by constitutively transcribed *lcrF*. (A) Map of the *lcrF*::*lacZ'* construct in pUM4186. Note that this construct is identical to the constitutively expressed pUM4180 construct, except for deletion of *lac* sequences to inactivate *lacZ*. H, *Hin*dIII; D, *Dra*I; Bc, *Bcl*I. (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*.

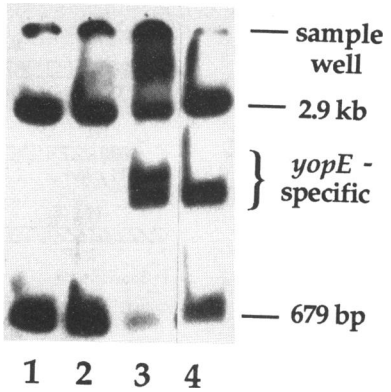

FIG. 5. Results of gel mobility shift assay. *Eco*RI-*Pst*I-digested pUM4200 was used to detect LcrF-specific binding. Lanes: 1, no extract added; 2, crude extract prepared from BL21(DE3, Bluescript KS M13⁻); 3, crude extract prepared from BL21(DE3, pNF409). The second shifted band in the region labeled *yopE* specific is observed only at high LcrF concentrations. Lane 4, LcrF-containing fraction from phosphocellulose column.

When placed in *trans* with the reporter pUM4090 in *Y. pestis* KIM6 (Fig. 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 *E. coli* CSH50 (Fig. 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.

A constitutively transcribed clone of *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 Ca^{2+} concentration. Thus, despite overproduction of *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 Ca^{2+}. However, at 37°C in the presence of Ca^{2+}, 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.

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. 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. 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 *Bam*HI-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. 6).

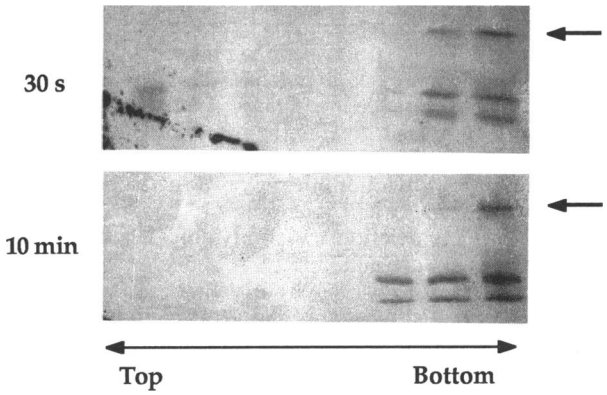

FIG. 6. Localization of LcrF in *E. coli*. Pulse-chase experiments were performed as described in Materials and Methods with K38(pGP1-2) containing pNF409. Cells were pulsed for 20 s with 2.5 mCi of [^{35}S]methionine and chased, and samples were withdrawn at 15 and 30 s and 1, 2, 5, and 10 min. The 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.

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.

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* (11). 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* (10, 11). 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 P_{tac}-*virF* fusion did not result in significant expression of *virB*, as observed by S1 nuclease protection assays (61). 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 (12), and the state of DNA supercoiling (23). 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 (10), 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 (18) is that compensating changes in linking number maintain a fixed degree of superhelical tension 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 (14). 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 (22); DNA loop formation can be either negatively or positively affected by bending proteins (32, 40) and is also known to be dependent on supercoiling (20). AraC activator activity is also modulated by interaction with another protein (cyclic AMP receptor protein [32]). 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 (10). 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 σ^{32}) (64) and the *dnaA* gene itself (16). This repression may not be significant at physiological DnaA levels, however (3). 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 (49). 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.

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ADDENDUM

During review of this report, two other relevant articles have been published. Lambert de Rouvroit et al. (29a) 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 (50a) report on the role of *Y. pseudotuberculosis* LcrF in regulation of the *yadA* gene.

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