The gram-negative bacterium *Yersinia pestis*, which undergoes an obligate flea-rodent-flea life cycle, causes bubonic plague, a rapid and highly fatal zoonotic disease that was responsible for at least three pandemics (in the 5–6th centuries, the 8–14th centuries, and the 19–21st centuries). During the mid-1940s, researchers began studying a series of *Y. pestis* virulence determinants to learn how this pathogen causes disease in its mammalian hosts. More recently over the past decade, researchers have renewed an interest in this pathogen’s interactions with its flea vector, seeking factors that enable the bacteria to persist in these insects.

This revived interest in plague-flea interactions helped lead researchers to identify two bacterial factors, called hemin storage phenotype (Hms) and *Yersinia* murine toxin (Ymt), that are important in maintaining the vector cycle of *Y. pestis*. Although much remains uncertain about these factors, ongoing efforts to identify and characterize flea survival and transmission factors eventually will illuminate flea-plague interactions that are proving nearly as complex as plague-mammalian host interactions.

**Fleas Play Key Role in Infectious Life Cycle of *Y. pestis***

Fleas become infected with *Y. pestis* after taking blood meals from septicemic animals. Even after ingesting a large number of bacteria, however, some fleas clear their infections. More commonly, the *Y. pestis* cells grow to high density in the normally sterile midgut (stomach) of fleas, without invading other flea tissues or individual cells.

After three days, the bacterial cells aggregate into brownish clumps that typically float freely in the midgut or attach to the proventriculus, a valve-like chamber between the midgut and esophagus. The interior of the proventriculus is lined with spine-like structures covered with cuticle, which is also found in insect exoskeletons. The proventriculus mechanically disrupts cells, allows entry of blood into the midgut, and prevents ingested blood from escaping the flea.

The bacterial aggregates, composed of microcolonies surrounded by a dense peripheral material, increase in size and density during the first week of infection, eventually blocking the proventriculus. Once this structure is blocked (Fig. 1), the fleas essentially become starved for blood, which no longer can reach the midgut, and thus the insects attempt to feed more often. During these futile feeding attempts, mammalian blood is pumped into the esophagus, where it dislodges bacteria growing there and in the proventriculus. This infected blood flows back into the wound from the flea bite.

Fleas with a blocked proventriculus eventually die, presumably of starvation and dehydration. Experiments indicate that only blocked fleas effectively transmit plague to mammals. They also indicate that fleas do not become blocked at higher temperatures. For instance, if held at 30°C, fleas survive *Y. pestis* infections in an unblocked state, perhaps explaining why human bubonic plague epidemics often end after the onset of warmer temperatures. In mammals, infections with *Y. pestis* need to cause a sustained high-level bacteremia or septicemia to transmit the organism back into the flea vector.
Several Bacterial Factors Needed To Maintain this Microbe in Fleas

Researchers have identified two bacterial factors that play important roles in Y. pestis-infected fleas. The first is called the hemin storage (Hms) phenotype, while the other, called Yersinia murine toxin (Ymt), is a cytoplasmic protein with phospholipase D activity. The Yops and LcrV effector proteins of Y. pestis, which down-regulate immune responses in mammals, do not appear important for maintenance or survival of these bacteria in fleas, according to B. Joseph Hinnebusch of the National Institute of Allergy and Infectious Diseases (NNIAID) Rocky Mountain Laboratories in Hamilton, Mont., who conducted studies with the oriental rat flea (Xenopsylla cheopis). In those experiments, some fleas were fed infected blood, then all of them were maintained on uninfected blood while they were monitored for blockage over a four-week period.

The Hms phenotype, named for adsorption of hemin and Congo red (CR), is required for Y. pestis cells to colonize and block the proventriculus (Fig. 1). However, it is not involved in survival and growth of these bacteria in the flea midgut, and it does not contribute to virulence of these bacteria when they infect mammals. Although Hms+ cells are highly aggregative and hydrophobic under some conditions, they and Hms- mutant cells adhere equally to the cuticle lining of the proventriculus. Furthermore, Hms+ and Hms- cells are equally sensitive to the antibacterial peptide Cecropin A as well as to phenoloxidase-generated cytotoxic intermediates that are generated by insect cuticle cells. How the Hms phenotype promotes colonization and blockage of the proventriculus is unknown.

Moreover, although it is generally accepted that blocking the flea is important for transmitting plague epidemics in mammals, researchers in Russia report that fleas can transmit some Hms- mutant strains, a finding that appears contradictory and thus needs to be resolved.

Hms Shows Intriguing Activities In Vitro

In vitro, Hms+ cells adsorb large amounts of exogenous hemin and CR that is associated with outer membrane (OM) fractions. These cells form greenish-brown or red colonies at 26°C but not at 37°C on solidified media containing these dyes (Fig. 2). With rising temperatures, cells gradually lose their CR binding capacity, forming white colonies at temperatures above 34°C. The CR-binding trait correlates with iron-independent virulence in mice and was originally termed the pigmentation (Pgm+) phenotype.

Spontaneous nonpigmented (Pgm-) mutants lose this CR-binding phenotype along with their ability to cause disease in mice from peripheral routes of infection. These mutants arise from large chromosomal deletions (102 kb in strain KIM), and they appear to be mediated by recombination between two flanking IS100 elements. The 102-kb pgm locus in Y. pestis KIM contains some of the genes necessary for the Hms phenotype as well as a high-pathogenicity island encoding the yersiniabactin (Ybt) siderophore-dependent iron transport system. When the bacteria lose the Ybt system, they are no longer virulent in mice following subcutaneous injection. Thus, in nature, the pgm deletion is lethal since bacteria carrying this mutation cannot block the flea proventriculus and cannot cause bubonic plague in mammals.

Five proteins are essential for producing the Hms+ phenotype. These proteins are encoded by two operons, designated hmsHFRS, which is located in the pgm locus, and hmsT, which is at a considerable distance from hmsHFRS in the Y. pestis chromosome (Fig. 3). HmsH and HmsF are OM proteins, while HmsS, HmsT, and probably also HmsR are located in the inner mem-
brane (IM). These proteins show some similarity to glycosyltransferases, polysaccharide synthesis enzymes, and the GGDEF family of proteins that contain putative adenylyl and guanylyl cyclase and HAMP signaling domains. These activities are implicated in biofilm formation in other bacteria. Formation of an extracellular matrix by *Y. pestis* in fleas was noted in the early 1900s.

It is intriguing that the *ycdS-T* genes of *Escherichia coli* K-12 encode proteins with similarities (ranging from 34%-83%) to all five Hms proteins. However, neither *E. coli* K-12 nor an Hms strain of *Y. pestis* carrying the *ycdS-T* locus can bind CR.

**Response of *Y. pestis* hms Genes to Temperature Shifts**

My laboratory is examining how changes in temperature regulate CR adsorption to *Y. pestis* cells. Using *lacZ* fusions to the *hmsHFRS* and *hmsT* promoters, we determined that neither of these promoters is transcriptionally regulated by growth temperature or population density. Because the *hmsF* gene contains a stem loop structure (Fig. 3) with some but not all of the characteristics of an antiterminator, we constructed a mutant in which the potential base pairing in the stem structure would be greatly reduced without altering the amino acid sequence of HmsF or introducing rare codons. This mutant retains the temperature-dependent CR-binding phenotype. Thus, temperature regulation of the Hms*+* phenotype does not seem to involve transcriptional initiation or termination.

An alternative way in which CR-binding can respond to changes in temperature would be for cells to produce more of a surface component that occludes the Hms complex at higher temperatures. One likely candidate in *Y. pestis* cells is the Fraction 1 glycoprotein capsule, called F1 or Caf1. F1 is highly expressed at 37°C but not at ambient temperatures. However, F1*−* mutant cells display a normal Hms phenotype—red colonies on CR plates incubated at 30°C and white colonies at 37°C.

When we use Western blots to look for possible posttranscriptional regulation, we find that the levels of HmsH, HmsR, and HmsT proteins are greatly reduced after growth at 37°C compared to 26°C, whereas HmsF and HmsS are only moderately affected. This finding suggests an alternating pattern of temperature effects on protein expression or stability within the *hmsHFRS* operon (Fig. 3), making mRNA stability or translational controls unlikely unless they differentially affect individual genes.

One other potential regulatory mechanism is selective proteolytic degradation at 37°C. We looked at Pla protease that is catalytically active at 37°C but not at 26°C. At 37°C, Pla activates plasminogen and cleaves several other host and *Y. pestis* proteins. Despite this temperature-dependent activity, however, mutational analysis indicates that this protein is not responsible for the lack of CR binding at 37°C. We are seeking factors that are involved in temperature-dependent Hms phenotype by using a random transposon-insertion mutagenesis screen to isolate mutants that are CR*+* at 37°C. This analysis has identified several new genes whose products (including a second GGDEF protein) are associated with biofilm formation or regulation in other bacteria. Our current hypothesis is that expres-
sion of the Hms phenotype is regulated in a manner similar to cellulose extracellular matrix in *Acetobacter* and *Agrobacterium*. In these organisms, GGDEF proteins and phosphodiesterases control the production and degradation of cyclic di-GMP. Further analysis of the temperature-constitutive Hms mutants should clarify the relevant mechanisms regulating the expression of this phenotype.

**Exploring How the ymt Gene Affects Y. pestis Maintenance in Fleas**

Four laboratories have collaborated in studying Ymt, one of the factors important for maintaining Y. *pestis* in fleas. Ymt was designated murine toxin because this protein is highly lethal for mice. Ymt is a cytoplasmic protein with phospholipase D activity, and altering histidine 188 in the HKD motif that is conserved in phospholipase D enzymes abrogates this activity.

While a Ymt⁻ mutant is as virulent as its parent in mice with plague, the phospholipase D activity of Ymt is required for survival of *Y. pestis* in the flea, and *Y. pestis* cells containing a mutation in the His-188 codon of *ymt* are rapidly eliminated from the flea midgut. The mutant cells appear to be converted to spheroplasts before they are eliminated. Ymt⁻ cells that survive appear only in the proventriculus, not the midgut.

When introduced into the midgut of fleas, cells of *E. coli* and *Yersinia pseudotuberculosis* are rapidly eliminated. However, when either of these two microorganisms is transformed with a recombinant *ymt* gene, survival in fleas dramatically increases. This finding suggests that Ymt is the only bacterial factor unique to *Y. pestis* that is absolutely required for survival in fleas. The antibacterial activity that kills Ymt⁻ cells and the mechanism by which Ymt protects bacterial cells will likely be unusual. Ymt is a cytoplasmic protein that presumably is not released unless cells lyse. When fleas are infected with an equal mixture of Ymt⁺ and Ymt⁻ *Y. pestis* cells, only Ymt⁺ cells survive and grow. Moreover, the bacteria do not release a Ymt-Gfp fusion protein. Finally, when purified Ymt is added by means of a blood meal to Ymt⁻ *Y. pestis* cells in the flea midgut, those mutant bacteria still cannot survive, suggesting that Ymt acts from an intracellular location to confer resistance to the antibacterial activity present in the flea midgut.

Producing this antibacterial factor requires plasma. Fleas fed filtered plasma instead of blood also eliminate Ymt⁻ bacteria, whereas fleas fed an artificial plasma supplemented with red blood cells do not kill the mutant. Ymt⁻ *Y. pestis* cells grow in plasma in vitro and in blood in vivo. Thus, the cytotoxic component is likely to be a degradation product of a plasma component produced in fleas by their digestive process. An alternative is that expression of an antibacterial factor, encoded by fleas, is induced by a plasma component.

Future studies will help to determine whether fleas modify one or more plasma components to target a bacterial structure that leads to spheroplast formation and lysis. In turn, the plague bacillus apparently counters by producing a phospholipase that either inactivates this cytotoxic agent or modifies its bacterial target.

**Evaluating Roles of other Gene Products in Maintaining Y. pestis in Fleas**

The role that Pla plays is far from certain. Researchers long have hypothesized that because it causes rabbit serum to coagulate at ambient temperatures, Pla may block the proventriculus.
in fleas and that this temperature-dependent activity could explain why plague epidemics subside with warmer weather.

Pla may play a role in killing infected fleas. For instance, one study, which did not monitor blockage, found that fleas fed a blood meal containing Pla+ Y. pestis cells have a higher mortality rate than those fed a meal with a Pla Y. pestis strain. In a separate study, however, the Pla status of a Y. pestis strain that was part of the blood meal did not affect the ability of Y. pestis to grow, cause blockage, or kill fleas. These two studies used different bacterial strains, mammalian blood sources, maintenance feeding procedures, and flea species, which may account for these conflicting results, leaving open the possibility that Pla plays some role in maintaining Y. pestis in some flea species but not in others.

Finally, DNA sequences common to the toxin complex (Tc) genes, which are present in insect pathogens such as Photobacterium luminescens, are also found in both Y. pestis KIM10+ (biotype medeavallis) and CO92 (biotype orientalis). P. luminescens produces at least four different, large toxin complexes (1 million Daltons) which contain numerous polypeptide components. These Tc complexes seem to require TcaAB-like, TcaC-like, and TccC-like proteins for full activity, with the TccC-like proteins possibly serving as the active toxic moieties.

The primary tc locus of Y. pestis KIM10+ encodes tcaABC-like genes and two tccC-like genes, while several other tccC-like genes are present elsewhere in the genome. Recognizing that the tcaABC genes might be involved in delivering active toxin, we constructed a deletion mutation encompassing tcaA and most of tcaB. In collaboration with Hinnebusch at the NIAID Rocky Mountain Laboratories, we tested the ΔtcaAB KIM strain in fleas and determined that this mutant strain is unaffected in its ability to survive and grow in fleas, to cause proventricular blockage, and to kill fleas. These results, in combination with the fact that one of the tc genes appears to have suffered a frameshift mutation in CO92 (but not in KIM10+), suggest that the tc genes are not important in the oriental rat flea.

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SUGGESTED READING


