INFECTION AND IMMUNITY, July 2003, p. 4159–4162 Vol. 71, No. 7
0019-9567/03/$08.00+0 DOI: 10.1128/IAI.71.7.4159–4162.2003

Yersinia pestis TonB: Role in Iron, Heme, and Hemoprotein Utilization

Robert D. Perry,* Jessica Shah, Scott W. Bearden,† Jan M. Thompson, and Jacqueline D. Fetherston

Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, Kentucky

Received 13 January 2003/Returned for modification 11 March 2003/Accepted 16 April 2003

In *Yersinia pestis*, the siderophore-dependent yersiniabactin (Ybt) iron transport system and heme transport system (Hmu) have putative TonB-dependent outer membrane receptors. Here we demonstrate that hemin uptake and iron utilization from Ybt are TonB dependent. However, the Yfe iron and manganese transport system does not require TonB.

Although outer membrane (OM) receptors serve to bind selected substrates in gram-negative bacteria, the substrate must still be translocated across the OM into the periplasm. For some OM receptors, TonB functions in concert with ExbB and ExbD to transmit the energy needed for this process (12, 17). To examine the role of TonB in the various iron and heme transport systems of *Yersinia pestis*, we have cloned and mutated the *tonB* gene. As expected, our results indicate that TonB is absolutely required for the utilization of the siderophore, yersiniabactin (Ybt), as well as hemin and all tested hemoproteins. TonB does not play a role in iron transport by either the Yfe system or a putative iron transport system which functions at 26°C. A second *tonB*-like gene was identified in the *Y. pestis* genome and designated *hasB* due to an association with the *Y. pestis has* locus (18). HasB does not appear to play a role in accumulation of iron, hemin, or hemoproteins.

Sequence analysis of *tonB*, *exbB*, and *exbD*. The *tonB* gene of *Y. pestis* KIM10+ (4) is predicted to encode a 252-residue, 27.5-kDa protein with a pI of 5.98. SignalP analysis (15a) suggested the presence of a signal sequence; as with *Escherichia coli*, the *Y. pestis* TonB may not be cleaved (17). A BLAST homology search indicates that the *Y. pestis* TonB protein is 80.8% identical and 85.6% similar to TonB from *Yersinia enterocolitica* (14). Y. *pestis* TonB possesses motifs common to other TonB proteins (13), including an N-terminal hydrophobic domain, a central region with EP repeats separated from a region with KP repeats, and a C-terminal domain with an amphiphilic β-strand. As with *E. coli*, the *Y. pestis tonB* gene appears to be monocistronic and is surrounded by similar sequences with the exception of an IS*285* element inserted downstream of *tonB* in *Y. pestis* KIM10+. *Y. pestis* TonB has four perfectly conserved amino acids (SHLS, residues 19, 23, 30, and 34) in the N-terminal hydrophobic domain that are predicted to form an α-helix (14). Visual inspection of the *tonB* promoter region revealed typical −10 and −35 regions and a strong putative ribosome binding site. Two potential Fur binding sites were identified; however, both of them are located well upstream of the putative promoter region.

Similarity searches identified a putative *exbBD* operon that is not closely linked to *tonB*. The *Y. pestis* KIM10+ *exbB* gene is predicted to encode a 251-residue, 26.8-kDa protein with a pI of 9.83. The *exbD* gene encodes a 143-residue, 15.6-kDa protein with a pI of 4.40. These proteins show a high level of similarity to ExbB (66.5% identity) and ExbD (67.8% identity) from *E. coli*. Contiguous genes encoding TolQ (228 residues, 25.4 kDa, pI of 6.17), TolR (142 residues, 15.5 kDa, pI of 5.55), and TolA (393 residues, 41 kDa, pI of 9.43), which are required by TolA-dependent OM receptors (15), were identified. No additional *exbB*- or *exbD*-like genes were detected.

Role of TonB in acquisition of inorganic iron by the Ybt-

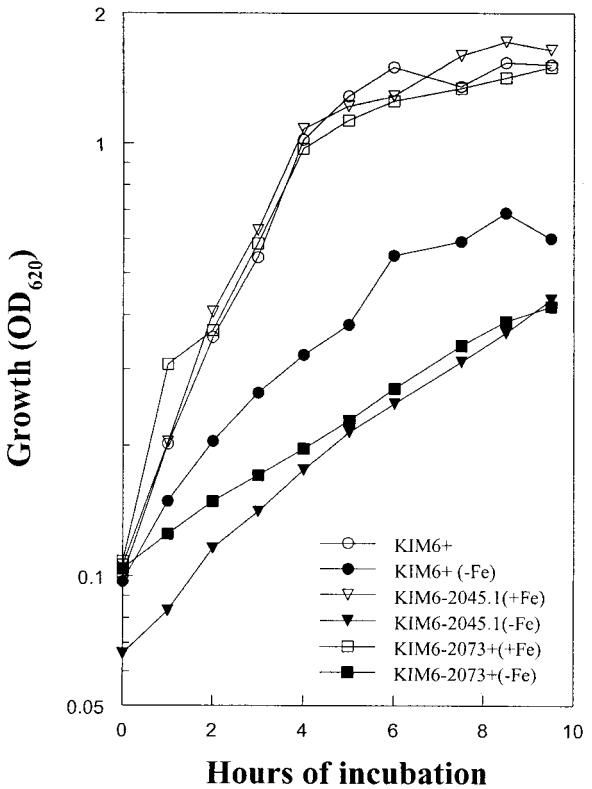

FIG. 1. Growth of *Y. pestis* strains KIM6+ (Ybt^+ $TonB^+$), KIM6-2045.1 (Psn^- $TonB^+$), and KIM2073+ (Ybt^+ $TonB^-$) at 37°C in deferrated PMH with (+Fe) and without (−Fe) $FeCl_3$ supplementation to 10 μM was performed as previously described (8, 19).

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Molecular Genetics, MS415 Medical Center, University of Kentucky, Lexington, KY 40536-0298. Phone: (859) 323-6341. Fax: (859) 257-8994. E-mail: rperry@pop.uky.edu.

† Present address: Centers for Disease Control, Division of Vector-Borne Infectious Diseases, Bacterial Zoonoses Branch, Fort Collins, Colo.

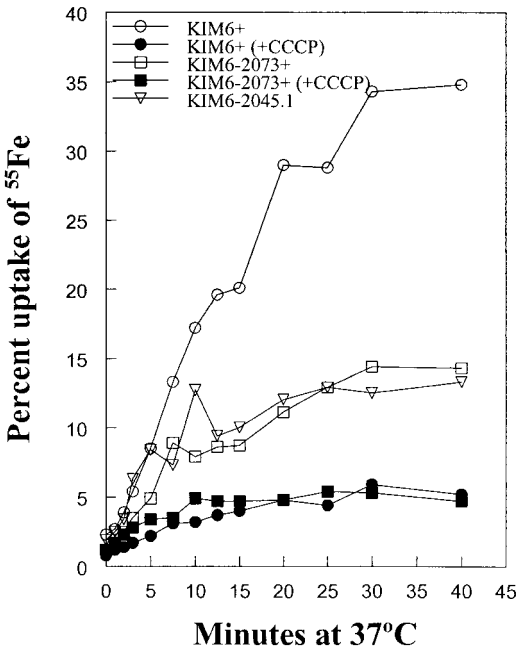

FIG. 2. Uptake of $^{55}FeCl_3$ by *Y. pestis* strains KIM6+ (Ybt^+ $TonB^+$), KIM6-2073+ (Ybt^+ $TonB^-$), and KIM6-2045.1 (Psn^- $TonB^+$) was monitored as described previously (1, 6). Where indicated (closed symbols), cells were metabolically poisoned by addition of 100 μM carbonyl cyanide *m*-chlorophenylhydrazone 10 min prior to addition of isotope. These data are from a single experiment but are representative of three independent assays.

dependent transport system. KIM6-2073+ possesses all of the iron and heme transport systems identified in *Y. pestis* but contains a kanamycin gene cassette inserted into *tonB*. Compared to the parent strain, KIM6+, KIM6-2073+ exhibited growth defects in a defined, iron-deficient medium (PMH) that are very similar to the defects seen with KIM6-2045.1 (Fig. 1), a *Y. pestis* strain carrying a deletion of the OM receptor (Psn) for Ybt (7). When surplus iron is present, the growth of all three strains is nearly identical (Fig. 1). Growth of KIM6-2073+ cells was inhibited on deferrated, solidified media (PMH-S). In addition, the $TonB^-$ mutant was unable to use Ybt produced by KIM6+. However, this mutant supports the growth of a Ybt-biosynthesis mutant (KIM6-2046.1) on PMH-S plates at 37°C. Similar growth-stimulation results were obtained using cell supernatants. These studies indicate that KIM6-2073+ still synthesizes and secretes Ybt, but it is unable to transport the Fe-Ybt complex back into the cell. KIM6-2073+ cells containing a plasmid encoding *Y. pestis* TonB (pYptonB) were able to grow on PMH-S plates, indicating that the observed growth defects were the result of the *tonB* mutation.

Compared to the KIM6+ parental strain, both the $TonB^-$ and Psn^- mutants are defective in iron uptake (Fig. 2). Thus, the $TonB^-$ mutant exhibits defects in both iron transport and growth under iron-deficient conditions, which very closely mimics the behavior of a Ybt receptor mutant, suggesting that the uptake of Fe-Ybt is indeed TonB dependent. This result was expected, since *Y. pestis* Psn has a TonB-dependent motif and TonB dependency of the homologous *Y. enterocolitica* Ybt system has been demonstrated (14).

Regulation of some iron transport systems involves signal transmission from the OM receptor (3, 9, 11). To determine whether the *tonB* mutation affected regulation of the Ybt system, we analyzed transcription from the *ybtP* promoter using a *ybtP*::*lacZ* fusion (6). The activity of this *lacZ* reporter was not significantly affected by the *tonB* mutation (data not shown). Thus, a surface signal-transduction system involving the OM receptor (5–7) and TonB (this study) does not seem to be responsible for controlling expression of the *Y. pestis* yersiniabactin system. Precisely how Ybt crosses the OM in a *tonB* mutant and interacts with the transcriptional regulator YbtA to induce the expression of *ybt* genes (5) is unknown.

Role of TonB in acquisition of inorganic iron by Ybt-independent transport systems. To assess the role of TonB in iron acquisition by the YfeABCD iron and manganese transport system (1, 2), the *tonB*::*kan* mutation was introduced into KIM6, a *Y. pestis* strain missing the Ybt system, and a derivative of KIM6 carrying a Δ*yfeAB* mutation (KIM6-2031.1). We compared the ability of these strains to grow at 30°C on solidified-PMH plates containing a 0 to 5 μM gradient of conalbumin. KIM6-2073 (a *tonB*::*kan* KIM6 strain) was as effective as KIM6 at acquiring iron from conalbumin (Fig. 3). Loss of the Yfe system caused a significant reduction in growth across the gradient (compare KIM6 to KIM6-2031.1 in Fig. 3). KIM6-

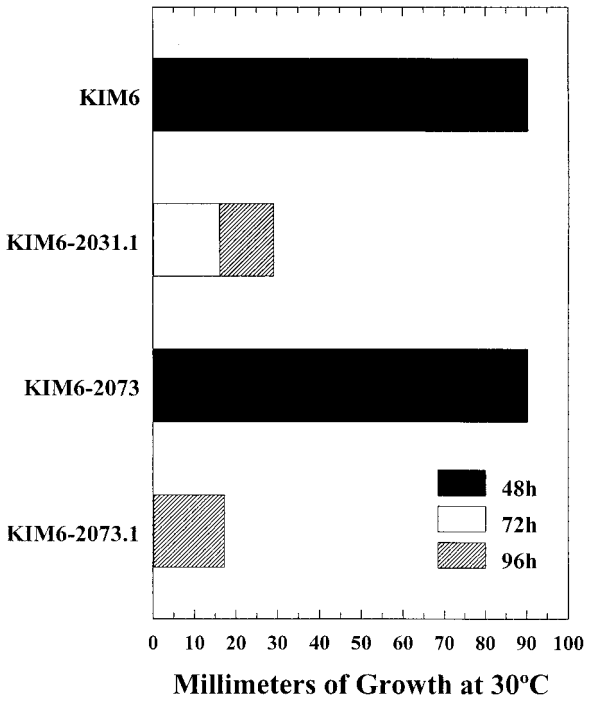

FIG. 3. Growth of *Y. pestis* KIM6 (Ybt^- Yfe^+ $TonB^+$ $HasB^+$), KIM6-2031.1 (Ybt^- Yfe^- $TonB^+$ $HasB^+$), KIM6-2073 (Ybt^- Yfe^+ $TonB^-$ $HasB^+$), and KIM6-2073.1 (Ybt^- Yfe^- $TonB^-$ $HasB^+$) at 30°C across PMH plates containing a gradient of the iron chelator conalbumin (0 to 5 μM) (1). Bars represent incremental growth against the concentration gradient over a 72-h period. Growth distances were recorded in millimeters from 0 (no growth) to 90 (confluent growth). The data shown are from a single growth assay but are representative of three independent experiments.

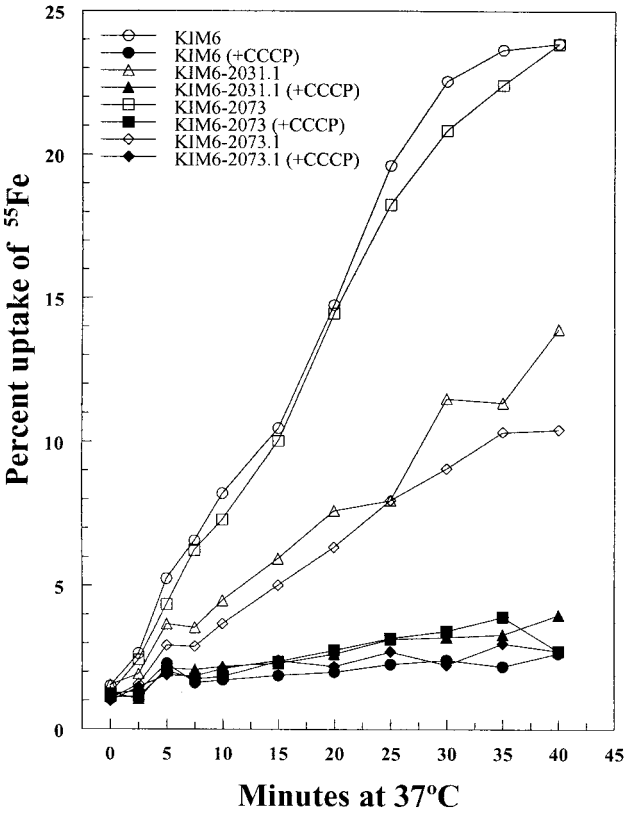

FIG. 4. Uptake of $^{55}FeCl_3$ by *Y. pestis* KIM6 (Ybt$^-$ Yfe$^+$ TonB$^+$ HasB$^+$), KIM6-2031.1 (Ybt$^-$ Yfe$^-$ TonB$^+$ HasB$^+$), KIM6-2073 (Ybt$^-$ Yfe$^+$ TonB$^-$ HasB$^+$), and KIM6-2073.1 (Ybt$^-$ Yfe$^-$ TonB$^-$ HasB$^+$) at 37°C. Where indicated (closed symbols), cells were metabolically poisoned by addition of 100 μM carbonyl cyanide *m*-chlorophenylhydrazone 10 min prior to addition of isotope. These data are from a single experiment but are representative of three independent assays.

2073 does not show a similar defect. Compared to KIM6-2031.1, the growth of KIM6-2073.1 (Yfe$^-$ TonB$^-$) was slightly delayed and inhibited. Iron transport assays (Fig. 4) also indicate that uptake of iron by the Yfe system does not depend upon TonB. Iron uptake by KIM6 and KIM6-2073 are nearly identical, while uptake by the Yfe$^-$ and Yfe$^-$ TonB$^-$ mutants is greatly reduced. Overall, these results suggest that the Yfe system is not TonB dependent.

A comparison of energy-dependent iron uptake by KIM6-2073 and KIM6-2073.1 indicates that the residual iron uptake activity is TonB independent. The mechanism of this residual iron uptake under these conditions is undetermined and may be due to one or more of the remaining seven potential inorganic iron uptake systems of *Y. pestis* (4, 16).

Role of TonB in heme transport. The effect of a *tonB* mutation on the ability of *Y. pestis* to utilize hemin and various hemoproteins as iron sources (10, 21) was tested on PMH plates containing 100 μM ethylenediamine-di(*o*-hydroxyphenyl) acetic acid (EDDA), a concentration that inhibits the growth of KIM6+. Similar to the case with strain KIM6-2060.1+ (in which *hmuP′RSTUV* genes have been deleted), KIM6-2073+ was unable to use hemin or any of the hemoproteins as iron sources (Table 1). KIM6-2073+ containing pYptonB was able to use hemin as an iron source. This confirms that the Hmu system is TonB dependent. TonB dependency of the highly homologous *Y. enterocolitica* Hem system has also been demonstrated (20).

TABLE 1. Analysis of hemin or hemoprotein utilization by various *Y. pestis* strains[a]

Heme source	Growth response of:		
	KIM6+ (Hmu$^+$ TonB$^+$)	KIM6-2060.1+ (Hmu$^-$ TonB$^+$)	KIM6-2073+ (Hmu$^+$ TonB$^-$)
500 μM hemin	3.1	NG	NG
50 μM hemin	1.9	NG	NG
50 μM hemin–200 μM albumin	1.6	NG	NG
200 μM albumin	NG	NT	NT
50 μM hemoglobin	3.7	NG	NG
10 μM hemoglobin	2.9	NG	NG
50 μM hemoglobin–100 μM haptoglobin	2.7	NG	NG
500 μM myoglobin	3.9	NG	NG
100 μM hemopexin	2.9	NG	NG
100 μM apohemopexin	NG	NG	NG

[a] Approximately 10^7 cells were overlaid onto deferrated PMH-100 μM EDDA agarose plates. Twenty microliters of the indicated compounds were added to 0.3-cm wells cut into the agarose plates, and growth around the wells (diameters in centimeters) was recorded after 3 days of incubation at 37°C. The results are averages from two independent experiments; NG, no growth in both independent experiments; NT, not tested.

Identification of a second *tonB*-like gene, *hasB*. Similarity searches identified a second *tonB*-like gene in *Y. pestis* KIM10+ that is immediately downstream of the *hasRADE* locus and is designated *hasB*. The predicted amino acid sequence of the *Y. pestis hasB* gene product (267 amino acids, 29.4 kDa) shows high similarities to TonB or TonB-like sequences in other bacteria and is 18.3% identical and 59.5% similar to *Y. pestis* TonB.

Our results with the TonB$^-$ mutant show that the *Y. pestis* HasB protein cannot substitute for TonB in the Ybt and Hmu transport systems. To determine whether HasB is required for TonB-independent inorganic iron uptake, we constructed a *Y. pestis* TonB$^-$ HasB$^-$ double mutant and tested its capacity for inorganic iron accumulation. The levels of energy-dependent iron accumulation by KIM6 (TonB$^+$ HasB$^+$), KIM6-2073 (TonB$^-$ HasB$^+$), KIM6-2080 (TonB$^+$ HasB$^-$), and KIM6-2073.2 (TonB$^-$ HasB$^-$) were nearly identical (data not shown), indicating that the observed iron uptake was not HasB dependent.

This work was supported by Public Health Service grants AI25098 and AI33481.

REFERENCES

1. **Bearden, S. W., and R. D. Perry.** 1999. The Yfe system of *Yersinia pestis* transports iron and manganese and is required for full virulence of plague. Mol. Microbiol. **32:**403–414.
2. **Bearden, S. W., T. M. Staggs, and R. D. Perry.** 1998. An ABC transporter system of *Yersinia pestis* allows utilization of chelated iron by *Escherichia coli* SAB11. J. Bacteriol. **180:**1135–1147.
3. **Braun, V.** 1997. Avoidance of iron toxicity through regulation of bacterial iron transport. Biol. Chem. **378:**779–786.
4. **Deng, W., V. Burland, G. Plunkett III, A. Boutin, G. F. Mayhew, P. Liss, N. T. Perna, D. J. Rose, B. Mau, S. Zhou, D. C. Schwartz, J. D. Fetherston, L. E. Lindler, R. R. Brubaker, G. V. Plano, S. C. Straley, K. A. McDonough, M. L. Nilles, J. S. Matson, F. R. Blattner, and R. D. Perry.** 2002. Genome sequence of *Yersinia pestis* KIM. J. Bacteriol. **184:**4601–4611.
5. **Fetherston, J. D., S. W. Bearden, and R. D. Perry.** 1996. YbtA, an AraC-type regulator of the *Yersinia pestis* pesticin/yersiniabactin receptor. Mol. Microbiol. **22:**315–325.

6. **Fetherston, J. D., V. J. Bertolino, and R. D. Perry.** 1999. YbtP and YbtQ: two ABC transporters required for iron uptake in *Yersinia pestis*. Mol. Microbiol. **32:**289–299.
7. **Fetherston, J. D., J. W. Lillard, Jr., and R. D. Perry.** 1995. Analysis of the pesticin receptor from *Yersinia pestis*: role in iron-deficient growth and possible regulation by its siderophore. J. Bacteriol. **177:**1824–1833.
8. **Gong, S., S. W. Bearden, V. A. Geoffroy, J. D. Fetherston, and R. D. Perry.** 2001. Characterization of the *Yersinia pestis* Yfu ABC iron transport system. Infect. Immun. **67:**2829–2837.
9. **Heinrichs, D. E., and K. Poole.** 1996. PchR, a regulator of ferripyochelin receptor gene (*fptA*) expression in *Pseudomonas aeruginosa*, functions both as an activator and as a repressor. J. Bacteriol. **178:**2586–2592.
10. **Hornung, J. M., H. A. Jones, and R. D. Perry.** 1996. The *hmu* locus of *Yersinia pestis* is essential for utilization of free haemin and haem-protein complexes as iron sources. Mol. Microbiol. **20:**725–739.
11. **Kim, I., A. Stiefel, S. Plantör, A. Angerer, and V. Braun.** 1997. Transcription induction of the ferric citrate transport genes via the N-terminus of the FecA outer membrane protein, the Ton system and the electrochemical potential of the cytoplasmic membrane. Mol. Microbiol. **23:**333–344.
12. **Klebba, P. E., and S. M. C. Newton.** 1998. Mechanisms of solute transport through outer membrane porins: burning down the house. Curr. Opin. Microbiol. **1:**238–247.
13. **Klebba, P. E., J. M. Rutz, and C. K. Murphy.** 1993. Mechanisms of TonB-catalyzed iron transport through the enteric bacterial cell envelope. J. Bioenerg. Biomembr. **25:**603–611.
14. **Koebnik, R., A. J. Bäumler, J. Heesemann, V. Braun, and K. Hantke.** 1993. The TonB protein of *Yersinia enterocolitica* and its interactions with TonB-box proteins. Mol. Gen. Genet. **237:**152–160.
15. **Lloubès, R., E. Cascales, A. Walburger, E. Bouveret, C. Lazdunski, A. Bernadac, and L. Journet.** 2001. The Tol-Pal proteins of the *Escherichia coli* cell envelope: an energized system required for outer membrane integrity? Res. Microbiol. **152:**523–529.
15a. **Nielsen, H., J. Engelbrcht, S. Brunak, and G. von Heijne.** 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Eng. **10:**1–6.
16. **Perry, R. D., S. W. Bearden, and J. D. Fetherston.** 2001. Iron and heme acquisition and storage systems of *Yersinia pestis*. Recent Res. Dev. Microbiol. **5:**13–27.
17. **Postle, K.** 1993. TonB protein and energy transduction between membranes. J. Bioenerg. Biomembr. **25:**591–601.
18. **Rossi, M.-S., J. D. Fetherston, S. Létoffé, E. Carniel, R. D. Perry, and J.-M. Ghigo.** 2001. Identification and characterization of the hemophore-dependent heme acquisition system of *Yersinia pestis*. Infect. Immun. **69:**6707–6717.
19. **Staggs, T. M., and R. D. Perry.** 1991. Identification and cloning of a *fur* regulatory gene in *Yersinia pestis*. J. Bacteriol. **173:**417–425.
20. **Stojiljkovic, I., and K. Hantke.** 1994. Transport of haemin across the cytoplasmic membrane through a haemin-specific periplasmic binding-protein-dependent transport system in *Yersinia enterocolitica*. Mol. Microbiol. **13:**719–732.
21. **Thompson, J. M., H. A. Jones, and R. D. Perry.** 1999. Molecular characterization of the hemin uptake locus (*hmu*) from *Yersinia pestis* and analysis of *hmu* mutants for hemin and hemoprotein utilization. Infect. Immun. **67:**3879–3892.

Editor: J. T. Barbieri