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Yersinia enterocolitica ClpB Affects Levels of Invasin and Motility

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Expression of the *Yersinia enterocolitica inv* gene is dependent on growth phase and temperature. *inv* is maximally expressed at 23°C in late-exponential- to early-stationary-phase cultures. We previously reported the isolation of a *Y. enterocolitica* mutant (JB1A8v) that shows a decrease in invasin levels yet is hypermotile when grown at 23°C. JB1A8v has a transposon insertion within *uvrC*. Described here is the isolation and characterization of a clone that suppresses these mutant phenotypes of the *uvrC* mutant JB1A8v. This suppressing clone encodes ClpB (a Clp ATPase homologue). The *Y. enterocolitica* ClpB homologue is 30 to 40% identical to the ClpB proteins from various bacteria but is 80% identical to one of the two ClpB homologues of *Yersinia pestis*. A *clpB*::Tn*Max2* insertion mutant (JB69Qv) was constructed and determined to be deficient in invasin production and nonmotile when grown at 23°C. Analysis of *inv* and *fleB* (flagellin gene) transcript levels in JB69Qv suggested that ClpB has both transcriptional and posttranscriptional effects. In contrast, a *clpB* null mutant, BY1v, had no effect on invasin levels or motility. A model accounting for these observations is presented.

Yersinia enterocolitica is a food-borne pathogen that causes a variety of intestinal syndromes (6, 8). One of the key features of the disease process is the ability of the bacteria to translocate through the intestinal epithelium and proliferate within the Peyer's patches (13, 37). Invasin, an outer membrane protein of *Y. enterocolitica*, is required for efficient translocation of the bacteria from the intestinal lumen to the Peyer's patches (37). Expression of the *inv* gene, which encodes invasin, is influenced by temperature and growth phase. *inv* expression is maximal when *Y. enterocolitica* is in late exponential to early stationary phase at 23°C (35, 38). In addition to temperature and growth phase, other environmental factors affect *inv* expression. Notable expression of *inv* is observed when the bacteria are grown at 37°C in L broth (LB) buffered at pH 5.5. Furthermore, an increase in Na^+ ion concentration elicits a slight increase in *inv* expression at 37°C (35).

Expression of other identified *Y. enterocolitica* virulence factors is also thermally regulated in vitro. For example, *ail* encodes a protein that mediates *Y. enterocolitica* adherence, invasion, and serum resistance (5, 28, 39, 49). *ail* is highly expressed when bacteria are grown at 37°C, while at lower temperatures *ail* expression is repressed (39). Recently it was demonstrated that ClpP plays a role in repression of Ail synthesis at lower temperatures (34). In *Escherichia coli*, ClpP is the proteolytic subunit of an ATP-dependent serine protease (Clp) (11, 47).

Motility is also optimally expressed at 23°C (17, 20, 43). *Y. enterocolitica* possesses three flagellin structural genes (*fleA*, *fleB*, and *fleC*) (20). The *fleA* and *fleB* genes are transcribed at 25°C, while no flagellin transcripts were detected at 37°C (20). As in other *Enterobacteriaceae*, expression of the *Y. enterocolitica* flagellin genes has been shown to be dependent on the master regulators FlhDC (57) and alternative sigma factor FliA (σ^F) (20, 21). *Y. enterocolitica* also possesses the anti-sigma factor FlgM, which negatively regulates FliA activity (21). *fliA* and *flgM* expression is coordinately regulated by temperature; expression of *fliA* and *flgM* is observed principally at lower temperatures (21). The mechanism for activation or repression of these genes in response to temperature has not been elucidated. However, FlhDC and FliA are required for the expression and secretion of at least one virulence determinant, the phospholipase YplA (56).

Little is known about the molecular basis for the environmental regulation of *inv* expression. However, we recently reported the isolation and characterization of two *Y. enterocolitica* invasin regulatory mutants. Both of the mutants, JB1A8v and JB16v, show a significant decrease in invasin expression but are hypermotile compared to the wild type when grown at 23°C. The first *inv* regulatory mutant, JB1A8v, has an mTn*5*Km transposon insertion that disrupts *uvrC*. It is not known at this time how the mTn*5*Km insertion mutation results in the observed phenotypes of JB1A8v (1). The second regulatory mutant, JB16v, was generated from a targeted disruption of the *sspA* locus in wild-type *Y. enterocolitica* (1). It was determined that the *uvrC* and *sspA* genes are separated by approximately 16 kb of DNA. In *E. coli*, *sspA* encodes the stringent starvation protein SspA, which has been shown to bind RNA polymerase (19, 54). In addition, *E. coli* SspA has been shown to negatively and positively regulate the expression of several proteins during the exponential- and post-exponential-growth phases (54). The expression of *sspA* itself is induced by entry into stationary phase as well as by starvation for phosphate, glucose, nitrogen, and amino acids (54). The exact mechanism for *E. coli* SspA regulation is presently unknown, but it is thought to be related to the ability of SspA to bind RNA polymerase.

Concurrently with the above studies, attempts were made to isolate a clone that could complement the phenotype of the *Y.*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
Y. enterocolitica		
8081v	Serogroup O:8; pYV8081 Nal^r	R. Martinez
JB580v	Derivative of 8081v; R^- M^+ Nal^r	22
JB41v	JB580v with wild-type *inv*; *inv*::*phoA*; Nal^r Cm^r	1
JB1A8v	JB580v *uvrC*::mTn*5*Km; regulatory mutant; Nal^r Cm^r Km^r	1
JB69Qv	JB580v *clpB*::Tn*Max2*; Nal^r Erm^r	This work
BY1v	JB580v *clpB*::pEP185.2; Nal^r Cm^r	This work
E. coli		
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-TC::Mu; Km^r	46
DH5α	*supE44* [*lacU169*(ϕ80*dlacZ*ΔM15)] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	15
E131	F^- ϕ(80*dlacZ*ΔM15)Δ(*lacZYA argF*)*U169 deoR recA1 endA1 hsdR17 supE44* λ-*thi-1 gyrA96 relA1*λ-CH616	14
LE392	F^- *hsdR514 supE44 supF58*	25
Plasmids		
pLAFR2	Mobilizable cosmid vector; Tet^r	S. Libby
pJQ200KS	Mobilizable suicide vector; Gm^r	41
pTM100	Mobilizable derivative of pACYC184; Tet^r Cm^r	27
pCR2.1	PCR product cloning vector; Amp^r Km^r	Invitrogen
pEP185.2	Mobilizable suicide vector; Cm^r	22
pRK2013	ColE1 Tra $(RK2)^+$; Km^r	10
pUC19	Cloning vector; Ap^r	New England Biolabs
pTn*Max2*	Tn*Max2* delivery plasmid; Erm^r	14
pWSK129 and pWSK130	Low-copy-number cloning vectors with pSC101 *ori*; Km^r	51
pJB32	pLAFR2 containing an ~25-kb insert; Tet^r	This work
pJB69	pTM100 containing a 5.4-kb *Eco*RI fragment from pJB32 carrying *clpB*; Tet^r	This work
pJB69-Q	pJB69 *clpB*::Tn*Max2*; Tet^r Erm^r	This work
pCRII-Q	pCR2.1 containing *clpB*::Tn*Max2* PCR product; Ap^r Km^r Erm^r	This work
pEP185.2-Q	pEP185.2 containing *clpB*::Tn*Max2* PCR product; Cm^r Erm^r	This work
pJB302	pCR2.1 containing *clpB* PCR product; Ap^r Km^r	This work
pJB305	pTM100 containing *clpB* PCR product; Tet^r	This work
pAVKF	pACY184 containing a 2.2-kb *Eco*RV fragment carrying *fliA* and upstream sequences; Cm^r	21
pVM112	pMT11HC containing *inv*; Ap^r	36
pKVS2	pBluescript containing a 2.2-kb *Sac*II fragment of the 3′ end of *fleA*, all of *fleB*, and the 5′ end of *fleC*; Ap^r	20
pBY38	pEP185.2 with a 744-bp *Sal*I-*Sac*II fragment from pAJD70	This work
pAJD69	pWSK129 containing a 3.6-kb *Eco*RI-*Pst*I fragment of pAJD11 carrying *clpB*	This work
pAJD70	pWSK130 containing a 3.6-kb *Eco*RI-*Pst*I fragment of pAJD11 carrying *clpB*	This work
pAJD11	pWSK129 containing a 5.4-kb *Eco*RI fragment carrying *clpB*; Km^r	This work

enterocolitica mutant JB1A8v. Thus, a clone was isolated that suppressed the phenotypes of decreased invasin and increased flagellin levels in JB1A8v. We report here the isolation and characterization of this suppressing clone, and we show evidence that ClpB (encoded on the suppressing clone) can play a role in the expression of invasin and the motility regulon.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Y. enterocolitica* strains 8081v and JB580v (nalidixic acid resistant) are referred to as wild type. The latter is an R^- M^+ derivative of 8081v (22) and retains full virulence in BALB/c mice (unpublished data). A schematic of strain lineages for strains used or constructed in this study is shown in Fig. 1. The "v" designation refers to *Y. enterocolitica* strains harboring the virulence plasmid pYV8081. Expression of the *inv* gene was examined in *Y. enterocolitica* strains grown aerobically for 16 to 18 h at 23 or 37°C in LB. *E. coli* SM10λ*pir* (46) was used to deliver mobilizable plasmids into *Y. enterocolitica*, and when necessary pRK2013 was used as as a conjugation helper plasmid (10). Antibiotics were used as necessary at the following concentrations (in micrograms per milliliter): nalidixic acid, 25; chloramphenicol, 25 for multicopy and 10 for single copy; tetracycline, 7.5; kanamycin, 50; ampicillin, 100; gentamicin, 100; and erythromycin, 150 for multicopy and 50 for single copy.

DNA manipulations. The chromosomal DNA library of *Y. enterocolitica* strain 8081v was constructed as follows. Chromosomal DNA was partially digested with the restriction enzyme *Sau*3AI and ligated into a *Bam*HI site in the cosmid cloning vector pLAFR2 (obtained from S. Libby), a derivative of pLAFR1 (3). The ligation mixture was packaged into λ phage by use of a λ packaging mix (Promega, Madison, Wis.); the packaged DNA was transfected into *E. coli* strain LE392, (25). The complementing clone obtained from the library was designated pJB32.

To generate pJB69, pJB32 was partially digested with *Eco*RI and the fragments were ligated into pTM100 digested with *Eco*RI. The resulting recombinant plasmids were screened for the ability to complement JB1A8v. A plasmid with a 5.4-kb *Eco*RI insert was found to suppress both the invasin and motility phenotypes of mutant JB1A8v; this plasmid was designated pJB69.

pJB305 was constructed as follows. The *Y. enterocolitica clpB* locus was amplified by PCR using pJB32 as a template. PCR amplification was performed using oligonucleotide primers 691 (5′ GCTGATATACACATGTAG 3′) and 69Nr (5′ CCAATGAATGCTGCTG 3′), which anneal 5′ and 3′ of *Y. enterocolitica clpB*, respectively. The resulting ca. 3.5-kb PCR product was cloned into pCR2.1, generating a plasmid designated pJB302. pJB302 was subsequently digested with *Eco*RI, and insert DNA was isolated and ligated into pTM100 digested with *Eco*RI to yield pJB305. The insert in pJB305 does not carry any open reading frames other than *clpB*.

Construction of the *clpB* mutants JB69Qv and BY1. JB69Qv was constructed as follows. Oligonucleotide primers used for PCR amplification of *clpB*::Tn*Max2*-Q were 69GG (5′ CCTCAGGTCGATGATGAACCTG 3′) and 69Nr (5′ CCAATGAATGCTGCTG 3′), which anneal within and 3′ of the *clpB* gene, respectively. Template DNA used for PCR amplification was plasmid pJB69-Q, which contains *clpB* with a Tn*Max2* insertion in the 3′ end of the *clpB* gene. The

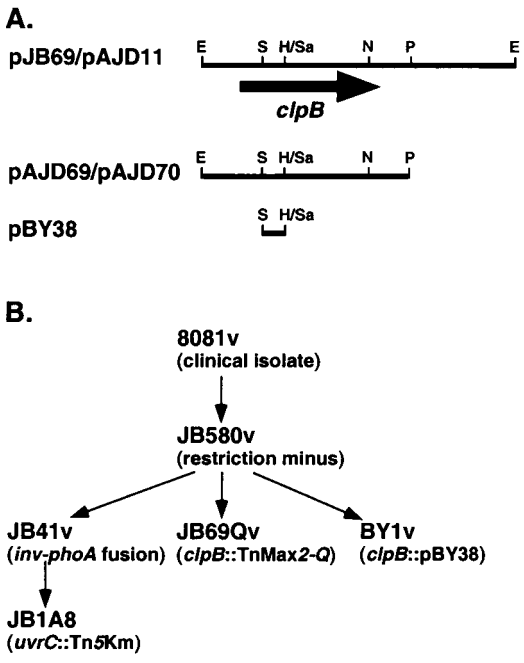

FIG. 1. Physical maps of various clones used this study. (A) Restriction endonuclease sites: H, *Hin*dIII; R, *Eco*RI; S, *Sal*I; Sa, *Sac*II; P, *Pst*I; N, *Nar*I. (B) Schematic of strain lineages for strains used and/or constructed in this study.

resulting PCR product, ca. 3.8 kb, was cloned into pCR2.1, generating plasmid pCRII-Q. pCRII-Q was subsequently digested with *Ava*I and *Spe*I, and insert DNA was isolated and ligated into pEP185.2 digested with *Xho*I and *Xba*I. The resulting plasmid, pEP185.2-Q, was conjugated into wild-type *Y. enterocolitica* JB580v and recombined onto the chromosome by allelic exchange as described elsewhere (35). Double-crossover events at the homologous chromosomal locus were confirmed by Southern analysis using *clpB*-specific sequences as a probe (data not shown). BY1 was constructed as follows. A *Sal*I/*Sac*II internal fragment (744 bp) of pAJD70 was subcloned into pEP185.2 digested with *Sal*I and *Sac*II. This plasmid, pBY38, was conjugated into JB580v, and Nal^r Cm^r exconjugants were selected. The integration of the plasmid into the chromosome was confirmed by Southern analysis using *clpB*-specific sequences as a probe (data not shown). This mutation (*clpB*::pBY38) should create a truncated ClpB (364 amino acids of ClpB).

Cloning the *clpB* gene from the *Y. enterocolitica* chromosome. Chromosomal DNA from *Y. enterocolitica* strain JB580v was digested with *Eco*RI. Fragments of approximately 5 to 6 kb were purified from a 0.8% (wt/vol) agarose gel and ligated into plasmid pWSK129. This library was then used to transform *E. coli* strain DH5α to kanamycin resistance. One hundred ninety-two transformants were grown directly on nylon filters on the surfaces of LB agar plates containing kanamycin (75 μg/ml) at 37°C overnight. The filters were then removed, dried for 10 min at room temperature, and placed on Whatman 3MM paper soaked with 0.4 M NaOH for 8 min. The filters were rinsed in 0.5 M Tris-HCl (pH 7.0) for 5 min and then in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for a further 5 min. The DNA was then cross-linked to the filters by UV fixation. The approximately 3.5-kb *Eco*RI insert fragment of plasmid pJB305 was labeled as described elsewhere (9) and used to probe the filters. A single clone that hybridized with the probe was identified. The plasmid had a single *Eco*RI insert fragment of approximately 5.4 kb and was designated pAJD11.

Western (immunoblot) analysis. All the strains analyzed in this study had the same growth rates, and comparable culture densities were reached after overnight growth at 23°C. Whole-cell lysates were prepared from bacteria grown to early stationary phase in LB at either 23 or 37°C as described previously (35). Equal amounts of whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Blots were then prepared for Western (immunoblot) analysis with a polyclonal anti-invasin antibody and visualized as described previously (2, 35). Coomassie-stained gels run in parallel confirmed that equivalent amounts of protein were loaded in each lane. Invasin levels as detected by immunoblot analysis were estimated by densitometry with the GDS2000 gel documentation system (UVP International). Cell surface-associated flagellin was obtained from the supernatants of vigorously vortexed cultures as described previously (1) and was detected by immunoblotting with monoclonal antibody 15DB (anti-*E. coli* flagellin) (Igen, Inc.) used at a dilution of 1:2,000 as described previously (1).

Phenotypic assays. Motility assays were conducted in LB with 0.3% agar and appropriate antibiotics as described previously (1). Invasion assays were performed with bacterial cultures grown aerobically for 16 to 18 h in LB at 23 or 37°C as indicated. Bacteria were added to subconfluent human laryngeal epithelial (HEp-2) cells at a multiplicity of infection of ca. 100, and the invasion assay was performed as described elsewhere (28). Results were calculated by the following equation: % invasion = 100 × (number of bacteria recovered/number of bacteria added). Alkaline phosphatase (AP) activity was measured in permeablized cells, and results are expressed in enzyme units per OD_{600} (optical density at 600 nm) unit as described elsewhere (26). Assays were performed on duplicate cultures grown 16 to 18 h aerobically in LB. *Y. enterocolitica* JB580v was always assayed and had low but detectable background levels of AP activity (~10 U) that were subtracted from the values presented.

mRNA slot blot analysis. Total cellular RNA was purified from late-log- to early-stationary-phase cultures using the TRIzol reagent (Gibco BRL). RNA samples were treated with RNase-free DNase. In addition, RNase controls were always run for detection of possible DNA contamination. Purified RNA (2.5 μg) was applied to nylon membranes (Pro-Nytran; Schleicher and Schuell) using a slot blot apparatus (Bio-Rad). Membranes were UV cross-linked and subjected to prehybridization for 2 h at 42°C in 50% formamide–5× SSC–0.5% SDS–1× Denhardt's solution–10% dextran sulfate–150 μg of salmon sperm DNA/ml. Radiolabeled probes were added directly to membranes in prehybridization buffer and incubated for 12 to 24 h at 42°C. Membranes were washed twice in preheated 65°C 0.1% SDS–5× SSC for 15 min. A final wash in 2× SSC was performed at room temperature for 5 min. mRNA slot blots were visualized and analyzed with a Molecular Dynamics PhosphorImager and the ImageQuant program. The density of each slot was determined by volume analysis. Wild-type transcript levels were set at 100%, and relative amounts of transcripts from other strains were determined. Each result presented represents the mean ± the range from duplicate samples of an individual representative assay. Probes used in mRNA slot blot analysis were as follows: a 1.2-kb *Nde*I-*Bgl*II internal fragment of *inv* (36), an 840-bp *Stu*I-*Xba*I fragment encompassing *fliA* (21), and a 460-bp *Acc*I-*Hpa*I fragment encompassing the 5′ and internal portions of *fleB* (20).

Nucleic acid purification and probe preparation. Plasmid DNA was isolated by the alkaline lysis method (25) or with Wizard Minipreps (Promega). DNA fragments used in plasmid construction and probe preparation were prepared by digestion with the appropriate restriction endonuclease. After digestion the resulting fragments were gel purified using Gene Clean (Bio 101, La Jolla, Calif.). The purified fragments used as probes were labeled with [^{32}P]dATP by the random primer method as previously described (9). DNA restriction enzymes, T4 DNA ligase, T4 DNA polymerase, and Klenow fragment were purchased from New England Biolabs (Beverly, Mass.) and used according to the manufacturer's instructions.

DNA sequence analysis. Sequencing across Tn*Max2* transposon-plasmid junctions was facilitated by two primers, RES1 (5′-CCTGACAGAAATGGGC-3′) and Tn*Max2* (5′-CCTAAAGGGATCCAAAAGCT-3′), which anneal at the 5′ and 3′ ends of Tn*Max2*, respectively. For each Tn*Max2* insertion, the DNA sequence of 200 to 400 bp in each direction from the site of insertion was determined. The nucleotide sequence was obtained by the dideoxynucleotide chain termination method (44) with the Sequenase sequencing kit (U.S. Biochemical Corp.). The DNA sequence of the insert in pAJD69 (complete *clpB* clone), from the *Eco*RI site (356 bp upstream of the putative *clpB* start codon) to the end of the *clpB* open reading frame, was determined. DNA sequencing was carried out using an Applied Biosystems DNA sequencing system and the Big-Dye terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions. Some of the sequencing was performed by the Murdock Molecular Biology automated sequencing facility (University of Montana). Sequences were analyzed using the BLAST X program available in the NCBI database (www.ncbi.nlm.nih.gov).

Nucleotide sequence accession number. The *clpB* sequence (3,025 bp) was deposited in GenBank under accession no. AF285784.

RESULTS

Isolation and characterization of a clone that suppresses the phenotypes of the JB1A8v mutant. *Y. enterocolitica* strain JB41v carries wild-type *inv* and an *inv*::*phoA* translational fusion on the chromosome and is genotypically and phenotypically Inv$^+$ (Table 1). Thus, modulation of *inv* expression can be monitored by AP activity, Western analysis, primer extension, or tissue culture invasion assays. Analysis of *inv* expression in JB41v by any of these assays showed the same pattern: expression of the *inv* gene and invasin levels were elevated at 23°C, while expression was reduced at 37°C (1). An mTn*5*Km insertion mutant of JB41v, JB1A8v, that demonstrated a significant reduction in *inv* expression and an increase in *fleABC* (flagellin) expression (1) was isolated. These phenotypes could be detected by both protein and RNA analyses (1). Sequencing of

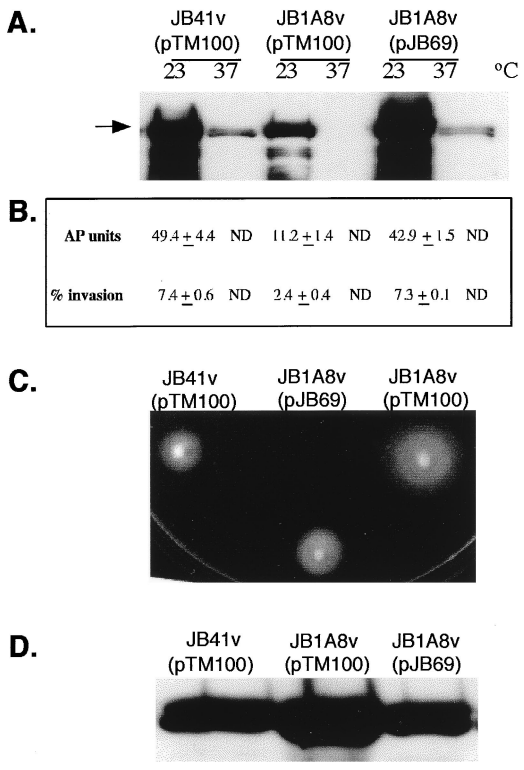

FIG. 2. Suppression of JB1A8v invasin motility phenotypes by pJB69. (A) Western analysis of invasin expression by JB41v(pTM100), JB1A8v(pTM100), and JB1A8v(pJB69). Whole-cell extracts were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with a rabbit polyclonal anti-invasin antibody as described in Materials and Methods. The arrow points to the band representing full-length invasin. It is typical to see full-length invasin along with several breakdown products. (B) Invasion phenotype and AP activity exhibited by JB41v(pTM100), JB1A8v(pTM100), and JB1A8v(pJB69). AP activities were assayed in duplicate and are represented as means ± ranges. Invasion assays with HEp-2 cells were performed in duplicate, and data represent means ± ranges as described in Materials and Methods. (C) Motility assays were performed with JB41v(pTM100), JB1A8v(pTM100), and JB1A8v(pJB69) in 0.3% motility soft agar plates incubated at 23°C for 16 h. (D) Western analysis of flagellin preparations probed with monoclonal antibody 15D8 as described in Materials and Methods. Data shown are from a single experiment and are representative of several experiments performed with similar results.

the insertion junction demonstrated that the disrupted gene was *uvrC*, but several different approaches taken to clone the wild-type locus disrupted by the mTn*5*Km were unsuccessful (1). However, we were able to isolate a low-copy-number cosmid clone that complemented the phenotypes of JB1A8v. This cosmid, pJB32, contains ca. 25 kb of *Y. enterocolitica* chromosomal DNA in pLAFR2. When bacteria were grown at 23°C, wild-type JB41v and mutant JB1A8v containing the cloning vector pLAFR2 showed 19.7 and 7.2 AP units, respectively. JB1A8v harboring pJB32 restored AP levels to 23.0 AP units. Western analysis and tissue culture invasion assays showed similar suppression of the JB1A8v invasin phenotype by pJB32 (data not shown). In addition, pJB32 restored motility and flagellin expression to wild-type levels (data not shown).

To determine the minimal region responsible for suppression of the JB1A8v mutant phenotypes, subclones from the insert of pJB32 were constructed and tested. A resulting subclone, pJB69, had a 5.4-kb *Eco*RI insert within the cloning vector pTM100 (Fig. 1). pJB69 restored normal invasin expression as verified via AP assays, Western analysis, and tissue culture invasion assays (Fig. 2A and B). In addition, the suppressing clone pJB69 restored flagellin synthesis and the motility phenotype to wild-type levels (Fig. 2C and D). JB41v harboring pJB69 showed a motility pattern similar to that of JB41v containing the cloning vector pTM100 (data not shown).

Sequence analysis of the suppressing clone pJB69. To identify the genetic determinant encoded on the suppressing clone pJB69, the nucleotide sequences of portions of the insert DNA of pJB69 were determined. To this end, Tn*Max2* insertions into pJB69 were isolated and mapped. Primers complementary to the ends of Tn*Max2* were used to obtain the nucleotide sequences upstream and downstream of the site of insertion. Nucleotide sequence analysis revealed that plasmid pJB69 encodes the *Y. enterocolitica* homologue ClpB from *E. coli* (33 to 50% amino acid identity depending on the section of sequence). Subsequently the DNA sequence of the insert from the *Eco*RI site (356 bp upstream of the putative *clpB* start codon) to the end of the *clpB* open reading frame was determined and deposited in GenBank (3,025 bp; accession no. AF285784). Note that the *clpB* gene apparently has a GTG translation initiation codon (the first ATG codon is more than 350 bp after the start of the open reading frame).

The predicted amino acid sequence of the *Y. enterocolitica* ClpB protein is 30 to 40% identical to those of the ClpB proteins from a variety of bacteria (data not shown) but is more than 80% identical to an open reading frame in *Yersinia pestis* (The Sanger Center, www.sanger.ac.uk/Projects/Microbes/). Homologues of the *Y. enterocolitica* ClpB include the ClpB protein of *E. coli* (Fig. 3). The *Y. enterocolitica* ClpB protein has several features in common with the Clp family of proteins (Fig. 3). There are apparently two nucleotide binding domains consisting of the conserved A and B consensus sequences (50). The first nucleotide binding region contains a second B segment, as is found in other Clp proteins (12, 47). The two nucleotide binding regions are separated by a large spacer region, a characteristic found only in the ClpB proteins (12, 47). The *clpB* gene of *E. coli* has an internal GTG initiation codon that leads to the synthesis of a truncated ClpB protein, the function of which is not known (33). This GTG codon is not conserved in the *Y. enterocolitica* gene (Fig. 3).

Construction and characterization of a *Y. enterocolitica clpB* mutant. We constructed a *clpB* mutant of *Y. enterocolitica* to more clearly determine if ClpB plays a role in invasin expression and motility (flagellin expression). For this purpose, the plasmid with a Tn*Max2* insertion within the last one-third of *clpB*, pJB69-Q, was used as a template for a PCR to amplify *clpB*::Tn*Max2*-Q; pJB69-Q does not suppress the phenotypes of JB1A8v (data not shown). The resulting PCR product, containing approximately 1.3 kb upstream and 1.0 kb downstream of the Tn*Max2* insertion site, was subcloned into the suicide vector pEP185.2. This clone was then recombined onto the chromosome of JB580v by allelic exchange. The subsequent homologous recombination leading to the replacement of *clpB* by *clpB*::Tn*Max2*-Q was confirmed by Southern hybridization analysis with *Y. enterocolitica clpB* as a probe (data not shown). The resulting *clpB*::Tn*Max2*-Q mutant, designated JB69Qv, has the same growth rate as JB580v at either 26 or 37°C in LB (data not shown). However, JB69Qv exhibited a significant decrease in invasin expression at 23°C compared to the wild-type strain JB580v, as analyzed by immunoblot analysis (Fig. 4A). In contrast to the mutant strain JB1A8v, the *clpB*::Tn*Max2*-Q mutant JB69Qv was nonmotile at 23°C (Fig. 4B). Western analysis with a monoclonal anti-flagellin antibody showed that isolated cell surface flagellin protein levels were significantly decreased in the *clpB*::Tn*Max2*-Q mutant JB69Qv compared to the wild-type strain JB580v (Fig. 4C).

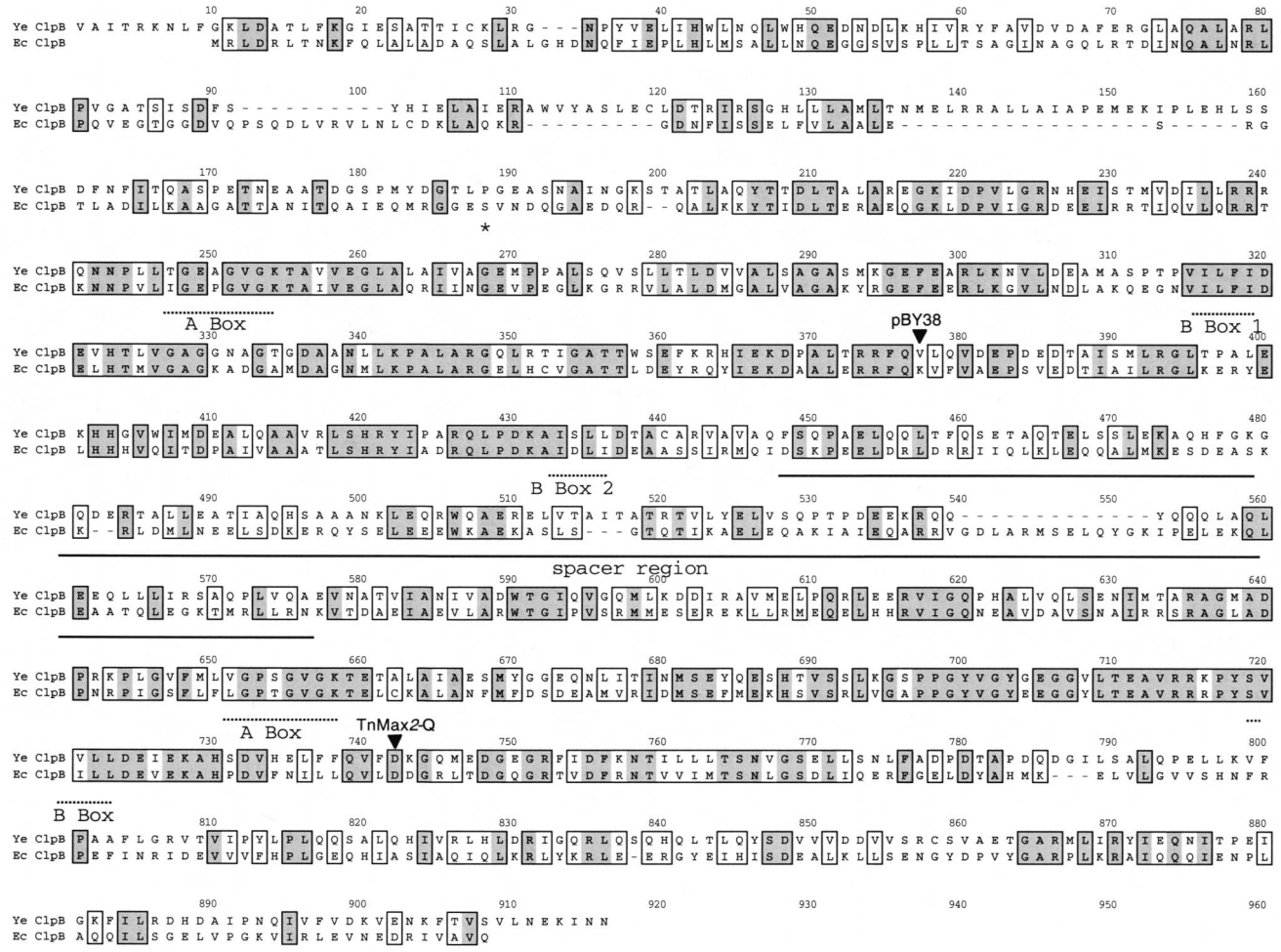

FIG. 3. Comparison of the ClpB proteins of *E. coli* and *Y. enterocolitica*. The predicted amino acid sequence of the *Y. enterocolitica* (Ye) ClpB protein was aligned to that of the *E. coli* (Ec) ClpB protein (GenBank accession no. PO3815) using the ClustalW alignment software (MacVector) and the identity alignment matrix. Similar residues are boxed; identical residues are boxed and shaded. The Walker A and B boxes are underlined with dotted lines, and the spacer region is solid underlined. The asterisk indicates the position of an amino acid translated from a GTG codon that initiates the synthesis of a truncated ClpB protein in *E. coli*. Arrowheads point to the positions of the pBY38 and Tn*Max2*-Q insertions (giving mutants BY1v and JB69Qv, respectively).

Complementation of the *clpB* mutant JB69Qv. To more definitively assess complementation of the *clpB* mutant JB69Qv and suppression of mutant JB1A8v by *clpB*, a smaller subclone containing only *clpB* was generated. For this purpose *Y. enterocolitica clpB* was PCR amplified using pJB32 as a template, and the resulting PCR product was cloned into pCR2.1 and pTM100. The plasmids obtained were designated pJB302 and pJB305, respectively. When the high-copy-number *clpB* plasmid, pJB302, was introduced into the wild-type strain JB580v and the *clpB*::Tn*Max2*-Q mutant JB69Qv, we observed decreased motility and decreased invasin levels (Fig. 5A), suggesting that overexpression of *clpB* negatively affects expression of *inv* and motility. Therefore the moderate-copy-number *clpB* plasmid, pJB305, was introduced into the mutant strains and assayed for invasin expression and motility. In contrast to the high-copy-number *clpB* plasmid, pJB305 suppressed the JB1A8v phenotype of decreased invasin levels, as analyzed by immunoblot analysis (Fig. 5B). In addition, JB1A8v harboring the moderate-copy-number complementing clone (pJB305) produced normal amounts of flagellin and showed a wild-type motility pattern (Fig. 5B; also data not shown). However, the *clpB* mutant JB69Qv containing pJB305 demonstrated only partial complementation of invasin expression and motility (Fig. 5B). Two other *clpB* complementing clones, pAJD69 and pAJD70, were independently constructed, but these also failed to fully complement the invasin and motility phenotypes of the *clpB* mutant JB69Qv (data not shown). This could potentially be due to a dominant negative effect of the *clpB*::Tn*Max2* mutation. In *E. coli*, *clpB* encodes two proteins, a full-length ClpB and a truncated form. The ClpB proteins have been shown to form the tetrameric ATPase in addition to heterooligomeric complexes (33). The *clpB* mutant JB69Qv was generated by a Tn*Max2* insertion in the carboxyl terminus of *Y. enterocolitica* ClpB; thus, if a truncated ClpB is synthesized, the *clpB*::Tn*Max2*-Q mutation could have a *trans*-dominant effect. This possibility is supported by the observation that the expression of *inv-phoA* in JB41v carrying the *clpB* clone pJB69 is more than twice the expression of *inv-phoA* in JB41v carrying the *clpB*::Tn*Max2*-Q mutant clone pJB69-Q (23.2 versus 9.8 U, respectively). It is also possible that ClpB is not expressed at sufficient levels from pJB305 for full complementation or that genes downstream of *clpB* are affected. To address this issue, a new *clpB* mutant, BY1v, was generated by plasmid insertion early in the gene; this mutation should create a truncated ClpB

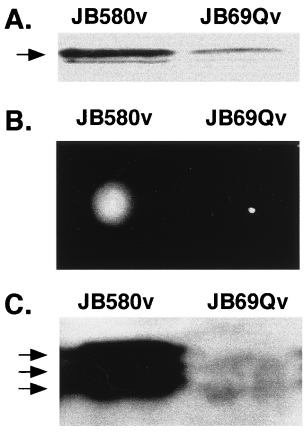

FIG. 4. Invasin and motility phenotypes of the *clpB* mutant JB69Qv. (A) Whole-cell extracts of indicated strains grown at 23°C were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with a rabbit polyclonal anti-invasin antibody as described in Materials and Methods. The arrow points to the band representing full-length invasin. (B) Motility assays were performed with JB580v and JB69Qv in 0.3% motility soft agar plates incubated at 23°C for 16 h. (C) Western analysis of flagellin preparations probed with monoclonal antibody 15D8 as described in Materials and Methods. Arrows point to the three flagellin subunits in *Y. enterocolitica*. Data shown are from a single experiment and are representative of several experiments performed with similar results.

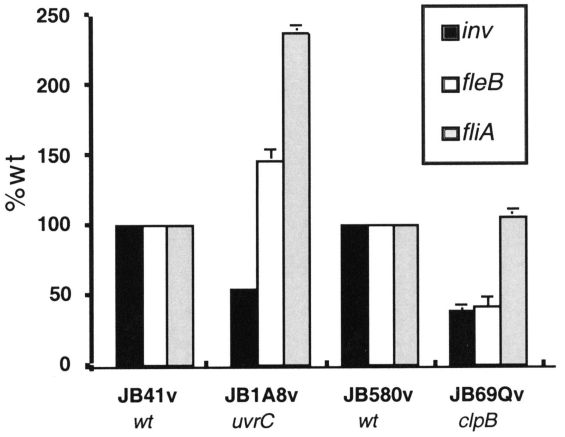

FIG. 6. Analysis of transcript levels and summary of phenotypes of the various mutants. Transcription of *inv*, *fleB*, and *fliA* was measured by RNA slot blot analysis. RNA was prepared from late-log- to early-stationary-phase cells grown at 23°C. Wild-type transcript levels were set at 100%, and relative amounts of specific mRNA from other strains were determined. For mutant JB1A8v, transcript levels are shown relative to the parental strain JB41v (wild-type *inv* and *inv*::*phoA*). Transcript levels for JB69Qv are shown relative to the parental strain JB580v. Data presented represent the mean ± the range from an individual representative experiment containing duplicate samples and reflect the results from several experiments using independently prepared RNA samples.

(364 amino acids). However, BY1v showed the same invasin and motility phenotypes as the wild-type strain JB580v (data not shown). Thus, the invasin and motility defects of JB69Qv may be specific to the *clpB*::Tn*Max2*-Q allele.

mRNA transcript levels in various mutants. To determine if the changes in invasin and flagellin levels in the *clpB*::Tn*Max2*-Q mutant JB69Qv were due to changes in the level of transcript, we performed RNA slot blot analysis as described in Materials and Methods. We first analyzed mRNA transcripts for *fliA* levels; *fliA* encodes a sigma factor required for expression of the flagellin genes (18, 21). *fliA* transcript levels in the *clpB*::Tn*Max2*-Q mutant JB69Qv were comparable to wild-type JB580v levels. In contrast, the other *inv* regulatory mutant, JB1A8v, showed a significant increase in *fliA* transcript levels (Fig. 6). RNA transcripts were then analyzed using a *fleB*-specific probe; *fleB* encodes one of the flagellins. JB1A8v showed increased *fleB* transcription compared to that in the wild type, which correlates with the observed increase in motility and flagellin expression in this strain. In contrast, the *clpB*::Tn*Max2*-Q mutant JB69Qv showed significantly lower *fleB* transcript levels compared to those in the wild type, consistent with the nonmotile phenotype of this mutant (Fig. 6). The increase in *fliA* transcript levels for JB1A8v suggests that the hypermotility phenotype of JB1A8v is due to increased expression of *fliA* and subsequent increased expression of *fleABC*. In contrast, the effect on motility observed for the *clpB*::Tn*Max2*-Q mutant JB69Qv does not appear to work through alterations in *fliA* mRNA levels.

We next analyzed *inv* transcript levels in these mutants. The *clpB*::Tn*Max2*-Q mutant JB69Qv and the *inv* regulatory mutant JB1A8v demonstrated significant decreases in *inv* transcript levels. The decrease in *inv* transcript levels for JB1A8v correlates with *inv*-specific primer extension analysis reported previously (1). Although RNA analysis may suggest that an effect is at the transcriptional level, we cannot at this time distinguish between transcription initiation and stability of the transcript.

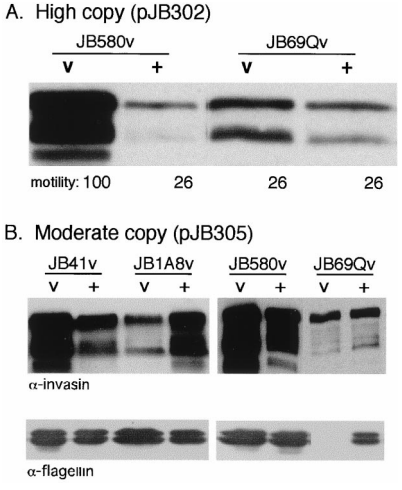

FIG. 5. Complementation of the *clpB* mutant JB69Qv and the *uvrC* mutant JB1A8v. (A) Western analysis of whole-cell extracts of indicated strains grown at 23°C using an antibody to invasin. The numbers listed at the bottom (motility) indicate the percentage of the size of the motility zone in soft agar seen with the wild type (i.e., zone for JB580v pCR2.1 = 100). The "v" indicates the presence of vector pCR2.1, and "+" indicates the presence of the *clpB* plasmid pJB302. (B) (Top) Western analysis of whole-cell extracts of indicated strains grown at 23°C using an antibody to invasin. (Bottom) Western analysis of flagellin preparations from the same strains probed with monoclonal antibody 15D8 as described in Materials and Methods. The "v" indicates the presence of vector pTM100, and "+" indicates the presence of the *clpB* plasmid pJB305. Data shown are from a single experiment and are representative of several experiments performed with similar results.

DISCUSSION

In a previous report we described the isolation and characterization of an invasin regulatory mutant, JB1A8v, that demonstrates decreased invasin levels and increased flagellin levels. JB1A8v has an mTn*5*Km insertion into *uvrC*. The *uvrC* mutant JB1A8v demonstrated a significant decrease in *inv*-

specific transcript levels (1) and an increase in *fleB*-specific transcripts consistent with the observed expression levels of invasin and flagellin, respectively. It is not known at this time how the mTn*5*Km insertion mutation results in the observed phenotypes of JB1A8v, due to the inability to clone the wild-type locus (1). Interestingly, a clone encoding the *Y. enterocolitica* homologue of ClpB suppresses the mutant phenotypes of this mutant.

ClpB belongs to a recently identified family of proteins (Clp ATPases). Clp ATPases (ClpA, ClpX, and ClpY) were originally identified as regulatory components for ATP-dependent Clp proteases and have been shown to exist in organisms from all three kingdoms (12, 47). ClpB was initially identified as a heat shock protein (48) and has been shown to have ATPase activity (33, 55). Unlike other members of the Clp family (i.e., ClpA and ClpX), ClpB has yet to be shown to associate with the ATP-dependent protease subunit ClpP. In *E. coli*, *clpB* encodes two proteins, a full-length ClpB and a truncated form. The ClpB proteins form a tetrameric ATPase in addition to hetero-oligomeric complexes (33, 55). It has been suggested that the two forms of ClpB may provide the ClpB protein with separate functions (47). Recent data also suggest that Clp ATPases may act as chaperones independently of ClpP or other catalytic subunits (53).

The ClpB identified in this study as a suppressor of the mutant phenotypes of the *inv* regulatory mutant JB1A8 shows a relatively low level of identity to the ClpB of *E. coli*. This low level of identity with the *E. coli* protein is surprising given the close relationship of the two organisms. Conserved proteins of *Y. enterocolitica* and *E. coli* are often more than 80% identical. For example, the ClpX and ClpP proteins of *E. coli* and *Y. enterocolitica* are 94 and 90% identical, respectively (34). One possible explanation for the low identity of the ClpB proteins is that the *clpB* gene of *Y. enterocolitica* identified in this study was acquired by horizontal transfer. This explanation is supported by the observation that the *Y. pestis* chromosome encodes a close homologue of the *Y. enterocolitica* ClpB protein (more than 80% identical) in addition to a separate close homologue of the *E. coli* ClpB protein (more than 80% identical) (The Sanger Center, www.sanger.ac.uk/Projects/Microbes/). It is possible that the same is true for *Y. enterocolitica*.

What is the role of ClpB in *Y. enterocolitica inv* and *fleABC* (flagellin) expression? Two *clpB* mutants were constructed; one of these mutants, JB69Qv, shows decreased levels of invasin and is nonmotile. From densitometry analysis, the *clpB*::Tn*Max2*-Q mutant JB69Qv shows 40% of wild-type *inv* message levels yet only 6% of wild-type protein levels. In addition, JB69Q shows 50% of wild-type *fleB* (flagellin) message levels, yet flagellin is virtually undetectable (Fig. 4C and 5B). These results suggest that the *clpB* mutation may affect *inv* expression both transcriptionally and posttranscriptionally. This mechanism of a Clp ATPase regulator working at several levels in the same genetic pathway is not uncommon. In *Bacillus subtilis*, MecB (a ClpC ATPase homologue) is a pleiotropic regulator of competence genes (29). In addition, it was recently determined that *B. subtilis* MecB/ClpC positively regulates autolysin gene expression and differentially regulates SigD (alternative sigma factor)-dependent genes, including flagellin and motility genes. The MecB/ClpC regulation of SigD-dependent genes has been demonstrated to act in both a SigD-dependent and a SigD-independent manner (42).

In addition, RNA analysis suggests that the *clpB*::Tn*Max2*-Q mutation in JB69Qv affects motility at some point beyond transcription of *fliA*. By analogy to the ability of ClpA to activate and degrade the DNA binding protein RepA (53), one could postulate that ClpB may be necessary for the stability/activation and degradation of FliA. In addition, ClpB might be required for activation/stability of a yet-to-be-identified regulator of *inv* expression. Complicating the interpretation of these results are the observations that a *clpB* mutation early in the gene (probably a null mutation, yielding mutant BY1v) shows the same *inv* expression pattern and motility as the wild type.

ClpB has two different ATP binding domains, and there is evidence in the literature that Clp proteins can have both positive and negative effects (52). Thus, one model to explain these results would be that each ATP binding domain of ClpB has a different function. In this model, ATP binding domain 1 functions to downregulate expression of *inv* and motility, and ATP binding domain 2 functions to upregulate expression of *inv* and motility; these effects could be direct or indirect. Therefore, under standard lab conditions, the functions of domain 1 and domain 2 cancel each other out. This would explain why the null mutant BY1v (ClpB1) phenotypically resembles the wild type under these conditions. Similarly, expression of *inv* and motility would be downregulated in JB69Qv because ClpB69 contains only a functional domain 1. This also would explain the partial complementation of JB69Qv by wild-type ClpB; the ratio of domain 1 to domain 2 would be greater than 1, and therefore expression of *inv* and motility would still be reduced relative to those in the wild type. Any condition that differentially affected the activity of domain 1 relative to that of domain 2 would alter the expression pattern of *inv* and motility.

In addition, the copy number of *clpB* also could affect expression of *inv* and motility. Consistent with this hypothesis, when *Y. enterocolitica clpB* is supplied in *trans* on a high-copy-number plasmid (pJB302), invasin levels are dramatically decreased. This phenomenon is seen in all strains tested, including the wild-type strain JB580v (J. L. Badger and V. L. Miller, unpublished data). In contrast, providing ClpB in *trans* on a moderate-copy-number plasmid (pJB305) restores invasin production and motility to wild-type levels in the *uvrC* mutant JB1A8v, partially complements the *clpB* mutant JB69Qv, and has a moderate effect on invasin levels and motility in the wild-type strains. This apparent requirement for *Y. enterocolitica* to maintain ClpB at appropriate levels in order to preserve proper expression is reminiscent of what has been observed for *Saccharomyces cerevisiae*. In *S. cerevisiae* a yeast non-Mendelian factor, [psi$^+$], is suggested to be a self-modified protein analogous to mammalian prions, and recently it has been shown that intermediate amounts of the chaperone protein ClpB (Hsp104) are required for propagation of the [psi$^+$] factor. It has also been observed that either overexpression or inactivation of ClpB (Hsp104) in *S. cerevisiae* leads to the same phenotype of being cured of the prion-like factor (7).

Interestingly, Clp ATPase homologues have been emerging as important factors for virulence. For example, Pederson et al. recently demonstrated that the repression of *Y. enterocolitica ail* expression at low temperatures is dependent on ClpP (34). In *Salmonella enterica* serovar Typhimurium, the alternative sigma factor σ^S (RpoS) regulates genes involved in the environmental stress response in addition to regulating many virulence properties (16, 23, 24, 31, 32). In *E. coli*, σ^S is negatively regulated by ClpXP-mediated degradation (45). MviA, a two-component response regulator homologue (also known as SprE in *E. coli*), plays an essential role in RpoS turnover (4, 30, 40). It has been demonstrated that MviA/SprE influences the susceptibility of RpoS to ClpXP-mediated degradation (40, 45). More recently, the *Listeria monocytogenes* MecB/ClpC homologue was determined to be necessary for macrophage survival and virulence in mice (44).

From evidence presented in this communication, it is apparent that ClpB plays a role in the regulation of the virulence factor invasin and of motility in *Y. enterocolitica*. Furthermore, it appears that the effects of ClpB take place at multiple levels. Based on evidence presented here, there are several levels at which expression of invasin and motility are cocontrolled, as follows. (i) A locus of JB1A8v (possibly *uvrC*) is necessary for proper expression of *inv* and *fliA*, and this effect occurs at the level of the *inv* and *fliA* transcripts. Overexpression of ClpB can compensate for this defect. (ii) ClpB-dependent effects on the *inv* and *fleB* transcripts are observed when only the N-terminal two-thirds of ClpB is expressed. (iii) ClpB-dependent regulation of FliA and invasin protein levels may take place. The complexity of the adaptive process by which *Y. enterocolitica* regulates invasin and motility may reflect the need to coordinate multiple functions associated with the pathogenic life cycle. It remains to be determined whether other factors associated with the pathogenicity or virulence of *Y. enterocolitica* are affected by ClpB.

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