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18. We believe that the inconsistency between the O_2-derived estimates of I_C and those presented here (and elsewhere) is due to fundamental differences between the processes regulating annual O_2 cycles and those responsible for initiating spring phytoplankton blooms. R. G. Najjar and R. F. Keeling [*J. Mar. Res.* **55**, 117 (1997)] related the depth where the vertical O_2 profile switches sign from net production to net consumption as the compensation depth, by using a harmonic analysis over the annual cycle. This "nodal depth" is influenced by a variety of processes not directly relevant to the initiation of a spring bloom including diapycnal mixing, degassing, and particle sinking and remineralization. The harmonic analysis uses O_2 observations from throughout the annual cycle, not all of which are relevant for spring blooms. Last, typical values of the photosynthetic quota (carbon assimilated by phytoplankton per O_2 evolved) will be smaller for phytoplankton grown on ammonia (recycling communities) than those grown on nitrate (bloom communities). This should result in a 20% or so underestimate in the I_C value estimated using the seasonal O_2 cycle. In short, we believe for a number of reasons that analyses of the seasonal O_2 profile will not provide I_C estimates appropriate for diagnosing the initiation of a spring bloom. Clearly, resolution of this issue is beyond the scope of the present contribution, and it is likely that this issue is best resolved via a detailed modeling evaluation.
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26. The depth penetration of PAR is calculated using $Z_{1\%PAR} = 38.0\ Chl_{BL}^{-0.428}$, where $Z_{1\%PAR}$ is the 1% PAR isolume depth and Chl_{BL} is the surface chlorophyll concentration (mg m^{-3}) as the spring bloom starts. Note that $Z_{1\%PAR} = 4.6/K$ where K is the diffuse attenuation coefficient [A. Morel, *J. Geophys. Res.* **93**, 10749 (1988)].
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Role of Yersinia Murine Toxin in Survival of *Yersinia pestis* in the Midgut of the Flea Vector

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Transmission by flea bite is a relatively recent adaptation that distinguishes *Yersinia pestis*, the plague bacillus, from closely related enteric bacteria. Here, a plasmid-encoded phospholipase D (PLD), previously characterized as Yersinia murine toxin (Ymt), was shown to be required for survival of *Y. pestis* in the midgut of its principal vector, the rat flea *Xenopsylla cheopis*. Intracellular PLD activity appeared to protect *Y. pestis* from a cytotoxic digestion product of blood plasma in the flea gut. By enabling colonization of the flea midgut, acquisition of this PLD may have precipitated the transition of *Y. pestis* to obligate arthropod-borne transmission.

Y. pestis, the highly virulent flea-borne agent of bubonic plague, is a recently emerged clone of *Yersinia pseudotuberculosis*, which causes a relatively mild food- and water-borne enteric disease (*1*). A major genetic difference between them is the presence of two *Y. pestis*–specific plasmids, one of which contains the gene for Ymt (*2–5*), a phospholipase D (PLD) (*6*). Murine toxin was characterized as a protein fraction of *Y. pestis* in the 1950s (*7*) and later shown to have β-adrenergic blocking ability (*8*). Toxicity is manifested by hypotension and vascular collapse when Ymt is released from lysing bacteria at the terminal stage of septicemic plague (*9*, *10*). *Y. pestis* Ymt has since been shown to belong to a family of PLD enzymes, characterized by conserved HKD ($HXKX_4DX_6GG/S$; X, any amino acid) catalytic motifs, found in plants, animals, fungi, bacteria, and eukaryotic viruses (*6*, *11–13*). Because Ymt is toxic to mice and rats but not to other animals (*9*, *10*), it has been presumed that the high lethality of plague for mice is partly attributable to Ymt. However, the lethal dose of *Y. pestis* in mouse infection models is not changed significantly by deletion of *ymt* (*14–16*). This, and the fact that *ymt* expression is greater at 26°C than at 37°C (*15*), prompted us to investigate a role for Ymt in the insect vector. We infected *X. cheopis* fleas with Ymt^+ or isogenic Ymt^- *Y. pestis* and monitored them for 4 weeks after the infectious blood meal (*17*). To produce a transmissible infection, wild-type *Y. pestis* multiplies in the flea midgut to form cohesive aggregates. In some fleas, bacteria eventually fill the proventriculus (a valve that connects the esophagus to the midgut) and block normal blood feeding. Blocked fleas transmit plague efficiently because during their persistent efforts to feed, plague bacilli are dislodged from the proventriculus into the bite site (*18*). *Y. pestis ymt*H188N, a strain that synthesizes a mutant form of Ymt in which a single amino acid change in one of the two HKD catalytic motifs reduces PLD activity by >99% (*6*), did not block any of 319 fleas examined (Table 1). In contrast, the isogenic parent *Y. pestis* KIM6+ blocked 24 to 38% of fleas and caused high mortality due to blockage-induced starvation. Complementation of the *ymt*H188N mutant by transformation with pCH16, a plasmid that contained a wild-type copy of *ymt*, restored the normal blockage rate. Similarly, a *ymt* deletion mutant of *Y. pestis* that blocked only 3 of 310 fleas regained normal blockage capability when complemented with *ymt*.

Because blockage depends on prior colonization of the digestive tract, we assessed the infection rate and bacterial load of fleas at various times after a single infectious blood meal (*17*) (Fig. 1). *Y. pestis* KIM6+ established chronic infection in 50 to 80% of fleas, the normal infection rate (*19*, *20*), but the *ymt*H188N mutant was eliminated from 80 to 95% of fleas within the first 24 hours, and the average number of viable bacteria per positive flea decreased from 4.7×10^4 to 250 during the first 24 hours. Similar results were obtained from fleas infected with the *Y. pestis ymt* dele-

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Table 1. Flea blockage and mortality during a 4-week period after infection with Ymt$^+$ or Ymt$^-$ *Yersinia pestis*. The results of three experiments with each bacterial strain are shown. The *ymt*H188N allele (*6*) was introduced into *Y. pestis* KIM6+ by allelic exchange (*27*) and confirmed by DNA sequence analysis. *Y. pestis* KIM5Δ*ymt* has a 1320–base pair in-frame deletion of *ymt* (*14*). *Y. pestis* KIM6+*ymt*H188N was complemented by transformation with pCH16, which contains a wild-type copy of *ymt* (*14*). *Y. pestis* KIM5Δ*ymt* was complemented by reinserting a full-length copy of *ymt* downstream of the deleted allele. *Y. pestis* KIM5 has a deletion of the chromosomal pigmentation locus that contains the *hms* genes previously shown to be required by *Y. pestis* to block fleas (*19*). Therefore, KIM5 strains were transformed with pHMS1, which contains *hms*HFRS (*28*). All of the *Y. pestis* strains used lack the 70-kb virulence plasmid, which is not required to infect or block fleas (*19*).

Fleas infected with	Fleas blocked (%)	Excess mortality* (%)	Fleas (*n*)
Y. pestis KIM6+	24	39	100
	38	46	100
	34	30	103
Y. pestis KIM6+ *ymt*H188N	0	0	109
	0	3	103
	0	0	107
Y. pestis KIM6+ *ymt*H188N(pCH16)	32	44	109
	23	42	107
	29	38	109
Y. pestis KIM5 (pHMS1)	27	50	106
	32	60	109
	36	44	108
Y. pestis KIM5Δ*ymt* (pHMS1)	3	0	109
	0	7	89
	0	0	112
Y. pestis KIM5Δ*ymt*::*ymt* (pHMS1)	22	55	106
	29	37	109
	30	46	108

*Percent mortality of infected fleas minus percent mortality of uninfected control fleas.

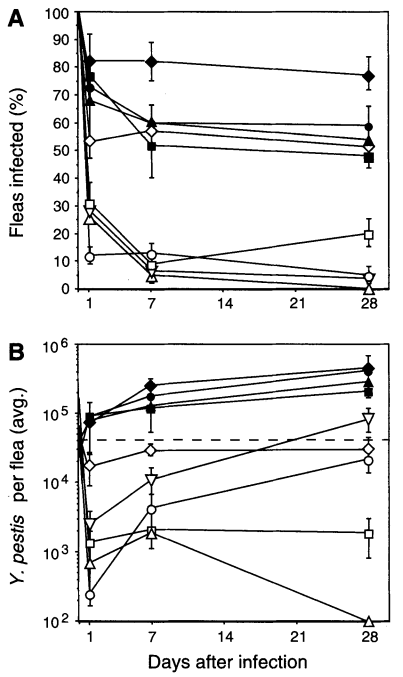

Fig. 1. Infection of *X. cheopis* fleas with Ymt$^{+/-}$ *Y. pestis*, *Y. pseudotuberculosis*, and *E. coli*. (**A**) Percentage of fleas infected, and (**B**) average number of bacteria per infected flea at different times after a single infectious blood meal containing *Y. pestis* strains KIM6+ (●), KIM6+*ymt*H188N (○), KIM6+*ymt*H188N (pCH16) (▲), KIM5Δ*ymt* (△), KIM5Δ*ymt*(pHMS1) (▽); *Y. pseudotuberculosis* strains O1:b (◇), O1:b(pCH16) (◆); or *E. coli* strains F470 (□), or F470(pCH16) (■). The results (mean ± SEM) of three separate experiments with each strain are shown. The dashed line indicates the average number of bacteria taken up by the fleas in the infectious blood meal on day 0 of infection. pCH16 contains a wild-type copy of *ymt* (*14*), and pHMS1 contains the *Y. pestis hms* locus required for proventricular blockage (*19*). *E. coli* F470 has a defined mutation in the lipidA core:surface polymer ligase gene (*waaL::aacC1*) so that its outer-membrane lipopolysaccharide (LPS), like that of *Y. pestis*, lacks O-polysaccharide (*29*). The wild-type *Y. pseudotuberculosis* PB1 strain used expresses LPS O-polysaccharide of serotype O1:b.

tion mutant. Thus, the greatly reduced ability of Ymt$^-$ *Y. pestis* strains to initially colonize the flea digestive tract accounted for the subsequent low incidence of proventricular blockage.

We also tested the effect of Ymt on the ability of *Escherichia coli* and *Y. pseudotuberculosis* to colonize the flea gut (Fig. 1). One to 4 weeks after an infectious blood meal containing *E. coli*, only 9 to 20% of fleas remained infected, containing an average of 1.9×10^3 bacteria. Transformation of the *E. coli* with *ymt*, however, resulted in an increase in the chronic infection rate to 50% ($P < 0.01$), and the average bacterial load increased over 100-fold to 2.1×10^5. These infection levels match those of Ymt$^+$ *Y. pestis*, suggesting that Ymt may have a similar substrate and mechanism of action in both *Y. pestis* and *E. coli*. *Y. pseudotuberculosis*, which does not contain *ymt*, persisted in 54% of fleas for 4 weeks after an infectious blood meal. However, the number of bacteria per flea did not increase from the initial infectious dose during this period. Transformation of *Y. pseudotuberculosis* with *ymt* increased the infection rate to 80% ($P < 0.01$), and the average number of bacteria per infected flea increased by a factor of 16.

Examination of flea midgut contents by indirect fluorescent antibody (IFA) (*21*) demonstrated that the *Y. pestis ymt*H188N mutant assumed a spherical shape within 24 hours after ingestion (Fig. 2). This aberrant spheroplast form presumably preceded lysis or elimination, because the mutant was not detected by IFA after 1 week in the midgut of any of 20 fleas dissected. In contrast, Ymt$^+$ *Y. pestis* maintained its normal bacillary shape and was detected in 80% of fleas during the first week. Thus, PLD activity of Ymt was necessary for *Y. pestis* to survive in the flea midgut; however, up to 10% of fleas were infected (Fig. 1), and 0.4% became blocked (Table 1), with Ymt$^-$ *Y. pestis*. Microscopic examination of digestive tracts dissected from the few fleas that were colonized by Ymt$^-$ *Y. pestis* indicated that the proventriculus, but not the midgut, was infected. In *X. cheopis*, the proventriculus is part of the foregut and is physically separated from the midgut except when the flea feeds. The proventriculus is usually colonized secondarily by spread from the midgut, but it can sometimes be the primary site of infection (*22*). The *Y. pestis* hemin storage (*hms*) locus, which encodes outer-surface proteins responsible for bacterial pigmentation and autoaggregation phenotypes, is required for infection of the proventriculus, but not the midgut, of *X. cheopis* (*19*). If, conversely, Ymt is required for infection of the midgut but not the proventriculus, then a *Y. pestis hms*$^-$*ymt*$^-$ double mutant should be unable to establish a chronic infection at either site and be eliminated from every flea. This was the observed result (Fig. 1).

Ymt is not a conventional secreted exotoxin, but a cytoplasmic protein that is released upon bacterial lysis (*10*, *14*). *Y. pestis* containing an in-frame translational fusion between full-length *ymt* and the gene for green fluorescent protein (*gfp*) was used to visualize Ymt localization in infected fleas. The Ymt-GFP chimeric protein retained normal PLD activity in vitro and toxicity to mice; this *Y. pestis* strain also infected and blocked fleas at normal efficiency (*23*). Ymt was present in the cytoplasm of *Y. pestis* in all midgut preparations (Fig. 3). No evidence for extracellular Ymt was seen by this method, or by IFA (*21*). Nevertheless, to test whether Ymt released from wild-type bacteria could rescue a Ymt mutant, we fed fleas blood containing an equal mixture of *Y. pestis* KIM6+ and KIM6+*ymt*H188N(pGFP) (*17*). The Ymt$^-$ strain was eliminated from 19% of infected fleas, which contained only *Y. pestis* KIM6+ as indicated by the absence of GFP-fluorescent bacteria. In the remaining coinfected fleas, Ymt$^-$ bacteria were present in the proventriculus but not the midgut in 25%, in both proventriculus and midgut in 51%, and only in the

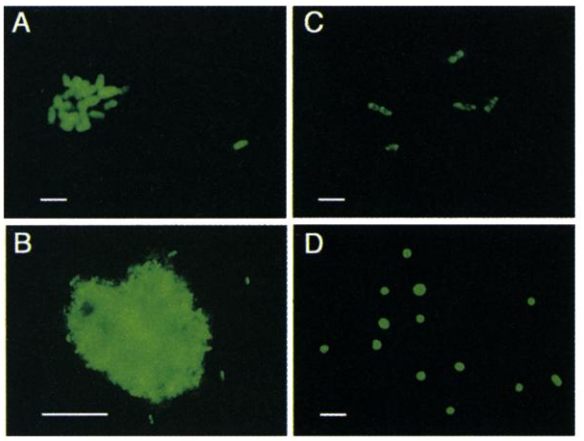

Fig. 2. Ymt$^-$ *Y. pestis* changes rapidly to an aberrant spheroplast-like form in the flea midgut. Normal bacillus-shaped Ymt$^+$ *Y. pestis* forming typical aggregates in the flea midgut 2 days (**A**) and 4 days (**B**) after infection. Normal bacillus-shaped Ymt$^-$ *Y. pestis ymt*H188N from a flea dissected immediately after infection (**C**), and aberrant spherical forms present 1 day after infection (**D**). Bacteria were detected in individual triturated flea midguts by IFA assay with anti-Ymt antibody and by fluorescence microscopy. Bar, 5 μm (A, C, D); 25 μm (B).

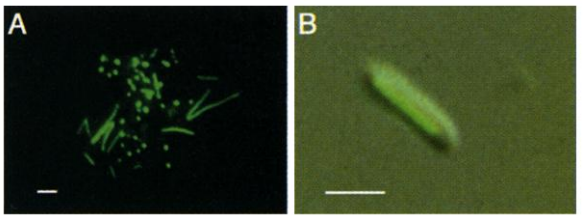

Fig. 3. Localization of Ymt-GFP fusion protein in the cytoplasm of *Y. pestis* infecting the flea midgut. (**A**) Cluster of bacteria in the intact midgut of a flea dissected 4 days after infection and examined directly by fluorescence microscopy, and (**B**) higher magnification of an individual cell by fluorescence plus phase microscopy. Bar, 2.5 μm.

midgut in 5%. However, whereas the mutant *Y. pestis* was distributed evenly throughout the proventriculus of coinfected fleas, when present in the midgut it occurred only in weakly fluorescent clusters contained within larger masses of wild-type bacteria. The nonrandom pattern indicated that Ymt$^-$ *Y. pestis* did not tolerate direct exposure to the midgut environment, and further suggests that the protective PLD activity occurs intracellularly, at the individual cell level. Consistent with this interpretation, exogenous purified Ymt protein added to the blood meal did not enhance the survival of *Y. pestis ymt*H188N in the flea gut (*17*).

Thus, the flea midgut contains an agent that induces spheroplast formation and lysis of *Y. pestis* cells that lack intracellular Ymt. An intracellular PLD conceivably could protect *Y. pestis* in the flea gut either by modifying an endogenous membrane component to make the bacteria impervious to the cytotoxic agent (prophylaxis model), or by neutralizing the agent, directly or indirectly, after it interacts with the bacteria (antidote model). Although the protective mechanism remains to be determined, the cytotoxic agent appeared to derive from plasma, because when bacteria added to filtered mouse plasma was used to infect fleas (*17*), the results 1 day after infection were the same as those for whole blood. However, when fleas were infected with lipid-free artificial plasma, or artificial plasma to which mouse red blood cells were added (*17*), the *ymt*H188N mutant survived as well as Ymt$^+$ *Y. pestis*. Because Ymt$^-$ *Y. pestis* grows well in plasma in vitro and produces septicemia in mice (*14–16*), and because fleas begin to digest their meals immediately after feeding (*24*), the results suggest that a digestion product of one or more plasma components in the flea gut is toxic to the mutant.

Y. pestis is a clonal variant of *Y. pseudotuberculosis* that emerged only 1500 to 20,000 years ago (*1*). The close phylogenic relationship implies that relatively few genetic changes in *Y. pestis* are responsible for its radically different route of transmission and virulence. DNA sequence analysis of *ymt* indicates that it was acquired by *Y. pestis* through horizontal transfer from an unrelated bacterium or eukaryote (*4*, *5*, *12*), a mechanism by which many pathogenic bacteria have obtained virulence factors that enhance survival in their hosts (*25*). Although originally discovered as a murine toxin, our results indicate that a major biological function of Ymt is to enable infection of the vector. For the *Y. pestis* progenitor, therefore, an important effect of acquiring *ymt* was to extend host range to include the flea, a common ectoparasite of the usual rodent host. Introduction of this single gene into *Y. pestis* was likely a crucial step in the recent evolutionary process that led, uniquely among the enteric bacteria, to arthropod-borne transmission. Adapting to transmission by a blood-feeding insect may, in turn, have favored the selection of more virulent strains, capable of producing high-density septicemia, that were responsible for the emergence of plague.

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17. We infected *X. cheopis* fleas by allowing them to feed on heparinized mouse blood containing ~5.0 × 10^8 bacteria per milliliter using a membrane feeder apparatus (*19*, *26*). In three replicate experiments with each bacterial strain, equal numbers of male and female fleas that took the single infectious blood meal were kept at 21°C in an atmosphere of 75% relative humidity and subsequently fed twice weekly on uninfected mice. Fleas were monitored for 4 weeks for mortality and proventricular blockage as described (*19*). Infection rates and levels were determined by colony-forming unit counts (*19*) from 20 individually triturated female fleas collected at 1 hour, 1 day, 7 days, and 28 days after the infectious blood meal. A mixed infection with *Y. pestis* KIM6+ and with *Y. pestis* KIM6+*ymt*H188N that had been transformed with pGFP (Clontech) was established by feeding fleas on blood containing 4.0 × 10^8 of both bacteria per millileter. The pGFP plasmid carries the *gfp* gene and is stable in *Y. pestis* even in the absence of selective pressure (*19*). One to 3 weeks after the infectious blood meal, dissected digestive tracts of 64 fleas that contained typical bacterial aggregates were examined by light and fluorescence microscopy. To test the effect of exogenous Ymt in the flea gut, we infected fleas using mouse blood containing 140 μg of Ymt protein per millileter (*6*, *13*). To test the effect of blood components, we infected fleas with filtered mouse plasma (Millipore Millex-GS, 0.45-μm pore diameter), or artificial plasma [phosphate-buffered saline (PBS, pH 7.4) containing 7% bovine serum albumin (BSA), 6 mM glucose, 12 mM sodium bicarbonate, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 1 mM citric acid] with or without an equal volume of washed mouse red blood cells.
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