

A Plasminogen-Activating Protease Specifically Controls the Development of Primary Pneumonic Plague

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Primary pneumonic plague is transmitted easily, progresses rapidly, and causes high mortality, but the mechanisms by which *Yersinia pestis* overwhelms the lungs are largely unknown. We show that the plasminogen activator Pla is essential for *Y. pestis* to cause primary pneumonic plague but is less important for dissemination during pneumonic plague than during bubonic plague. Experiments manipulating its temporal expression showed that Pla allows *Y. pestis* to replicate rapidly in the airways, causing a lethal fulminant pneumonia; if unexpressed, inflammation is aborted, and lung repair is activated. Inhibition of Pla expression prolonged the survival of animals with the disease, offering a therapeutic option to extend the period during which antibiotics are effective.

Of the three species of *Yersinia* pathogenic to humans, *Y. pestis* is infamous owing to its ability to cause plague. Although usually transmitted by an arthropod vector, it is during cases of secondary pneumonic plague that *Y. pestis* can be spread from person to person through the inhalation of respiratory droplets carrying the bacteria (1). This method of transmission may initiate an epidemic of primary pneumonic plague, which is fatal if not treated early. Pneumonic plague is the most likely form to result in the event of a biowarfare attack with aerosolized *Y. pestis* (2).

Yersinia virulence in mammals requires the Ysc type III secretion system (T3SS), which is shared among all three pathogenic species (3). Unlike *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis* are foodborne pathogens and usually result in self-limiting gastrointestinal infections (4–6). Thus, the presence of the Ysc T3SS alone is not sufficient to cause a rapidly progressing respiratory infection.

Y. pestis also carries pPCP1, a 9.5-kb plasmid that encodes the plasminogen activator Pla, a surface protease that is thought to promote plasmin degradation of fibrin clots (1, 7). In models of bubonic plague, Pla promotes the invasion of *Y. pestis* from subcutaneous sites of inoculation into the lymphatic system and deeper tissues but is dispensable for growth at the site of inoculation (8, 9). When introduced by aerosol, *Y. pestis* lacking Pla was reported to be equivalent or near equivalent in virulence to wild-type by median lethal dose (LD₅₀) analysis (10, 11), but the progression of lung and systemic disease has never been evaluated in a model of primary pneumonic plague. On the basis of these studies, we predicted that respiratory infection with a strain of *Y. pestis* lacking Pla would proceed nor-

mally in the lungs and result in a lethal pneumonia but that fibrin deposition would restrict the ability of bacteria to escape the respiratory system.

We infected C57BL/6 mice intranasally with wild-type *Y. pestis* CO92, an isogenic *Y. pestis* strain lacking Pla (CO92 Δ pla), or the Δ pla strain complemented with the coding sequence for Pla. Mice given wild-type *Y. pestis* CO92, a strain isolated from a fatal case of pneumonic

plague (12), succumbed to the infection in a highly synchronous manner. In contrast, only 50% of the mice infected with the Δ pla strain developed terminal plague after 7 days, and the rate at which the mice died was less synchronous than the rate of those infected with the wild-type strain (Fig. 1A). Complementation of the mutant with the coding sequence for Pla fully restored virulence. Thus, the lack of Pla substantially delayed the time to death resulting from respiratory infection.

Although the kinetics of bacterial growth during infection with CO92 proceeded as expected (13), bacterial outgrowth in the Δ pla-infected mice was significantly altered (Fig. 1, B and C). After 24 hours, 100- to 1000-fold fewer bacteria were recovered from the lungs of Δ pla-infected mice compared with the lungs with wild-type infection. Over the next 2 days, the numbers of Δ pla bacteria in the lungs did not substantially change, whereas wild-type bacteria increased by almost 6 logs. In contrast, we detected bacteria in the spleens of all mice by 72 hours, indicating that escape of the Δ pla strain from the lungs to distal organs still occurred (Fig. 1C). Indeed, at later times, one of five mice had a bacterial burden approaching 10⁸ to 10⁹ colony-forming units (CFU) in the spleen. This corresponded with increased numbers of bac-

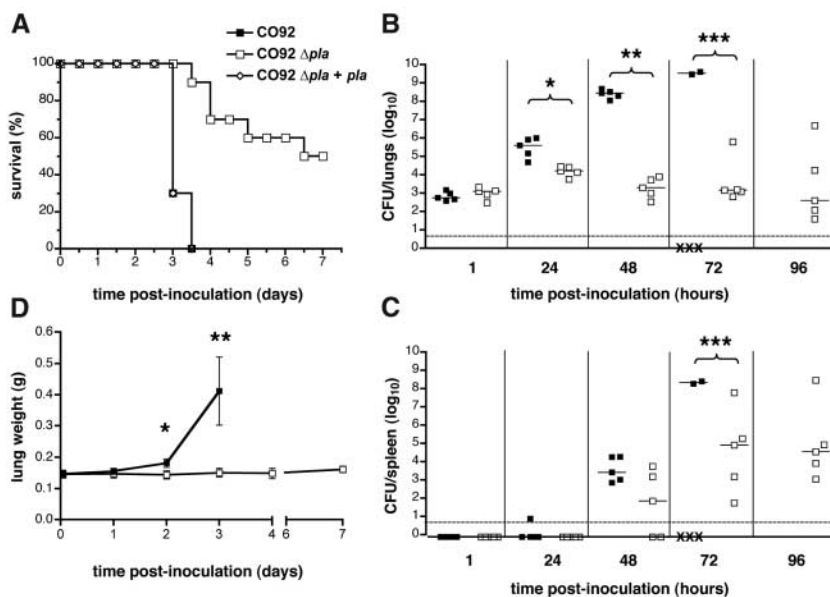


Fig. 1. Pla is required for *Y. pestis* to cause a fulminant infection of the lungs. **(A)** Survival of C57BL/6 mice infected intranasally with *Y. pestis* CO92 (black squares), CO92 Δ pla (white squares), or CO92 Δ pla complemented with *pla* (white diamonds). **(B and C)** Kinetics of infection with *Y. pestis* CO92 (black) or CO92 Δ pla (white). Bacteria were introduced intranasally, and at various times CFU per organ in the lungs (B) and spleen (C) were determined. Each point represents the numbers recovered from a single mouse. The limit of detection is indicated by a dashed line. Symbols below the limit of detection represent mice that survived but did not have detectable numbers of bacteria; an "X" indicates a mouse that succumbed to the infection. A solid line indicates the median of CFU recovered. * $P = 0.037$, ** $P = 0.002$, and *** $P < 0.001$ (unpaired *t* test). **(D)** Gross weight of lungs from mice infected with *Y. pestis* CO92 (black) or CO92 Δ pla (white). C57BL/6 mice were infected as above, and at various times lungs were excised and weighed. * $P = 0.01$ and ** $P < 0.001$. Each experiment was repeated twice. Error bars represent standard deviation.

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teria in the lungs; however, this is likely due to the recirculation of systemic organisms back into the lungs (13) rather than outgrowth of bacteria in this organ.

These data show that Pla controls the proliferation of *Y. pestis* in the lungs but is not essential for bacteria to disseminate. This is a distinct phenotype from that attributed to Pla in models of bubonic plague: When Pla-negative *Y. pestis* is introduced subcutaneously, dissemination is dramatically reduced, but bacterial outgrowth at the local site of infection is unaffected (8, 9). Indeed, we show that rates of dissemination from the initial site of colonization to the spleen were substantially increased when Pla-negative bacteria were introduced intranasally compared with the subcutaneous route (table S2). This may be due to the highly vascularized nature of the lung, allowing escape of a few Pla-negative bacteria through an alveolar capillary and thus initiating systemic infection.

A hallmark of fatal bacterial pneumonia is the development of edema in the lungs, which can be measured by a change in gross lung weight as fluid and cells contribute to increased mass of the organ. Although mouse lungs infected with wild-type *Y. pestis* weighed significantly more than uninfected lungs, the lungs of mice infected with the Δ pla strain of *Y. pestis* showed no change in weight, even after 7 days (Fig. 1D), suggesting that the death of mice infected with this strain is not due to pneumonia but rather is caused by systemic infection. Our results, therefore, may explain the similar LD₅₀ values for wild-type and Pla-negative strains when inhaled, even though *Y. pestis* requires Pla to cause a severe pneumonia.

We also examined lung histology of wild-type and mutant *Y. pestis*-infected mice to resolve an existing controversy regarding pathology and inflammation at the site of infection (8, 9, 13). An influx of inflammatory cells was detected in the lungs in both wild-type and Δ pla infections 36 hours after inoculation; in both cases, the predominant infiltrating cell type was polymorphonuclear (Fig. 2). Although the size of the pulmonary lesions in the wild-type infection increased over time, resulting in tissue destruction and hemorrhage, the foci of inflammation in mice infected with CO92 Δ pla remained relatively constant and restricted. In addition, we examined infected lung sections by using immunofluorescence with an antibody against *Y. pestis*. Numerous extracellular bacteria were associated with inflammation in the lungs of wild-type-infected mice, but relatively few bacteria were detected in the Δ pla infection and were restricted to the much smaller inflammatory lesions (Fig. 2). Thus, both bacterial outgrowth and subsequent inflammation in the lungs were dependent on the Pla surface protease.

These results suggest that host immunity controls the pulmonary infection without developing an overwhelming inflammatory reaction to Pla-negative bacteria. Therefore, we assessed

the amount of immune activation in the lungs by using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to determine changes in transcript quantities of multiple inflammatory mediators. Consistent with our previous observations (13), mice infected with CO92 remained unresponsive early in the infection but showed significant cytokine up-regulation by 48 hours (Fig. 3A). Similarly, the cytokine transcript numbers in the lungs of mice infected with Δ pla were also relatively unchanged early in the infection. After 48 hours, however, cytokine transcript numbers in response to Δ pla were unchanged or only slightly increased, and

by the following day of infection transcript numbers for most cytokines decreased, suggesting down-regulation of the inflammatory response to Δ pla. Thus, the data reveal that, in the absence of Pla, an anti-inflammatory state is maintained in the lungs and the infection is unable to progress to the pro-inflammatory phase that we described previously (13).

That cytokine transcript amounts appeared to stabilize and then decrease in the Δ pla-infected mice suggested the pulmonary inflammatory lesions were resolving. We immunostained infected lungs for the proliferating cell nuclear antigen (PCNA), a marker for host cellular

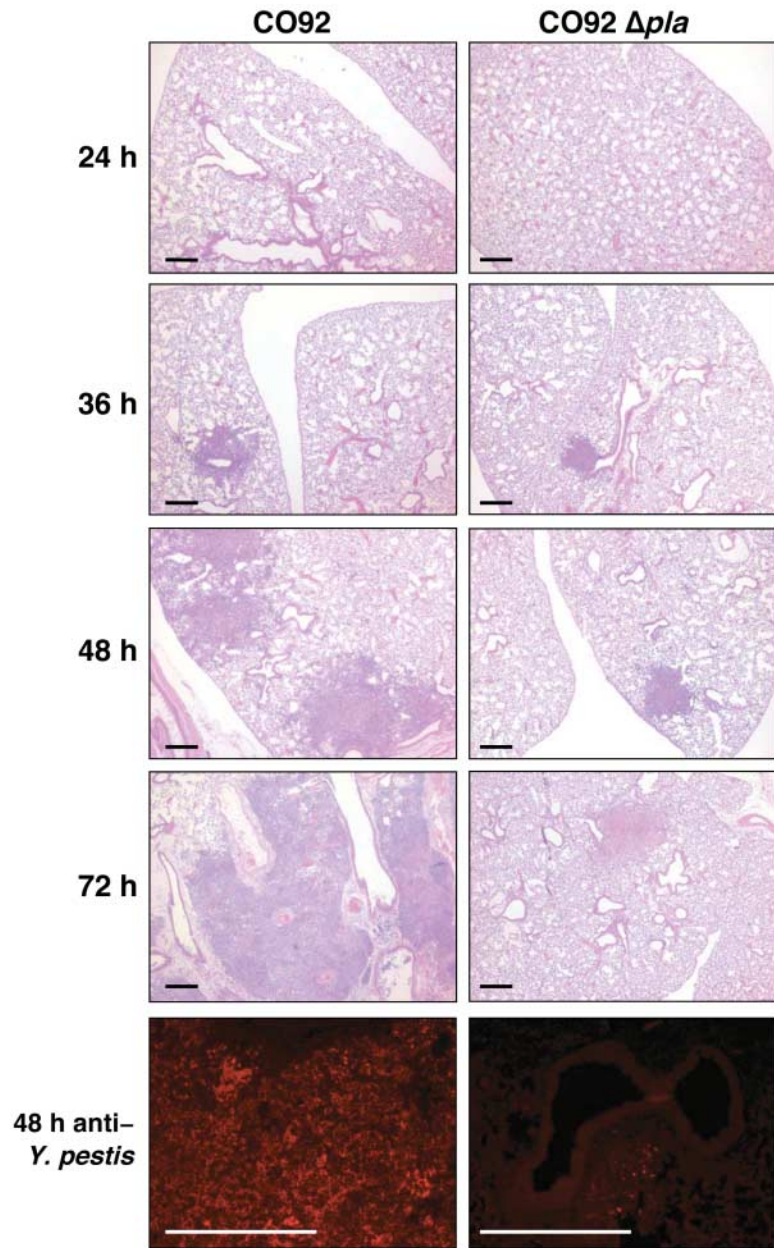
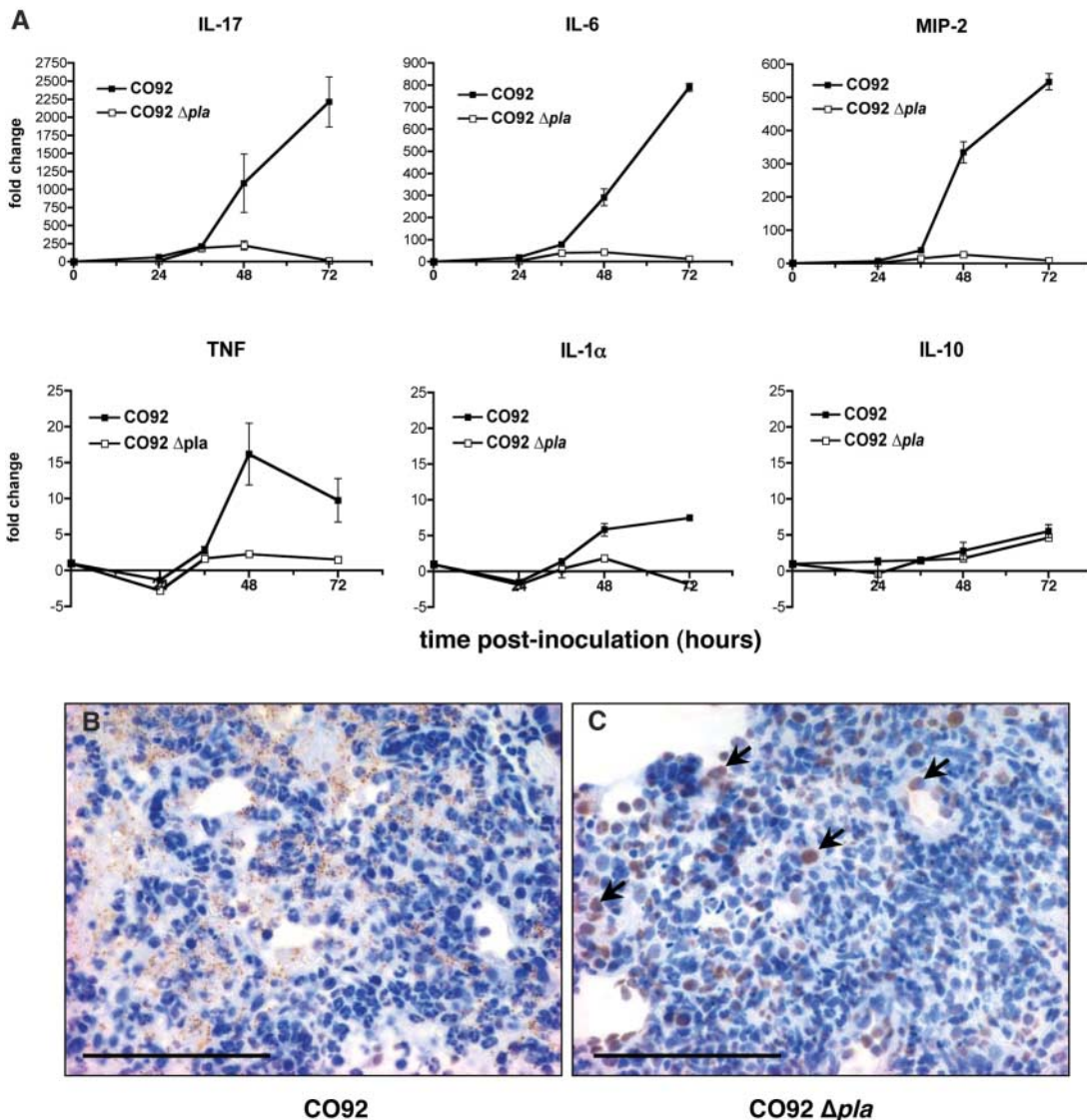


Fig. 2. Histology and presence of bacteria in the lungs of mice during the progression of pneumonic plague. Mice were infected intranasally; at various times, lungs were inflated and fixed with 10% neutral buffered formalin and embedded in paraffin, and 5- μ m sections were stained with hematoxylin and eosin or a *Y. pestis* antibody. The images shown are representative of experiments repeated twice. Scale bars indicate 200 μ m.

Fig. 3. Progression of the inflammatory response to *Y. pestis* CO92 or CO92 Δpla . (A) Mice were uninfected or infected intranasally, and at various times lungs were removed and immersed in RNAlater (Ambion, Woodward, TX). RNA was extracted and reverse-transcribed, and relative cytokine transcript quantities compared with those of uninfected mice were determined by qRT-PCR using the $\Delta\Delta C_t$ method (22) and normalized to glyceraldehyde-3-phosphate dehydrogenase for *Y. pestis* CO92 (black) or Δpla -infected mice (white). IL, interleukin; MIP, macrophage inflammatory protein; and TNF, tumor necrosis factor. (B and C) PCNA antibody stain and hematoxylin counterstain of lungs infected with *Y. pestis* CO92 or CO92 Δpla after 48 hours. PCNA-positive cells (arrows) are present in the lungs of Δpla -infected mice but largely absent in the wild-type infection. Extracellular granular staining in the CO92-infected lungs correlate with the presence of bacteria. Scale bars, 50 μm .



DNA synthesis (14). Whereas the cells of wild-type-infected lungs were almost uniformly PCNA-negative (Fig. 3B), large numbers of PCNA-positive cells were present in Δpla -infected mice (Fig. 3C), indicating active cell proliferation and regeneration of tissue in the lungs. Lung repair at this stage of the infection is further evidence that fatalities among Δpla -infected mice are not a consequence of airway inflammation or damage but instead are the result of systemic spread of the bacteria.

If Pla alone controls the ability of *Y. pestis* to cause pneumonic plague, we hypothesized that experimental induction of *pla* expression midway during the aborted pulmonary disease would be sufficient to turn the nonpneumonic infection into a pneumonic one. To test this, we adapted the tetracycline-responsive promoter system (15) to exogenously control gene expression in *Y. pestis* during infection (Materials and Methods and figs. S1 and S2). We cultured the Δpla strain of *Y. pestis* carrying *pla* under control of the tetracycline-responsive promoter

(*Y. pestis* CO92 Δpla $P_{tet-pla}$, strain YP138) in *pla*-repressing conditions (i.e., absence of anhydrotetracycline, or ATC). Thirty-six hours after intranasal inoculation, we induced *pla* expression by injecting ATC and then followed the progression of the infection. Bacteria in the *pla*-repressed state established a nonprogressive lung infection in a manner similar to that of the Δpla -infected mice. However, once ATC was administered and Pla expression was up-regulated, the condition of these mice quickly converted to a disease with all the features of pneumonic plague: rapid proliferation of bacteria with development of visible microcolonies (Fig. 4, A and B), unrestricted inflammatory infiltrate, tissue damage (Fig. 4C), and shortened time to death. Thus, the absence of Pla stalls the development of disease in the early anti-inflammatory phase but does not eliminate the potential of these organisms to cause pneumonic plague. Ultimately, the block in the progression of infection by the respiratory route is completely reversible by the expression of Pla.

One hypothesis of the mechanism by which Pla facilitates the invasive nature of *Y. pestis* is that the protease converts host plasminogen into plasmin while degrading the plasmin inhibitor α_2 -antiplasmin, releasing bacteria from the entrapment of fibrin clots (16, 17). Indeed, recent evidence has shown that fibrin deposition is an important means of immune control for a variety of pathogens (18–20), and thus the subversion of the coagulation cascade may be a notable virulence mechanism. Consistent with this, we show that the plasminogen-activating activity of Pla is essential to *Y. pestis* virulence in the pulmonary system (Materials and Methods and fig. S3). Additionally, fibrin(ogen) deposition can be detected in the lungs of mice infected with either the wild-type or the Δpla strain, but the pattern and extent of fibrin(ogen) immunostaining is substantially altered (fig. S4). We cannot exclude, however, the possibility that other targets of Pla activity may also contribute to the development of primary pneumonic plague. Nonetheless, the role of Pla during pneumonic

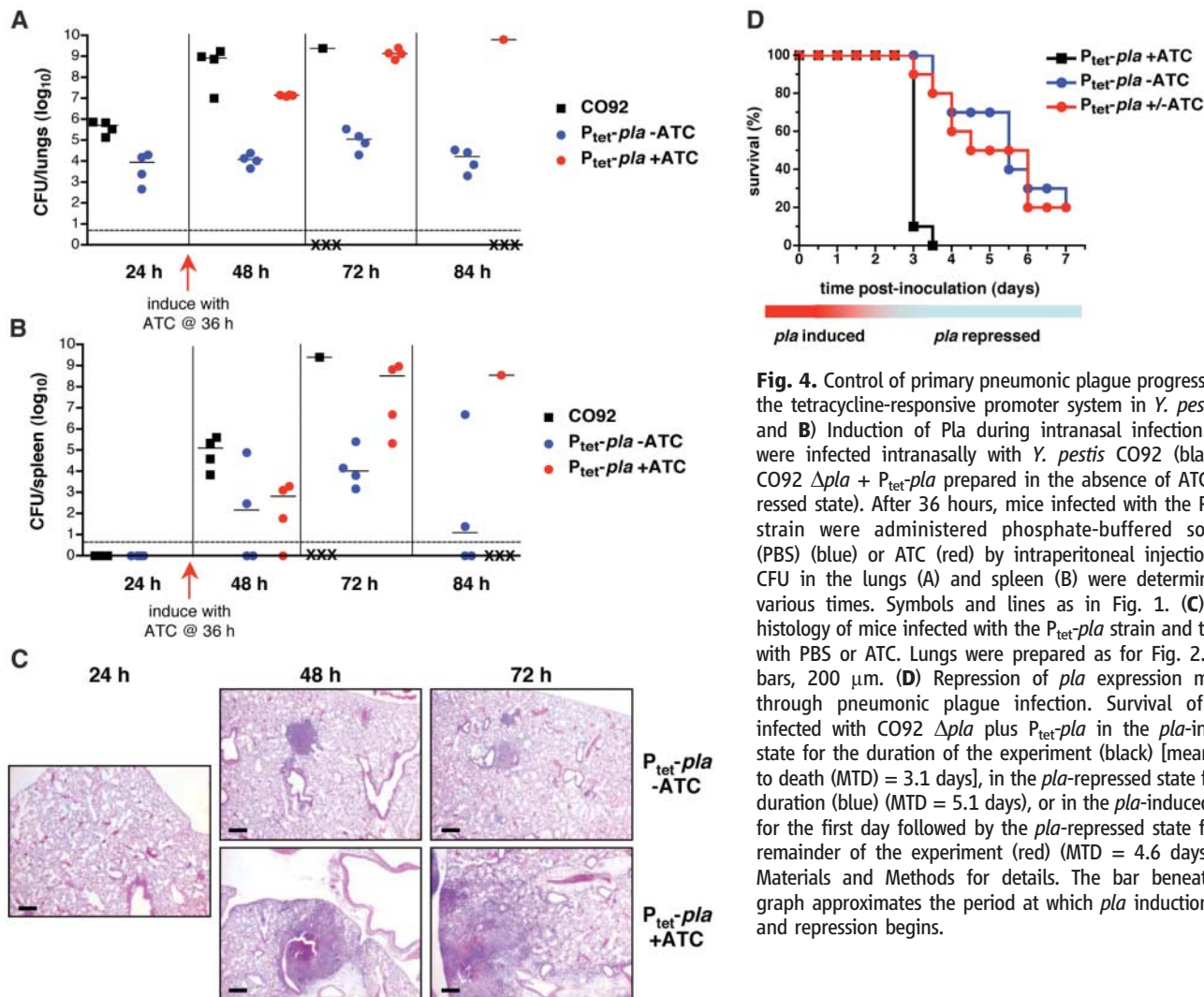


Fig. 4. Control of primary pneumonic plague progression by the tetracycline-responsive promoter system in *Y. pestis*. (A and B) Induction of Pla during intranasal infection. Mice were infected intranasally with *Y. pestis* CO92 (black) or CO92 Δ pla + P_{tet} -pla prepared in the absence of ATC (repressed state). After 36 hours, mice infected with the P_{tet} -pla strain were administered phosphate-buffered solution (PBS) (blue) or ATC (red) by intraperitoneal injection bid. CFU in the lungs (A) and spleen (B) were determined at various times. Symbols and lines as in Fig. 1. (C) Lung histology of mice infected with the P_{tet} -pla strain and treated with PBS or ATC. Lungs were prepared as for Fig. 2. Scale bars, 200 μ m. (D) Repression of *pla* expression midway through pneumonic plague infection. Survival of mice infected with CO92 Δ pla plus P_{tet} -pla in the *pla*-induced state for the duration of the experiment (black) [mean time to death (MTD) = 3.1 days], in the *pla*-repressed state for the duration (blue) (MTD = 5.1 days), or in the *pla*-induced state for the first day followed by the *pla*-repressed state for the remainder of the experiment (red) (MTD = 4.6 days). See Materials and Methods for details. The bar beneath the graph approximates the period at which *pla* induction ends and repression begins.

plague may help explain how *Y. pestis* acquired the ability to cause a rapid, severe respiratory infection and be transmitted from person to person by the aerosol route, whereas *Y. pseudotuberculosis* and *Y. enterocolitica* did not. Interestingly, the altered syndrome we observed with a Pla-negative strain is similar to case descriptions from the early 20th-century Manchurian epidemics in which aerosol-acquired plague resulted in fatal sepsis before a local lung disease could develop (21).

The critical role for Pla suggests that its inhibition could offer a therapeutic advantage, particularly because the rapid progression of pneumonic plague leaves little time for effective treatment. We tested this experimentally by infecting mice with the Pla-inducible *Y. pestis* strain YP138 prepared in the presence of ATC. We then provided ATC to the animals for only the first day of the infection, allowing the ATC to be cleared and *pla* expression to be repressed for the remainder of the experiment. The time until death was delayed when the Pla-induced state was switched to a Pla-repressed state dur-

ing the infection (Fig. 4D); in fact, the kinetics more closely resemble those of a Δ pla infection. This suggests that exogenous inhibition of Pla during primary pneumonic plague may indeed prolong the survival of the affected individual, expanding the window during which antibiotics could be successfully administered to treat the disease.

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Supporting Online Material

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Materials and Methods

SOM Text
Figs. S1 to S4
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A Virus in a Fungus in a Plant: Three-Way Symbiosis Required for Thermal Tolerance

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A mutualistic association between a fungal endophyte and a tropical panic grass allows both organisms to grow at high soil temperatures. We characterized a virus from this fungus that is involved in the mutualistic interaction. Fungal isolates cured of the virus are unable to confer heat tolerance, but heat tolerance is restored after the virus is reintroduced. The virus-infected fungus confers heat tolerance not only to its native monocot host but also to a eudicot host, which suggests that the underlying mechanism involves pathways conserved between these two groups of plants.

Endophytic fungi commonly grow within plant tissues and can be mutualistic in some cases, as they allow plant adaptation to extreme environments (1). A plant-fungal symbiosis between a tropical panic grass from geothermal soils, *Dichanthelium lanuginosum*, and the fungus *Curvularia protuberata* allows both organisms to grow at high soil temperatures in Yellowstone National Park (YNP) (2). Field and laboratory experiments have shown that when root zones are heated up to 65°C, nonsymbiotic plants either become shriveled and chlorotic or simply die, whereas symbiotic plants tolerate and survive the heat regime. When grown separately, neither the fungus nor the plant alone is able to grow at temperatures above 38°C, but symbiotically, they are able to tolerate elevated temperatures. In the absence of heat stress, symbiotic plants have enhanced growth rate compared with nonsymbiotic plants and also show significant drought tolerance (3).

Fungal viruses or mycoviruses can modulate plant-fungal symbioses. The best known example of this is the hypovirus that attenuates the virulence (hypovirulence) of the chestnut blight fungus, *Cryphonectria parasitica* (4). Virus regulation of hypovirulence has been demonstrated experimentally in several other pathogenic fungi (5–8). However, the effect of mycoviruses on mutualistic fungal endophytes is unknown. There is only one report of a mycovirus from the well-

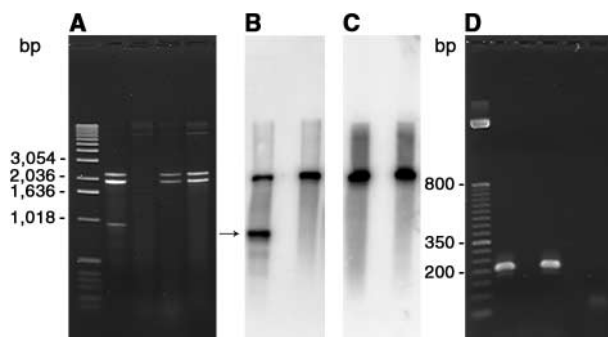
known mutualistic endophyte, *Epichloë festucae*, but no phenotype has been associated with this virus (9).

Fungal virus genomes are commonly composed of double-stranded RNA (dsRNA) (10). Large molecules of dsRNA do not normally occur in fungal cells and, therefore, their presence is a sign of a viral infection (9). Using a protocol for nucleic acid extraction with enrichment for dsRNA (11), we detected the presence of a virus in *C. protuberata*. The dsRNA banding pattern consists of two segments of about 2.2 and 1.8 kb. A smaller segment, less than 1 kb in length, was variable in presence and size in the isolates analyzed and, later, was confirmed to be a subgenomic element, most likely a defective RNA (fig. S1 and Fig. 1, A and B). Using tagged random hexamer primers, we transcribed the virus with reverse transcriptase (RT), followed by amplification and cloning. Sequence analysis revealed that each of the two RNA segments contains two open reading frames (ORFs) (fig.

S2). The 2.2-kb fragment (RNA 1) is involved in virus replication, as both of its ORFs are similar to viral replicases. The first, ORF1a, has 29% amino acid sequence identity with a putative RNA-dependent RNA polymerase (RdRp) from the rabbit hemorrhagic disease virus. The amino acid sequence of the second, ORF1b, has 33% identity with the RdRp of a virus of the fungal pathogen *Discula destructiva*. These two ORFs overlap and could be expressed as a single protein by frameshifting, a common expression strategy of viral replicases. The two ORFs of RNA 2 have no similarity to any protein with known function. As in most dsRNA mycoviruses, the 5' ends (21 bp) of both RNAs are conserved. Virus particles purified from *C. protuberata* are similar to those of other fungal viruses: spherical and ~27 nm in diameter (fig. S3). This virus is transmitted vertically in the conidiospores. We propose naming this virus *Curvularia thermal tolerance virus* (CThTV) to reflect its host of origin and its phenotype.

The ability of the fungus to confer heat tolerance to its host plant is related to the presence of CThTV. Wild-type isolates of *C. protuberata* contained the virus in high titers, as evidenced by their high concentration of dsRNA (~2 µg/g of lyophilized mycelium). However, an isolate obtained from sectoring (change in morphology) of a wild-type colony contained a very low titer of the virus, as indicated by a low concentration of dsRNA (~0.02 µg/g of lyophilized mycelium). These two isolates were identical by simple sequence repeat (SSR) analysis with two single-primer polymerase chain reaction (PCR) reactions and by sequence analysis of the rDNA ITS1-5.8S-ITS2 region (figs. S4 and S5). Desiccation and freezing-thawing cycles are known to disrupt virus particles (12); thus, mycelium of the isolate obtained by sectoring was

Fig. 1. Presence or absence of CThTV in different strains of *C. protuberata*, detected by ethidium bromide staining (A), Northern blot using RNA 1 (B) and RNA 2 (C) transcripts of the virus as probes, and RT-PCR using primers specific for a section of the RNA 2 (D). The isolate of the fungus obtained by sectoring was made virus-free (VF) by freezing-thawing. The virus was reintroduced into the virus-free isolate through hyphal anastomosis (An) with the wild type (Wt). The wild-type isolate of the fungus sometimes contains a subgenomic fragment of the virus that hybridizes to the RNA 1 probe (arrow).



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