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Identification and Cloning of a *fur* Regulatory Gene in *Yersinia pestis*†

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Yersinia pestis **is one of many microorganisms responding to environmental iron concentrations by regulating the synthesis of proteins and an iron transport system(s). In a number of bacteria, expression of iron uptake systems and other virulence determinants is controlled by the Fur regulatory protein. DNA hybridization analysis revealed that both pigmented and nonpigmented cells of *Y. pestis* possess a DNA locus homologous to the *Escherichia coli fur* gene. Introduction of a Fur-regulated β-galactosidase reporter gene into *Y. pestis* KIM resulted in iron-responsive β-galactosidase activity, indicating that *Y. pestis* KIM expresses a functional Fur regulatory protein. A cloned 1.9-kb *ClaI* fragment of *Y. pestis* chromosomal DNA hybridized specifically to the *fur* gene of *E. coli*. The coding region of the *E. coli fur* gene hybridized to a 1.1-kb region at one end of the cloned *Y. pestis* fragment. The failure of this clone to complement an *E. coli fur* mutant suggests that the 1.9-kb clone does not contain a functional promoter. Subcloning of this fragment into an inducible expression vector restored Fur regulation in an *E. coli fur* mutant. In addition, a larger 4.8-kb *Y. pestis* clone containing the putative promoter region complemented the Fur$^-$ phenotype. These results suggest that *Y. pestis* possesses a functional Fur regulatory protein capable of interacting with the *E. coli* Fur system. In *Y. pestis* Fur may regulate the expression of iron transport systems and other virulence factors in response to iron limitation in the environment. Possible candidates for Fur regulation in *Y. pestis* include genes involved in ferric iron transport as well as hemin, heme/hemopexin, heme/albumin, ferritin, hemoglobin, and hemoglobin/haptoglobin utilization.**

Both saprophytic and pathogenic bacteria often encounter iron-deficient growth conditions. At physiological pH, in an aerobic environment, ferric ions are essentially insoluble and unavailable for use by bacterial cells (11, 30, 59). Animal pathogens experience an additional level of difficulty in obtaining inorganic iron and hemin because mammalian iron-binding compounds, including transferrin, lactoferrin, hemopexin, albumin, hemoglobin, haptoglobin, and ferritin, control the availability of this essential trace nutrient within the host (11, 59). To obtain the quantities of iron necessary for growth, bacteria have developed diverse and complex iron acquisition systems. A number of siderophore-mediated iron transport systems have been described which allow bacteria to overcome iron deficiency in the host (11, 29, 30, 38, 45). These systems often require specific outer membrane receptors and a series of periplasmic and cytoplasmic membrane proteins to complete the transport circuitry. Because a number of components are required for iron acquisition, a significant investment of cellular energy is needed to fuel these systems. Consequently, the expression of iron acquisition genes is regulated by the availability of iron. In this manner, iron transport genes are not highly expressed in an iron-sufficient environment but are activated during iron deficiency (35).

In some pathogens, synthesis of toxins and virulence determinants unrelated to iron metabolism is also regulated by the iron status of the bacterial cell. Shiga toxin of *Shigella dysenteriae* (17), Shiga-like toxin of *Escherichia coli* (12), hemolysin of *Vibrio cholerae* (51), and diphtheria toxin of *Corynebacterium diphtheriae* (7, 15) are examples of iron-regulated gene products. Pathogenic bacteria in general exhibit iron-repressible membrane proteins which include outer membrane proteins in *E. coli* (32), *Salmonella typhimurium* (35), *Enterobacter cloacae* (36), *Shigella flexneri* (39), *Klebsiella aerogenes* (61), *Erwinia* species (20), and yersiniae (14, 49). The function of a number of these membrane proteins has not been determined.

In vitro growth of *Yersinia pestis* in iron-deficient medium results in expression of several iron-regulated membrane proteins (14, 49). Carniel et al. (14) have demonstrated the presence of two high-molecular-weight iron-regulated proteins and several smaller iron-regulated proteins in *Y. pestis* cells grown in iron-deficient medium at 26 and 37°C. Sikkema and Brubaker (49) demonstrated that four of the five major iron-regulated outer membrane proteins are specific to pigmented (Pgm$^+$) *Y. pestis* cells and may be required for the growth of Pgm$^+$ cells in iron-chelated medium at 37°C. The Pgm$^+$ phenotype of *Y. pestis* also confers the ability to store exogenous hemin at 26°C but not 37°C (26), to express unique, non-iron-regulated polypeptides (49, 54), and to be virulent in the mouse model (27). Due to a large chromosomal deletion (43), spontaneous nonpigmented (Pgm$^-$) mutants of *Y. pestis* do not express any of these characteristics and are avirulent. The mechanism by which this deletion prevents expression of some but not all iron-regulated peptides is unresolved (49) but may involve deletion of structural genes for these polypeptides. Although iron clearly affects the expression of genes in *Y. pestis*, nothing is known concerning the regulation of these iron-responsive proteins.

In a number of enteric organisms, the *fur* gene product is involved in the regulation of iron transport systems and other virulence determinants (19, 23, 24). A mutation in the

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† This paper is dedicated to the memory of Charles E. Lankford for his important work on bacterial siderophore transport systems.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevent characteristics	Reference
Y. pestis[a]		
KIM6+	Pgm$^+$ Pst$^+$ Lcr$^-$ Fra$^+$(pMT1, pPCP1)	49
KIM6	Pgm$^-$ Pst$^+$ Lcr$^-$ Fra$^+$(pMT1, pPCP1)	49
KIM10+	Pgm$^+$ Pst$^-$ Lcr$^-$(pMT1)	43
KIM10	Pgm$^-$ Pst$^-$ Lcr$^-$(pMT1)	43
Kuma+	Pgm$^+$ Pst$^+$ Lcr$^-$ Fra$^+$(pMT2, pPCP2)	40
Y. enterocolitica WA-LOX	Serotype O:8, Lcr$^-$	41
Y. pseudotuberculosis PB1/O	Type 1, Lcr$^-$	41
E. coli		
DH5α	*endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169*(ϕ80 lacZΔ*M15*)	22
HB101	*leuB6 proA2 recA13 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44 hsd20*	6
DHB24	Δ(*ara-leu*)*7697 araD139* Δ(*lac*)*X74 galE galK rpsL thi malF*Δ*3 phoR phoA*Δ*Pvu*II *zad*::Tn*10 pcnB* (F′ *lac*$^+$ *pro*$^+$ *lacI*^{q})	12
SBC24	*fur*::Tn*5* derivative of DHB24	12
SY327	*araD* Δ(*lac-pro*) *argE*(Am) *rif nalA recA56*	13
H1780	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR fur fiu*::λ*plac*Mu53	25
Plasmids		
pAT153	Cloning vector, Ap^r Tc^r	57
pBR322	Cloning vector, Ap^r Tc^r	2
pLG338	Low-copy-number cloning vector, Km^r Tc^r	52
pUC19	Cloning vector, Ap^r	2
pKK223-3	*tac* promoter expression vector, Ap^r	9
pRT240	Constitutive *lacZ* expression, Ap^r	13
pSC27.1	Fur-regulated *lacZ* expression, Ap^r	13
pMH15	Cloned *E. coli fur* gene, Cm^r	22
pMON2064	Cloned *E. coli fur* gene, Ap^r	58
pFURY2.1	2.1-kb *fur*-positive clone[b] in pBR322, Ap^r	This study
pFURY1.9	1.9-kb *fur*-positive clone[b] in pUC19, Ap^r	This study
pOEF2	1.9-kb *fur*-positive clone[b] in pKK223-3, Ap^r	This study
pFURY796	6.8-kb *fur*-positive clone[b] in pLG338, Km^r	This study
pFURY4.8	4.8-kb *fur*-positive clone[b] in pAT153, Tc^r	This study

[a] All KIM strains were derived from KIM6+; a "+" designates Pgm$^+$ strains. The three KIM plasmids have been designated pMT1 (110 kb, no known functions), pCD1 (75 kb, Lcr genes), and pPCP1 (9.5 kb, pesticin genes, plasminogen activator/coagulase gene [Pac$^+$]). Pst$^+$, Pesticinogenic; Lcr, low-Ca^{2+} response; Fra, fraction 1 antigen (53, 54).

[b] From *Y. pestis* KIM6+.

fur gene results in constitutive expression of outer membrane proteins and siderophores which are normally repressed at high iron concentrations and derepressed at low iron concentrations. The Fur (for Fe uptake regulation) protein acts as a negative regulator of transcription in vivo by complexing with ferrous iron (Fe^{2+}) to repress expression of iron-regulated operons. In the presence of ferrous iron, the Fur protein binds the promoter-operator region of Fur-regulated operons and interferes with the ability of RNA polymerase to transcribe the operon (3). Conversely, in the absence of ferrous iron, the Fur protein cannot repress transcription because it no longer associates with DNA. In bacterial pathogens, a Fur-like system may be important in regulating not only iron accumulation systems but also other virulence determinants. Gene regulation by this iron-sensitive surveillance system is likely a global mechanism which has been conserved throughout evolution, allowing bacteria to respond to fluctuations in iron conditions.

Because *Y. pestis* is very similar genetically to *E. coli* and iron-regulated expression of proteins and transport systems in *Y. pestis* has been demonstrated (14, 40, 49), we conducted this study to determine whether a Fur-like regulatory mechanism analogous to the one in *E. coli* is present in *Y. pestis*. By using an *E. coli fur* gene probe, *Y. pestis* was shown to contain a homologous sequence. A reporter gene fusion construct indicated that *Y. pestis* KIM possessed a functional Fur protein capable of interfacing with *E. coli* Fur binding sequences. The *Y. pestis fur* structural gene was cloned and shown to complement a Fur$^-$ mutant of *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids. Characteristics of the *Y. pestis* and *E. coli* strains and the plasmids used in this study are described in Table 1. All *Y. pestis* KIM strains are avirulent due to the loss of the Lcr plasmid pCD1 (42, 53, 54). Plasmid pCD1, which encodes the low-Ca^{2+} response (Lcr$^+$) virulence regulon of *Y. pestis* KIM strains, has no demonstrable effect on iron metabolism (10, 40). Spontaneous Pgm$^-$ mutants of *Y. pestis* KIM strains were isolated on Congo red agar (56). In plasmid pRT240, fusion of the β-galactosidase gene to the *E. coli* outer membrane protein F (OmpF) promoter results in constitutive expression of β-galactosidase regardless of iron concentration (13). In plasmid pSC27.1, a synthetic oligonucleotide corresponding to the *E. coli* consensus Fur binding sequence is inserted into the

TABLE 2. Composition of defined PMH medium[a]

Component	Final concn (mM)
HEPES[b]	25.0
K_2HPO_4	2.5
$MgCl_2$[c]	20.0
$MnCl_2$[d]	0.01
$Na_2S_2O_3$	2.5
NH_4Cl	10.0
D-Biotin	0.002
Calcium pantothenate	0.004
Thiamine	0.003
Glucose	10.0
DL-Alanine	2.5
L-Arginine hydrochloride	1.0
L-Asparagine	2.5
L-Aspartate	1.0
L-Glutamate	5.0
L-Glutamine	1.0
Glycine	5.0
L-Histidine	1.0
L-Isoleucine	1.0
L-Leucine	1.0
L-Lysine	1.0
L-Methionine	1.0
L-Phenylalanine	1.0
L-Proline	5.0
L-Serine	5.0
L-Threonine	2.5
L-Tryptophan	0.1
L-Tyrosine	1.0
L-Valine	1.0

[a] Medium prepared as a 2× stock; pH adjusted to 7.5 with 1 M NaOH; 2× stock deferrated with Chelex 100 and filter sterilized.
[b] HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.
[c] $MgCl_2$ added after Chelex 100 extraction.
[d] $MnCl_2$ omitted for analysis of the Fur regulatory system.

OmpF promoter region of pRT240. This insertion mediates negative transcriptional regulation of β-galactosidase synthesis by the Fur protein in direct response to the iron concentration in the growth medium (13). Each plasmid also encodes the β-lactamase gene originating from a promoter oriented in the opposite direction from the β-galactosidase gene. Plasmid pMON2064 contains the *E. coli fur* structural gene under the control of a promoter which can be induced to overexpress the Fur protein (58).

Cultivation of cells. All bacterial strains were stored at −20°C in buffered glycerol (4). For DNA isolation, *Y. pestis* cells were grown in liquid HIBX (heart infusion broth [Difco Laboratories, Detroit, Mich.] supplemented with 0.2% xylose) and plated on tryptose-blood-agar (TBA) base (Difco) plates. *E. coli* strains were grown in Luria broth (LB) and plated on LB solidified with agar or iron-supplemented MacConkey-lactose plates (Difco) plus IPTG (isopropylthio-β-galactoside). All bacteria showing antibiotic resistance were cultivated with the appropriate antibiotic at concentrations of 50 μg/ml for ampicillin, kanamycin, and chloramphenicol and 12.5 μg/ml for tetracycline.

For growth response studies under various iron conditions, all glassware was acid cleaned in 18% (vol/vol) HCl and copiously rinsed with distilled, deionized H_2O. All reagents were prepared in double distilled, deionized H_2O. Chemically defined PMH medium omitting $MnCl_2$ (Table 2) was deferrated with Chelex 100 resin (Bio-Rad, Richmond, Calif.) prior to filter sterilization (18). Deferrated PMH contained less than 0.3 μM residual iron as determined by furnace atomic absorption spectroscopy. Growth response studies were initiated by inoculating glycerol-stocked cells onto PMH agar (PMHA) slants supplemented with 1 μM $FeCl_3$. These cells were suspended in deferrated PMH and used to inoculate deferrated PMH with or without 20 μM $FeCl_3$ or 20 μM hemin. The cultures were grown with aeration at 37°C in a New Brunswick model G76 gyratory shaker waterbath (200 rpm) for approximately eight generations and transferred to fresh PMH medium containing appropriate iron supplements plus an additional culture containing the iron chelator EDDA (ethylene-di[*o*-hydroxyphenylacetic acid]; Sigma, St. Louis, Mo.) at a final concentration of 10 μM. Contaminating iron was removed from EDDA by the method of Rogers (45). At regular intervals, growth was monitored by determining the OD_{620} with a Stasar II Gilford spectrophotometer.

Assays. β-Galactosidase and β-lactamase activities were detected in cell lysates by cleavage of the chromogenic substrates ONPG (4-nitrophenyl-β-D-galactopyranoside) (33) and nitrocefin (BBL Microbiology Systems, Cockeysville, Md.) (53), respectively. Protein concentrations were determined by the BCA protein liquid assay system (Pierce, Rockford, IL) with bovine serum albumin (BSA) standards.

Utilization of host iron sources. As described above, *Y. pestis* KIM6+ (Pgm^+) and KIM6 (Pgm^-) cells were cultivated in deferrated PMH, pelleted by centrifugation, and resuspended in 33 mM potassium phosphate buffer (pH 7.0). Cells were added to 3.0 ml of molten, deferrated PMHA containing 100 μM EDDA and dispensed onto prepoured PMHA plates containing 100 μM EDDA. Sterile paper disks were saturated with various host iron- and heme-binding compounds. Hemoglobin (Sigma) was dialyzed to remove contaminating ions and filter sterilized (1, 55, 60). Fifty percent-saturated complexes of hemoglobin/haptoglobin, heme/albumin, heme/hemopexin, and $FeCl_3$/ferritin were generated by incubating purified components together prior to filter sterilization (1, 34, 37, 55). Haptoglobin, transferrin, lactoferrin, ferritin, and albumin were obtained from Sigma, and hemopexin was generously provided by J. Glass. Deferrated lactoferrin and transferrin were approximately 50% saturated with $FeCl_3$ in the buffer system of Mazurier and Spik (31), followed by dialysis and filter sterilization (31, 60). Ferric chloride (10 mM) was solubilized in 10 mM HCl and sterilized by filtration. Contaminating inorganic iron was removed from hemin by the procedure described below. Hemin powder (Sigma) was washed in 10 mM HCl, and the undissolved powder was collected by centrifugation and rinsed in doubly distilled, deionized H_2O. The precipitate was dissolved in 10 mM NaOH. The hemin concentration was determined by the OD_{580} against a hemin standard curve.

In vitro DNA manipulations. The rapid plasmid isolation techniques of Kado and Liu (28) or Birnboim and Doly (5) were used to verify plasmid content in various bacterial strains. Plasmids pSC27.1 and pRT240 were introduced into *Y. pestis* KIM6+ by a modified dimethyl sulfoxide (DMSO) transformation method (22). Briefly, *Y. pestis* cells were grown to early log phase in 20 ml of HIBX, harvested, washed in 1/2 volume of ice-cold 60 mM $MgCl_2$, and resuspended in 1/10 volume of ice-cold 75 mM $CaCl_2$. Cells were incubated on ice for 20 min before addition of 300 μl of high-pressure liquid chromatography (HPLC) grade DMSO. After a further incubation of 10 min on ice, DNA was introduced into 200-μl aliquots of cells by standard transformation techniques (2). Bacterial genomic DNA was isolated by a lysozyme-sodium dodecyl sulfate (SDS)-proteinase K

method (2) and further purified by phenol and chloroform extractions.

A *Y. pestis* genomic DNA library was generated by partial *Sau*3A digestion of KIM6+ genomic DNA and ligation of pooled fragments into the low-copy-number vector pLG338 (52). The resultant library (43) was screened by colony blot hybridization (2) with appropriate DNA probes to isolate colonies of interest.

DNA restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were used according to the manufacturers' specifications. Restriction enzyme-digested genomic DNA fragments of appropriate sizes were separated by sucrose density gradient centrifugation (2). Radiolabeled probes were generated by using a nick translation kit (Bethesda Research Laboratories, Gaithersburg, Md.) or random priming labeling kit (New England Nuclear [NEN] Research Products, Boston, Mass.). NEN Research Products was the source of [^{32}P]dCTP.

DNA fragments were resolved through 0.7% agarose gels, and DNA was transferred to nitrocellulose by the method of Southern (50). The entire translated region of the *E. coli fur* gene, consisting of a 443-bp *Hin*dIII-*Nco*I fragment from pMON2064, was isolated from low-gelling-temperature agarose (FMC, Rockland, Maine), purified by GeneClean (Bio 101, La Jolla, Calif.) and radiolabeled by random priming. The *Y. pestis fur* gene probe, consisting of the 1.9-kb *Eco*RI-*Pst*I fragment of pFURY1.9, was labeled by nick translation (44). This probe was composed of a 1.1-kb fragment exhibiting hybridization to the *E. coli fur* gene plus flanking sequences of *Y. pestis* chromosomal DNA which did not hybridize to the *E. coli* probe. For *E. coli* to *Y. pestis* hybridizations, low-stringency procedures were performed at 55°C in low-stringency hybridization buffer (1 M NaCl, 50 mM sodium phosphate, 5 mM EDTA, 0.5% SDS, 10× Denhardt's solution [1× Denhardt's solution contains 0.02% each BSA, polyvinylpyrrolidone, and Ficoll]). For *E. coli* to *E. coli* or *Y. pestis* to *Y. pestis* hybridizations, high-stringency hybridization buffer (50% formamide, 5× Denhardt's solution, 0.1% SDS, 5× SSPE [1× SSPE contains 0.15 M NaCl, 11.5 mM NaH_2PO_4, and 1 mM EDTA, pH 7.4]) was used at 42°C. After 18 h of hybridization, the final washes of high-stringency blots were done at 55°C in 0.1× SSC (standard saline citrate)–0.1% SDS, and washes of low-stringency blots were done in 3× SSC–0.1% SDS at 55°C. Hybridization was visualized by exposure of the blots to X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.) at −70°C.

RESULTS

Iron-deficient growth of *Y. pestis*. Cells of *Y. pestis* KIM6+ containing the Fur-regulated reporter gene construct (pSC27.1) grown in low-iron PMH medium exhibited a decreased growth rate and final cell yield. These growth data mimic those of *Y. pestis* KIM6+ alone indicating that the presence of the reporter plasmid, pSC27.1, did not affect the growth response of this strain (data not shown). The generation times of cells grown in the presence of 20 μM $FeCl_3$ or 20 μM hemin was ~1.25 h, versus 2 h for those grown in deferrated PMH with no iron supplement. The maximum OD_{620} of the $FeCl_3$ and hemin cultures was 2.56 and 1.65, respectively, while that of the low iron culture was 1.1 (data not shown). These results indicate that deferrated PMH is an iron-deficient growth medium for *Y. pestis* cells. Growth could be further inhibited by 10 μM EDDA, resulting in a generation time of ~5 h, reaching a final OD_{620} of 0.39. With 100 μM EDDA, growth was virtually eliminated in deferrated liquid PMH or PMHA plates. This effect of EDDA was reversed by addition of excess $FeCl_3$ to PMHA plates or to liquid cultures (data not shown).

TABLE 3. β-Galactosidase activities of *Y. pestis* KIM derivatives and *E. coli* H1780 and SY327 cells grown in defined PMH medium[a]

Strain	β-Galactosidase activity[b] (U)			Induction ratio	
	+Fe (20 μM $FeCl_3$)	−Fe (no added $FeCl_3$)	+EDDA (10 μM EDDA)	−Fe/+Fe	+EDDA/+Fe
KIM6+(pSC27.1)	112.7	233.9	530.0	2.08	4.70
KIM6+(pRT240)	1,735.0	2,023.0	ND[c]	1.17	
KIM6(pSC27.1)	198.3	383.4	586.7	1.93	2.96
SY327(pSC27.1)	363.0	1,537.0	1,495.0	4.23	4.12
KIM6+	ND	0	ND		
H1780(pFURY4.8)	1.04	24.8	ND	23.85	
H1780(pAT153)	95.10	130.0	ND	1.37	

[a] *Y. pestis* KIM and *E. coli* SY327 cells possess a single-copy chromosomal *fur* gene and a multicopy reporter gene on pSC27.1. *E. coli* H1780(pFURY4.8) cells possess a single-copy chromosomal reporter gene and a multicopy *Y. pestis fur* gene on pFURY4.8.
[b] Units are Miller units (33).
[c] ND, Not determined.

Since iron availability and not the presence of EDDA was responsible for inhibiting the growth of *Y. pestis* cells, PMHA-EDDA plates were used to test the ability of the organism to utilize various host iron sources at 26 and 37°C. The following compounds were saturated to 50% binding capacity with $FeCl_3$ or hemin and added to sterile paper disks in the following quantities: 0.05 μmol of transferrin, 1.0 μmol of lactoferrin, 0.01 μmol of hemoglobin, 0.5 nmol of hemoglobin/haptoglobin, 2.4 pmol of ferritin, 0.5 nmol of heme/albumin, 2.1 nmol of heme/hemopexin, 50 nmol of $FeCl_3$, and 0.1 nmol of hemin. Addition of these disks to PMHA–100 μM EDDA plates onto which approximately 10^7 cells of *Y. pestis* KIM6+ or KIM6 had been spread resulted in zones of bacterial growth at both 26 and 37°C surrounding each disk with the exception of those containing transferrin and lactoferrin (data not shown). These data confirm and extend those reported by Sikkema and Brubaker (49) and demonstrate that host iron source utilization is independent of pigmentation phenotype and growth temperature.

Fur-regulated β-galactosidase expression. Calderwood and Mekalanos (13) have previously demonstrated iron-repressible β-galactosidase production in *E. coli* SY327(pSC27.1) and constitutive synthesis in SY327(pRT240). Iron-regulated expression of β-galactosidase from plasmid pSC27.1 requires a functional Fur protein (13). To determine whether a functional Fur-like protein is present in *Y. pestis*, KIM6+ and KIM6 cells carrying pSC27.1 or pRT240 were grown as described above in deferrated PMH with or without 20 μM $FeCl_3$. Mid-log-phase cells were lysed, and β-galactosidase activities of cell extracts were quantitated (33). Since *Y. pestis* cells have no detectable β-galactosidase activity (21) (Table 3), all activity detected in the *Y. pestis* transformants was due to the plasmid-borne gene. As expected, *Y. pestis* KIM6+(pRT240) produced constitutively high levels of β-galactosidase (Table 3). Under iron-deficient conditions in PMH medium, *E. coli* SY327(pSC27.1) cells exhibited a 4.23-fold derepression of enzyme activity (Table 3), compared with the 4.4-fold derepression observed by Calderwood and Mekalanos (13), demonstrating that this multicopy

reporter system produces induction ratios which are lower than those typically seen for iron-regulated genes in *E. coli*. Although β-galactosidase activity was lower in our hands for *Y. pestis* grown in PMH medium than for *E. coli* grown in T medium by Calderwood and Mekalanos (13), these differences are not due to carbon source because both media are glucose based. A 2.08-fold derepression of β-galactosidase activity was observed in iron-limited cells of *Y. pestis* KIM6+(pSC27.1), while a similar 1.93-fold derepression was observed with KIM6(pSC27.1) cells (Table 3). Although the degree of regulation is smaller than that observed with cells of *E. coli*, it is significant. The values reported in Table 3 for cells cultured without added iron and with 20 μM $FeCl_3$ represent a compilation of results from duplicate assays of samples from four or more independent cell cultures.

The modest degree of regulation observed in *Y. pestis* may indicate sequence divergence in the *fur* gene. Since 10 μM EDDA further limited growth of *Y. pestis* cells via iron starvation, extracts of these cells were assayed for β-galactosidase activity. Compared with extracts from cells grown with 20 μM $FeCl_3$, KIM6+(pSC27.1) and KIM6(pSC27.1) cell extracts exhibited respective 4.7- and 2.96-fold derepressions of β-galactosidase activity (Table 3). *E. coli* SY327 (pSC27.1) did not demonstrate a similar increase in activity since EDDA is unable to withhold iron from the siderophore (enterochelin) synthesized by these cells.

The low β-galactosidase activities seen with low-iron cultures of KIM6+ and KIM6 carrying pSC27.1 compared with those in similar cultures containing pRT240 have also been reported for *E. coli* strains carrying these plasmids (13) and may involve alterations in the promoter as a result of the Fur binding sequence oligonucleotide insertion in pSC27.1. To ensure that the observed iron-dependent differences were not due to changes in plasmid copy number or transcriptional or translational efficiencies, β-lactamase production encoded by pSC27.1 and pRT240 was determined (53). The β-lactamase gene lies in the opposite transcriptional orientation from the β-galactosidase gene and originates at another promoter. β-Lactamase activities in *Y. pestis* KIM6+(pSC27.1) and KIM6+(pRT240) grown under various iron conditions were nearly identical (data not shown), indicating that differences in β-galactosidase activities are due to iron status (i.e., Fur regulation) rather than nonspecific effects. Consequently, iron regulation of β-galactosidase activity from pSC27.1 indicates that *Y. pestis* possesses a functional Fur protein which is capable of interaction with the consensus Fur binding sequence.

Detection of the *Y. pestis fur* gene by Southern blot analysis. *Cla*I-digested genomic DNA from various Pgm^+ and Pgm^- derivatives of *Y. pestis* KIM was electrophoresed through a 0.7% agarose gel, Southern blotted, and hybridized under low-stringency conditions (~30% mismatch) with a 443-bp *E. coli fur* probe containing only the translated region of the gene (58). *Y. pestis* DNA contained a common 1.9-kb hybridizing band (Fig. 1). The probe hybridized to a 9-kb band in *E. coli* DH5α (Fig. 1) as well as to 6.3-kb *Eco*RI fragments from *Yersinia pseudotuberculosis* PB1/O, *Yersinia enterocolitica* WA-LOX, and *Y. pestis* Kuma (data not shown). Hybridization to the 1.9-kb band of *Y. pestis* was not linked to either the pigmentation phenotype or the presence of any *Y. pestis* plasmid (Fig. 1, lane 3, and data not shown). Plasmid DNA from either pMT1 or pCD1 did not hybridize to the probe. Under low-stringency conditions, we obtained hybridization of the *E. coli fur* gene probe from pMON2064 and from the smaller *Alu*I-*Fsp*I *fur* fragment from plasmid pMH15 (24) to regions of plasmid DNA containing the replication origin of pPCP1 of *Y. pestis*, as well as pACYC184 and pBR322 (data not shown). However, hybridization to the 1.9-kb fragment of *Y. pestis* chromosomal DNA did not represent hybridization to pPCP1 since hybridization to *Y. pestis* strains lacking this plasmid also occurred (Fig. 1). Since *Y. pseudotuberculosis* PB1/0 and *Y. enterocolitica* WA-LOX are strain derivatives cured of their respective Lcr plasmids, the *E. coli fur* probe hybridizes to chromosomal DNA from these strains and not to plasmid DNA origins of replication. These results indicate that *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* contain chromosomally located DNA sequences related to the *fur* gene of *E. coli*. However, there may be a fair degree of divergence at the nucleotide level, since hybridization was not obtained under higher-stringency hybridization conditions in the presence of formamide.

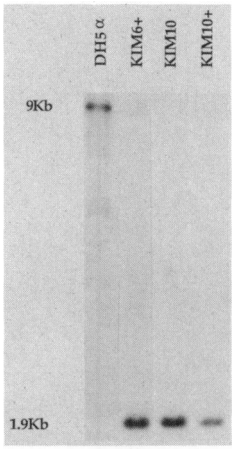

FIG. 1. Southern blot analysis of *Cla*I-digested genomic DNA from *E. coli* DH5α and representative *Y. pestis* KIM derivatives. The Pgm^+ phenotype is designated by +. KIM6+ carries pMT1 and pPCP1, while KIM10+ and KIM10 harbor only pMT1. The DNA probe was a *Hin*dIII-*Nco*I fragment of the *E. coli fur* gene from pMON2064.

Cloning and restriction analysis of the *Y. pestis fur* gene. KIM6+ genomic DNA was digested with the restriction enzyme *Cla*I and fractionated by sucrose density gradient centrifugation (2). A population of fragments from 1.3 to 2.3 kb were pooled and ligated into the *Acc*I site of pBR322 before introduction into *E. coli* DH5α by $CaCl_2$ transformation (2). Recombinant plasmids were isolated and screened for the presence of the *Y. pestis fur* gene by Southern blot hybridization (50) with the *Hin*dIII-*Nco*I *E. coli fur* gene probe from pMON2064. The recombinant plasmid pFURY2.1, containing a 2.1-kb insert of *Y. pestis* genomic DNA, was identified. Digestion with *Cla*I revealed the presence of two *Cla*I fragments, 1.9 and 0.2 kb. Only the 1.9-kb fragment hybridized to the *E. coli fur* probe and was subcloned into pUC19 to generate pFURY1.9. The restriction map of the 1.9-kb fragment is shown in Fig. 2. The *Y. pestis fur*-like structural gene was localized to a 1.1-kb fragment between the *Cla*I and *Sca*I sites as determined by Southern hybridization analysis (50) with the *E. coli fur* probe.

In addition, the 1.9-kb *Y. pestis* fragment was isolated, radiolabeled (44), and used to probe *Y. pestis* KIM6+ chromosomal DNA digested with a panel of restriction enzymes. An identical Southern blot was hybridized with the *E. coli fur* gene probe. Figure 3A shows the hybridization

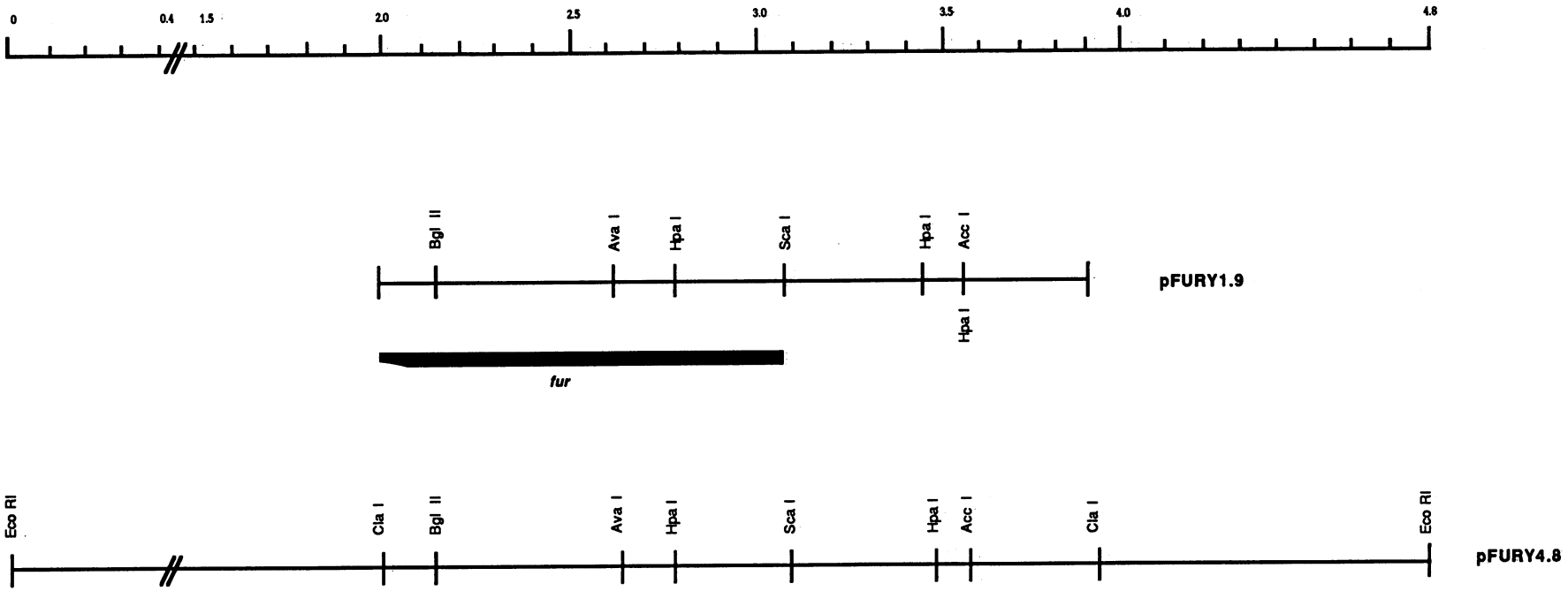

FIG. 2. Restriction endonuclease sites mapped within the 1.9-kb *Cla*I insert of pFURY1.9 and the 4.8-kb *Eco*RI insert of pFURY4.8. The 1.9-kb *Cla*I fragment was inserted into the *Acc*I site of pUC19, eliminating the *Cla*I sites. The figure illustrates the alignment of the 1.9-kb insert relative to the 4.8-kb fragment. The black bar represents the maximum region hybridizing to the *E. coli fur* probe.

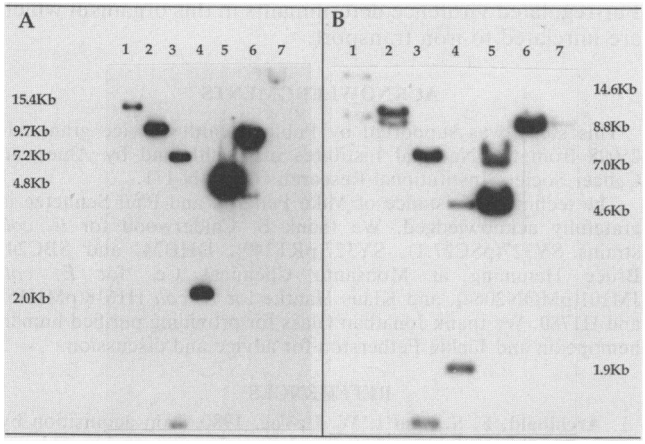

FIG. 3. Southern blot analysis of KIM6+ genomic DNA digested with various restriction endonucleases: lanes 1, *Pst*I; lanes 2, *Bgl*I; lanes 3, *Acc*I; lanes 4, *Cla*I; lanes 5, *Eco*RI; lanes 6, *Hin*dIII; lanes 7, *Bam*HI. (A) Probed with the 443-bp *Hin*dIII-*Nco*I fragment of the *E. coli fur* gene from pMON2064. (B) Probed with the 1.9-kb fragment of pFURY1.9.

pattern seen with the *E. coli fur* probe under low-stringency conditions. The Y-shaped hybridization pattern was duplicated when the *Y. pestis fur* gene probe was used under high-stringency conditions (Fig. 3B). There are some differences in the number of hybridizing bands between panels A and B of Fig. 3 which may be attributed to differences in the stringency of the hybridizations as well as to nonspecific hybridization of flanking *Y. pestis* sequences in the 1.9-kb probe (Fig. 2).

The *Y. pestis* and *E. coli* probes were also hybridized to Southern blots of *E. coli* SBC24 and DHB24 chromosomal DNA digested with various restriction enzymes. SBC24 is an *E. coli fur* mutant isogenic to DHB24 except for a transposon Tn*5* insertion into the *fur* gene (12). The transposon insertion results in altered migration of the *Pst*I-, *Bam*HI-, and *Eco*RI-hybridizing bands when either probe is used. Hybridizing fragments in Fig. 4 illustrate the results obtained with the *E. coli fur* probe. Similarly sized *Pst*I, *Bam*HI, and *Eco*RI fragments were also recognized by the 1.9-kb *Y. pestis fur* gene probe (data not shown). These results demonstrate that the 1.9-kb *Y. pestis fur* clone recognizes the same sequences in *Y. pestis* and *E. coli* chromosomal DNA as the *E. coli fur* probe.

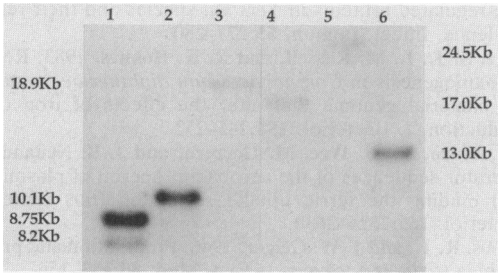

FIG. 4. Southern blot analysis of various digests of *E. coli* SBC24 (lanes 1 to 3) and DHB24 (lanes 4 to 6). Genomic DNA was digested with *Pst*I (lanes 1 and 4), *Bam*HI (lanes 2 and 5), and *Eco*RI (lanes 3 and 6). The DNA probe was the *Hin*dIII-*Nco*I fragment of the *E. coli fur* gene from pMON2064.

Complementation of an *E. coli* Fur$^-$ mutant. *E. coli* H1780 contains *fiu*::λ *plac*Mu53, an inframe insertion of lactose utilization genes into the Fur-regulated *fiu* gene (25). Since this strain is also Fur$^-$, expression of the single-copy β-galactosidase construct is constitutive and can be visualized on iron-supplemented MacConkey-lactose agar as red colonies (25). Complementation of the *fur* mutation was attempted by introducing pFURY1.9 into H1780; however, a Fur$^+$ phenotype was not restored, suggesting that the *Y. pestis* gene was not being expressed or was not functional. The 1.9-kb *Y. pestis fur* gene fragment was ligated into the IPTG-inducible expression vector pKK223-3 containing the *tac* promoter (9). The resulting plasmid, pOEF2, and pKK223-3 were introduced separately into *E. coli* H1780 and tested for complementation by plating on iron-supplemented MacConkey-lactose plates containing IPTG. While H1780 and H1780(pKK223-3) colonies were red, H1780(pOEF2) colonies appeared white on these plates, indicating that Fur-regulated expression of β-galactosidase was established by introduction of this clone. However, this construct proved unstable in this strain and did not allow quantitative β-galactosidase determinations.

Accordingly, a genomic library of *Y. pestis* DNA (43) was screened with the 1.9-kb *fur* fragment as a probe. A 6.8-kb clone, pFURY792, gave a strong hybridization signal and was used for further study. Restriction mapping revealed an internal 4.8-kb *Eco*RI fragment of pFURY792 containing DNA flanking each end of the 1.9-kb insert from pFURY1.9. This 4.8-kb fragment (Fig. 2) was inserted into pAT153, generating pFURY4.8, and was introduced into Fur$^-$ cells of H1780. H1780(pFURY4.8) colonies were white on iron-supplemented MacConkey-lactose plates, indicating that the Fur$^+$ phenotype had been restored and that the larger clone may contain the *Y. pestis fur* promoter which is functional in *E. coli*. β-galactosidase activity was determined for H1780(pFURY4.8) grown in PMH medium with or without 20 μM $FeCl_3$ as described above (Table 3). A 23.8-fold derepression of β-galactosidase activity was observed when H1780(pFURY4.8) was grown in the presence of 20 μM $FeCl_3$, while cells of H1780(pAT153) exhibited constitutively high β-galactosidase activity. These values represent the level of regulation of the single-copy β-galactosidase reporter gene by the *Y. pestis* Fur protein expressed in multicopy in *E. coli*. The level of β-galactosidase repression in *E. coli* H1780(pFURY4.8) grown in 20 μM $FeCl_3$ was much greater (Table 3) than that observed in *Y. pestis* KIM6+(pSC27.1) when the reporter construct was present in multicopy and the *fur* structural gene was present in single copy.

DISCUSSION

A number of siderophore-mediated iron accumulation systems comprising several coordinately regulated proteins are controlled by the Fur protein in response to iron availability. Aerobactin- (16, 38), enterobactin- (38, 45), ferrichrome- (8), coprogen- (8), and citrate-mediated (8) iron accumulation systems are among the most widely characterized. These siderophores effectively compete for iron with host iron-binding compounds, such as transferrin and lactoferrin, but cannot pull iron from heme compounds. *Y. pestis*, which is now well established as having siderophore-independent iron source utilization, cannot obtain iron from transferrin or lactoferrin (40, 49; this study). However, as determined previously (40, 49), we have confirmed that *Y. pestis* can utilize heme/hemopexin and hemoglobin as well as

hemin, ferritin, and ferric chloride as sole sources of iron. We have extended these nutritional sources of iron to include heme/albumin and hemoglobin/haptoglobin complexes. These findings indicate that cell-bound transport systems allowing utilization of host iron and heme compounds may play a significant role in the pathogenesis of *Y. pestis*.

The present study suggests that *Y. pestis* possesses a functional Fur protein. Under low-stringency conditions, we detected hybridization of the *E. coli fur* structural gene to *Y. pestis* DNA. The *Y. pestis fur* gene is chromosomally located and is not linked to the pigmentation phenotype. In addition, loci homologous to the *E. coli fur* structural gene are present in *Y. pestis* Kuma and plasmidless strains of *Y. pseudotuberculosis* PB1/0 and *Y. enterocolitica* WA-LOX. The Fur regulatory system was first identified in *S. typhimurium* and *E. coli* and was found to be involved in the regulation of siderophore-dependent iron transport systems (19, 23). It now appears that the Fur system is more widespread among bacteria than first proposed. A number of bacteria, including *Y. pestis* as well as *S. dysenteriae* (17), *V. cholerae* (51), and possibly *C. diphtheriae* (15), possess a Fur regulatory system. As in *E. coli* and *S. typhimurium*, the interaction of the *Y. pestis* Fur protein with the consensus Fur binding site appears to be regulated by the iron status of the cell in that iron-regulated β-galactosidase expression can be demonstrated by using the reporter plasmid pSC27.1 in *Y. pestis* KIM6+ cells. Finally, we have shown that the 4.8-kb *Y. pestis fur* clone encodes a Fur protein which complements the Fur^- phenotype in *E. coli* by repressing a Fur-regulated reporter gene 23.85-fold. These results indicate that *Y. pestis* expresses a functional Fur protein that interacts with the consensus Fur binding sequence.

The hybridization data in this study suggest significant divergence at the nucleotide level between the *fur* genes of *E. coli* and *Y. pestis*; whether a similar degree of divergence occurs at the amino acid level remains to be determined. While we have not demonstrated Fur regulation of specific *Y. pestis* operons, these results strongly suggest that *Y. pestis* possesses an iron-responsive Fur regulatory system. A likely candidate for Fur regulation is the inducible ferric iron transport system. Through ^{59}Fe uptake studies, Perry and Brubaker (40) have demonstrated increased ferric iron accumulation in *Y. pestis* Kuma cells grown in iron-deficient medium compared with that in cells grown in iron-sufficient medium. The inducibility of this system may be the result of gene expression in response to iron limitation which is encountered in vivo and may be particularly significant during intracellular growth. In addition, a number of iron-regulated proteins have been identified in *Y. pestis* (14, 49) which may be controlled by the Fur system. Finally, other systems with a possible link to Fur regulation in *Y. pestis* include heme compound transport systems and possibly other virulence determinants.

It will be of interest to determine whether utilization of host iron sources by *Y. pestis* is regulated by the Fur protein. In doing this we can determine whether multiple iron regulatory systems exist in this organism, as in *Vibrio* spp. (46, 51), in which multiple iron-regulatory systems have been identified. Multiple pathways for iron regulation are also possible in *C. diphtheriae*, in which a potential Fur binding sequence (17) as well as a unique repressor protein involved in Fur-independent iron regulation of toxin production (7) have been identified. The identification of Fur-regulated operons in *Y. pestis* may also lead to the characterization of Fur-regulated virulence determinants in this organism which are unrelated to iron transport.

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