

MINIREVIEW

Molecular Darwinian Evolution of Virulence in *Yersinia pestis*[∇]

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The genus *Yersinia* consists of 15 species (www.bacterio.cict.fr/xz/yersinia.html), and only three of them, *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, are pathogenic to mammals, including humans. The *Y. pseudotuberculosis*-*Y. pestis* evolutionary linkage diverged from *Y. enterocolitica* between 41 and 186 million years ago, while *Y. pestis* diverged from *Y. pseudotuberculosis* within the last 1,500 to 20,000 years (1, 65). In accordance with this evolutionary cascade, wide genetic diversity exists between *Y. pseudotuberculosis* and *Y. enterocolitica*, while very close genetic similarity is found between *Y. pseudotuberculosis* and *Y. pestis*. *Y. pseudotuberculosis* causes only nonfatal gastrointestinal disease in mammalian hosts, including humans, and the disease is transmitted by the food-borne route. *Y. pestis* causes plague, which is one of the most deadly diseases (47). Three pandemics of plague have been recorded in human history and have claimed hundreds of thousands of lives (47). Plague is a typical enzootic disease (an infection of the animal population[s] in one or more confined natural foci without the need for external inputs), and epidemics of rodent plague are restricted in various enzootic plague foci especially in Asia, the Americas, and Africa (80). Compared to its progenitor *Y. pseudotuberculosis*, *Y. pestis* utilizes a radically different mechanism of transmission in rodent reservoirs that relies primarily upon biting by flea vectors. This review deals with how genetic changes (gene inactivation, loss, and acquisition) and remodeling of gene regulation encourage *Y. pestis* to switch from an enteric lifestyle to a mammalian blood-borne lifestyle that relies on vector-borne transmission.

PROGRESSION OF PLAGUE INFECTION

Rodents and humans acquire *Y. pestis* by the bite of an infected flea, contact with infected tissues, or inhalation of respiratory droplets or aerosols, with manifestations of bubonic, septicemic, and pneumonic plague (47). After the flea bite, there is an initial subcutaneous and intradermal colonization, and then the bacteria migrate into the regional lymph nodes and inflammation, cellulitis, and occasionally large carbuncles develop around the bubo (bubonic plague) (60). Without timely effective treatment, the bacteria will rapidly escape from containment in the lymph node and spread systemically

through the blood to various organs, causing fatal sepsis (septicemic plague) (82). An intracellular growth of *Y. pestis* in macrophages at early stages of infection is thought to allow this pathogen to proliferate and to synthesize virulence determinants, enabling the releasing bacteria to acquire the ability to eliminate the host immune response (39). In addition, secondary pneumonic plague could result from hematogenous spread from the bubo to the lung, presenting in patients as severe bronchopneumonia, cavitation, or consolidation with production of bloody or purulent sputum (82). Primary pneumonic plague could be caused directly by the inhalation of infectious droplets or aerosols, with symptoms including acute pneumonia, intra-alveolar hemorrhage and edema, profound lobular exudation, fibrin deposition, and bacillary aggregation (33). Both primary and secondary pneumonic plagues are highly contagious for close contacts by airborne transmission.

ECOLOGICAL AND EPIDEMIOLOGICAL DIFFERENCES BETWEEN *Y. PESTIS* AND *Y. PSEUDOTUBERCULOSIS*

Animals, food, and the abiotic environment are *Y. pseudotuberculosis* reservoirs from which epizootic and human infection may arise, and the disease is mild and transmitted by the food-borne route. In humans, typical symptoms include fever and right-side abdominal pain. In rare cases the disease may cause skin complaints (erythema nodosum), joint stiffness and pain (reactive arthritis), or spread of bacteria to the blood (bacteremia).

Due to acute and systemic infection, the mortality rate of plague reaches 70 to 100% without treatment depending on routes of infection. *Y. pestis* has a limited ability to live in the environment, although there is evidence that *Y. pestis* can live in soil for up to 30 weeks (3). Maintenance of plague in enzootic plague foci is almost absolutely dependent upon cyclic transmission between fleas and mammals (80). Blocked fleas are important for transmission of plague (24). Blockage of fleas (heavy proliferation of bacteria in the adhesive biofilms in the proventriculus) makes them feel hungry and repeatedly attempt to feed, and the plague bacilli will be pumped into the host body during these futile feeding attempts (25). The development of heavy bacteremia (bacterial concentration reaching at least 10⁶ CFU/ml) in hosts is necessary to reliably infect the fleas (38). Such a high level of bacteremia raises the risk of hosts' rapid death. Nevertheless, once some fleas achieve feeding prior to the host's death, they will seek alternative hosts, thereby increasing the likelihood of transmission to other individuals from the hosts (15).

Generally, it takes about 2 weeks for blockage to develop,

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which is not sufficient to explain the high rate of spread that typifies plague epidemics. Various species of rodent fleas are immediately infectious after biting a septicemic host and transmit bacteria efficiently for at least 4 days postinfection, and accordingly the mode of “early-phase transmission” by unblocked fleas has been proposed (14). Early-phase transmission helps explain not only the rapid spread that typifies plague epidemics but also previous inconsistencies between the rates of pathogen spread expected from the blocked fleas. It was suggested that mechanical transmission by unblocked fleas is significant during epidemics that represent the periods when *Y. pestis* can spread rapidly across landscapes but that transmission by blocked fleas is important primarily during interepizootic transmission (15). In addition, a combination of early-phase transmission and blocking probably helps to explain the observed high mortality rates of susceptible host populations, including humans during the Black Death (74).

The rodent reservoirs, the flea vectors, and *Y. pestis* constitute a well-balanced biocommunity in the plague foci. *Y. pestis* possesses potential to attack humans, and human infection usually occurs with the transmission of the pathogen from rodents. Although cases of human plague can be well controlled by timely antibiotic administration, plague still remains a significant concern for public health because it can be transmitted from person to person through respiratory droplets and used for bioterrorism and biological warfare (68).

Y. PESTIS VIRULENCE DETERMINANTS SHARED BY Y. PSEUDOTUBERCULOSIS

Y. pestis has developed specialized strategies for virulence in hosts and transmission by fleas (Table 1), and many of these determinants are harbored in the genome of *Y. pseudotuberculosis*.

COLONIZATION AND DISSEMINATION

The major adhesin and invasin, YadA and Inv, respectively, specific for gastrointestinal infection are inactivated in *Y. pestis* (see below), but this pathogen still has additional proteins (Ail [31], YadBC [20], and YapE [35]) that account for bacterial colonization and dissemination during infection.

INTRACELLULAR GROWTH

The ability to replicate in macrophages is conserved in *Y. pestis* and *Y. pseudotuberculosis* (53). RipABC (54), MgtCB (22), Ugd (22), Yfe (48), and Feo (48) have been shown to be required for the replication of *Y. pestis* in macrophages. Both MgtCB and Ugd are positively regulated by the PhoP-PhoQ two-component system (37) that is important for survival under conditions of macrophage-induced stress and virulence in *Y. pestis* (44).

ELIMINATION OF HOST IMMUNE RESPONSE

The plasmid pCD1-borne type III secretion system (T3SS) is composed of a secretion machinery, a set of translocation proteins, a control system, and six Yop effector proteins (56). Through the T3SS, pathogenic yersiniae inject effectors into

the cytosol of eukaryotic cells when docking at the surface of host cells, and the injected Yops mediate suppression of phagocytosis and the inflammatory reaction (56). *Y. pestis* utilizes T3SS to selectively destroy innate immune cells that represent the first line of host defense, thereby preventing adaptive responses and precipitating the fatal outcome of plague (40). *Y. pestis* still employs pH 6 antigen fimbriae to function as an antiphagocytic factor independent of Yops (27).

The Tc genes were first identified in the insect pathogen and encode a protein complex toxic to insects. Tc proteins in *Y. pseudotuberculosis* and *Y. pestis* are not insecticidal toxins but have evolved toxicity to mammalian cells (23).

Heavy proliferation of *Y. pestis* in the bloodstream is essential for its transmission by fleas. Resistance to complement-mediated lysis (serum resistance) is required for bacterial survival in mammalian blood. The Ail protein (4) and lipopolysaccharide (LPS) (50) promote serum resistance, which appears to be a conserved mechanism in pathogenic yersiniae.

IRON UPTAKE

In mammals, iron is bound to Fe³⁺-binding proteins and hemoproteins, and thus free iron is too rare to sustain bacterial growth. Iron acquisition is critical for the survival of pathogenic bacteria during infection. A wide array of iron acquisition systems have been characterized or annotated for *Y. pestis* (21), and at least two (Ybt and Yfe) of them were proven to be required for full virulence (5). Ybt, also known as the high-pathogenicity island (59), is essential to iron acquisition at the site of the flea bite and in the lymphatic system, while Yfe is likely used in the later stages of the disease, i.e., blood-borne systemic dissemination (5).

LATERAL ACQUISITION OF NOVEL VIRULENCE DETERMINANTS BY Y. PESTIS

Lateral gene transfer directly introduces foreign DNA elements into the host genome, which will effectively alter the pathogenic characters of bacterial species (42). *Y. pestis* has acquired two unique virulence plasmids, pPCP1 and pMT1, through lateral gene transfer. pPCP1 encodes plasminogen activator (Pla), while pMT1 encodes murine toxin (Ymt) and F1 capsule (Table 1).

Pla is essential for bubonic and primary pneumonic plague (but not primary and secondary septicemic forms), since it specifically promotes *Y. pestis* dissemination from peripheral infection routes (34, 61). At 37°C but not 26°C, *Y. pestis* expresses a capsule-like antigen, called F1 antigen. F1 provides *Y. pestis* the ability to block phagocytosis by a mechanism different from those of T3SS and pH 6 antigen (13). Ymt does not play a role in mouse infection (57) but shows phospholipase D activity and is required for survival of *Y. pestis* in fleas (26). It was thought that intracellular phospholipase D activity appeared to protect *Y. pestis* from a cytotoxic digestion product of plasma in the flea gut (26).

Unexpectedly, only two chromosomal regions seem to be specific to *Y. pestis* (76) (Table 1). They are located in two different genomic islands probably acquired through lateral gene transfer (45). These two *Y. pestis*-specific chromosomal regions deserve more attention to investigation of their roles in

TABLE 1. Virulence determinants or functions involved in molecular evolution of *Y. pestis*

Function and protein name	Gene identifier(s)	Description	Status in organism:		Reference(s)	Label ^a
			<i>Y. pseudotuberculosis</i>	<i>Y. pestis</i>		
Flea transmission						
GmhA	YPO3243	Enzymes for biofilm formation	Present	Present	11	
SpeA and SpeC	YPO0929 and YPO1201	Enzymes for biofilm formation	Present	Present	46	
YrbH	YPO3577	Enzymes for biofilm formation	Present	Present	71	
Hms	YPO1951 to YPO1954	Enzymes for biofilm formation	Present	Inhibited	28	
NghA	YPO2632	Biofilm-inhibiting glycosyl hydrolase	Present	Inactivated	16	β
RcsA	YPO2449	Transcriptional repressor of biofilm formation	Present	Inactivated	69	β
Ymt	YPMT1.74	Phospholipase D required for survival in flea	Absent	Present	26	α
Colonization and dissemination						
Inv	YPO1793	Invasin	Present	Inactivated	63	β
YadA	YPCD1.87c	Adhesin and invasins	Present	Inactivated	45	β
Ail	YPO2905	Adhesin and invasins	Present	Present	31	
YadBC	YPO1387 to YPO1388	Invasin	Present	Present	20	
YapC	YPO2796	Autotransporter adhesin	Present	Present	18	
YapE	YPO3984	Autotransporter adhesin	Present	Present	35	
Pla	YPPCP1.07	Plasminogen activator promoting bacterial in vivo dissemination	Absent	Present	34, 61	α
Intracellular growth						
RipA	YPO1926	Putative acetyl coenzyme A transferase	Present	Present	54	
Ugd	YPO2174	LPS modification	Present	Present	22	
MgtCB	YPO1660 to YPO1661	Magnesium uptake	Present	Present	22	
Yfc	YPO2439 to YPO2442	ABC-type iron transporter	Present	Present	48	
FcoBA	YPO0132 to YPO0133	Ferrous iron transporter	Present	Present	48	
Elimination of host immune response						
YadA	YPCD1.87c	Serum resistance	Present	Inactivated	45	β
Ail	YPO2905	Serum resistance	Present	Present	31	
pH 6 antigen	YPO1301 to YPO1305	Resistance to phagocytosis	Present	Present	27	
T3SS		Elimination of innate immune cells	Present	Present	56	
Tc proteins		Toxicity to mammalian cells	Present	Present	23	
F1 capsule		Resistance to phagocytosis	Absent	Present	13	α
O-antigen genes	YPMT1.81c to YPMT1.84	Lack of O antigen is essential for Pla function	Present	Inactivated	32	β
Iron uptake						
Yfc	YPO2439 to YPO2442	ABC-type iron transporters	Present	Present	5	
High-pathogenicity island	YPO1906 to YPO1916	Siderophore yersiniabactin-based iron acquisition system	Present	Present	5	
Virulence-required regulators						
VirF	YPCD1.49	Positive regulator of T3SS	Present	Present	19	
CRP	YPO0175	Positive regulator of Pla	Present	Present	79	
PhoP-PhoQ	YPO1633 to YPO1634	Positive regulator of Ugd and MgtCB	Present	Present	44	
RovA	YPO2374	Positive regulator of pH 6 antigen	Present	Present	8	
Other virulence determinants						
Dam	YPO0154	DNA adenine methylation	Present	Present	58	
HtrA	YPO3382	Serine protease	Present	Present	78	
Lpp	YPO2394	Braun lipoprotein	Present	Present	62	
Yptφ	YPO2272 to YPO2281	Filamentous prophage	Absent	Present	12	α
<i>Y. pestis</i> -specific chromosomal regions						
	YPO3387 to YPO3396	Hypothetical proteins	Absent	Present	76	α
	YPO2087 to YPO2093	Prophage proteins	Absent	Present	76	α
<i>Y. pseudotuberculosis</i> -specific chromosome regions						
LpxL	YPTB2046	Loss of LpxL enables <i>Y. pestis</i> to evade LPS-induced inflammation	Present	Absent	41	γ
R1	YPTB0872 to YPTB0878	Methionine salvage pathway required for virulence of <i>Y. pseudotuberculosis</i>	Present	Absent	51	γ

ORF3 and ORF4 ORF2	YPTB1495 and YPTB3368 YPTB1058	Hypothetical proteins essential for viability of <i>Y. pseudotuberculosis</i> Putative pseudouridylylase synthase necessary for optimal growth of <i>Y. pseudotuberculosis</i>	Present Present	Absent Absent	51 51	γ γ
R3	YPTB2193 to YPTB2201	Hypothetical proteins necessary for optimal growth of <i>Y. pseudotuberculosis</i>	Present	Absent	51	γ
Genes linked to human-virulence attenuation of biovar Microtus strains AspA	YPO0348	Inactivated in typical <i>Y. pestis</i> rather than human-avirulent biovar Microtus or Pestoides strains	Present	Present	6, 75	
	YPM1143c	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO1956	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO1973	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2258	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2729	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2731	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO3049	Inactivated in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO1986 to YPO1987	Absent in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2096 to YPO2135	Absent in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2469	Absent in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO2487 to YPO2489	Absent in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	
	YPO3046 to YPO3047	Absent in biovar Microtus strains rather than typical <i>Y. pestis</i>	Present	Present	83	

^a α, acquired by *Y. pestis* through lateral gene transfer; β, conserved in *Y. pseudotuberculosis* but inactivated in *Y. pestis*; γ, conserved in *Y. pseudotuberculosis* but absent from *Y. pestis*.

virulence and/or transmission by fleas. However, it has been argued that analysis of more bacterial strains could further reduce the number of *Y. pestis*-specific chromosomal genes, perhaps to zero (7, 45).

DECAY OF REDUNDANT OR DELETERIOUS FUNCTIONS IN *Y. PESTIS*

About 13% of *Y. pseudotuberculosis* genes no longer function (inactivated or absent) in *Y. pestis* CO92 (45). Genome decay (gene loss and inactivation) appears to be closely linked to flea-borne transmission and increased virulence of *Y. pestis* (Table 1).

GENE INACTIVATION

yadA and *inv* encode the major adhesin and invasin, respectively, in *Y. pseudotuberculosis* and enable this enteropathogen to specifically adhere to surfaces of host intestines and invade lining epithelial cells. Both of them are inactivated in *Y. pestis* (30). Urease plays its role in using urea as a source of nitrogen. Production of urease by the *ure* operon is necessary for oral transmission of *Y. pseudotuberculosis*, but it is inactivated in *Y. pestis* due to the mutation causing a premature stop codon in *ureD* (33). Since *Y. pestis* spends its life almost exclusively in a flea-host-flea cycle, the organism can lose with impunity the function of urease needed for survival in natural environments.

Y. pestis expresses rough LPS lacking the O antigen, due to the inactivation of several genes in the O-antigen gene cluster (52). *Y. pseudotuberculosis* produces a rough LPS at 37°C but not at 26°C, and a variable number of LPS genes are seen to be defective when various biovars of *Y. pestis* are compared (64). Expression of rough LPS is essential for Pla activity and virulence in *Y. pestis* (32). A pathogenic advantage of rough LPS in *Y. pestis* is that it enables efficient Pla-mediated bacterial dissemination to cause systemic disease.

For the blocked fleas, *Y. pestis* synthesizes an attached biofilm in the flea proventriculus and in its midgut posteriorly (25). Three distinct operons, *hmsHFRS*, *hmsT*, and *hmsP*, are involved in the synthesis of bacterial extracellular matrix, which is the primary component of *Yersinia* biofilm (28). *Y. pseudotuberculosis* contains all of these *hms* genes, which are 99% identical to the *Y. pestis* homologues (9). In addition to Hms, several other proteins (GmhA, SpeAC, and YrbH) involved in biofilm formation by *Y. pestis* are harbored in *Y. pseudotuberculosis* (Table 1).

Only a small number of *Y. pseudotuberculosis* strains are able to form biofilm on *Caenorhabditis elegans*, and none of them has the ability to form adhesive biofilms in fleas (17). The transcriptional regulator RcsA (69) and the glycosyl hydrolase NghA (16) have been shown to inhibit *Yersinia* biofilm formation, but both of them are functional in *Y. pseudotuberculosis* but inactivated in *Y. pestis* (16, 69). Expression of functional RcsA or NghA in *Y. pestis* strongly represses biofilm formation and abolishes flea blockage (16). Therefore, *Y. pestis* has evolved the changes in regulatory functions on biofilm development to ensure stable biofilm formation in the flea proventriculus and to result in efficient arthropod-borne transmission.

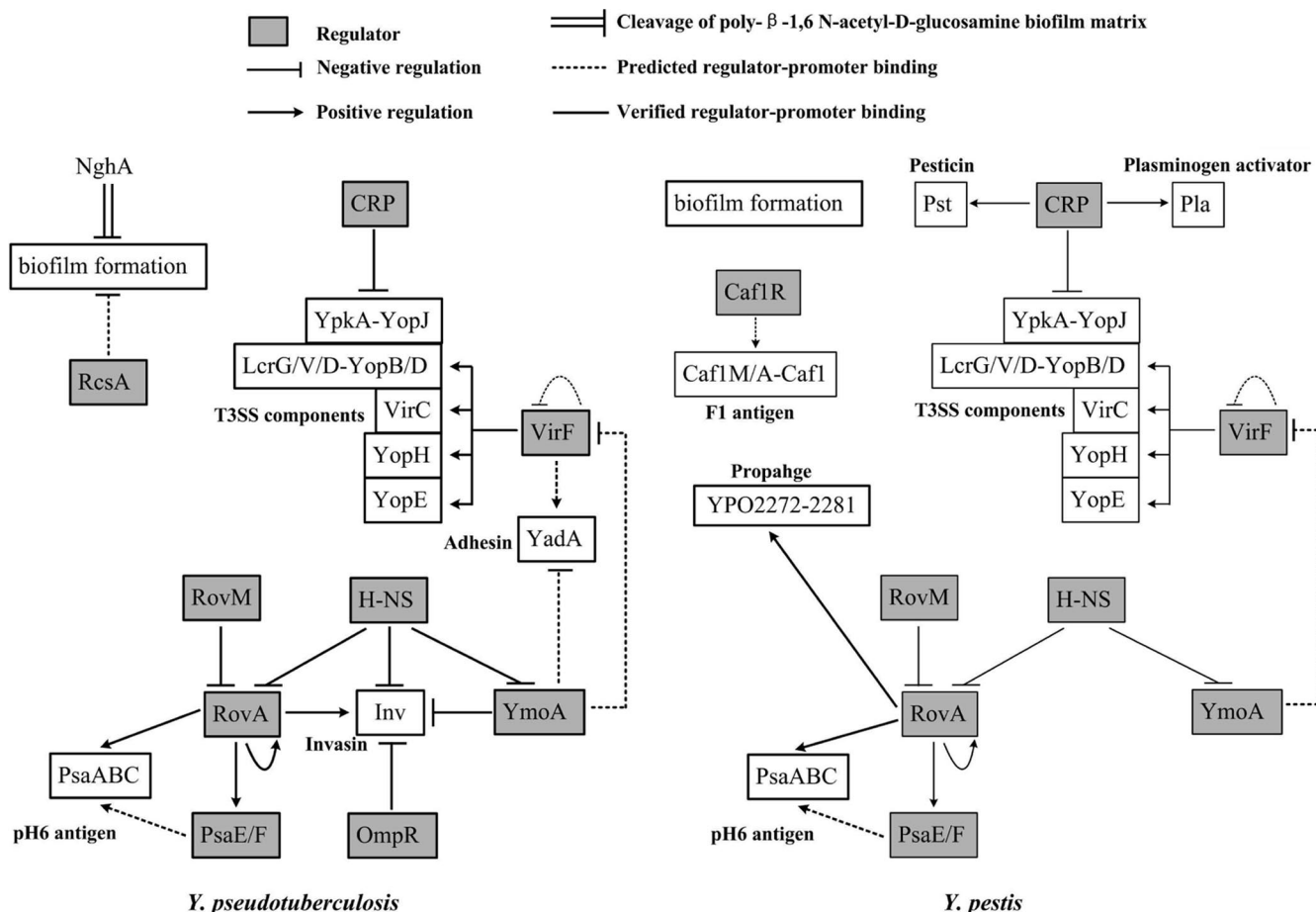


FIG. 1. Remodeling of gene regulation in *Y. pestis* and *Y. pseudotuberculosis*. Transcription regulators listed here bind to *cis*-acting DNA sequences within the promoters of their target genes and either activate or repress transcription initiation of these targets. Some regulators (e.g., CRP) can function as either activators or repressors according to the target promoters. Shown also is autoregulation of some regulators (e.g., RovA).

GENE LOSS

Hexa-acylated LPS observed in many gram-negative pathogens is able to activate TLR4 signaling and to further stimulate the host innate immune response (55). The acyltransferase LpxL is required for addition of the secondary acyl chains to the tetra-acylated precursor (55). The *lpxL* gene is absent from *Y. pestis*, leading this pathogen to produce tetra-acylated LPS that inhibits TLR4 activation, which allows this pathogen to evade the protective inflammatory response and establish fatal infection (41).

Five additional *Y. pseudotuberculosis*-specific chromosomal loci (R1, R3, ORF2, ORF3, and ORF4) required for its survival, optimal growth, or virulence are absent from *Y. pestis* (51) (Table 1). ORF3 and ORF4, with unknown function, are essential for the viability of *Y. pseudotuberculosis*, while ORF2 (a putative pseudouridylate synthase involved in RNA stability) and R3 (a genomic region composed mostly of genes of unknown functions) are necessary for its optimal growth in a chemically defined medium (51). Deletion of R1 (a genomic region responsible for the methionine salvage pathway) alters the mutant's virulence, suggesting that the availability of free methionine is severely restricted in vivo (51).

REMODELING OF GENE REGULATION

Virulence determinants are tightly and coordinately regulated during infection. Virulence-related regulators can sense host signals, e.g., changes in temperature, and then differentially regulate not only virulence genes but other large sets of genes required for adaptation to the host niche (84). A number of virulence-required regulators have been characterized in *Y. pestis* and *Y. pseudotuberculosis* (Table 1), indicating that remodeling of gene regulation contributes to the indicated differences between these two pathogens (Fig. 1).

INTEGRATION OF LATERALLY ACQUIRED VIRULENCE GENES

The global transcriptional regulators cyclic AMP receptor protein (CRP) (49, 79) and RovA (8, 73) are conserved and required for virulence in the three pathogenic *Yersinia* species. In addition, the *Y. pestis* CRP is 98.6% identical to the *Escherichia coli* one with the same length, and CRPs from these two bacteria share an identical consensus box sequence (TGTA-N₆-TCACA) that represents the conserved signals for CRP recognition of promoter DNA (79). Through regulator-pro-

moter DNA interaction in *Y. pestis*, CRP activates two laterally acquired plasmid genes, *pla* and *pst* (30, 79), while RovA up-regulates a genomic region, YPO2272 to YPO2281 (8). The prophage YPO2272 to YPO2281 is acquired by the *Y. pestis* ancestor, and its genome forms an unstable episome in biovars Antiqua and Medievalis whereas it is stably integrated in biovar Orientalis (12, 36). The acquisition of this prophage does not correlate with flea transmission but contributes to virulence in mice (12). These “newly” acquired virulence genes have evolved to integrate themselves into the “ancestral” *Yersinia* regulatory cascade. The plague pathogen integrates laterally acquired genes to coordinate virulence factor expression within global gene regulatory networks to maintain homeostasis through the infectious life cycle.

ADDITION OF LATERALLY ACQUIRED VIRULENCE GENE AND ITS OWN SPECIFIC REGULATOR

The pMT1-borne F1 antigen locus is transcribed as two operons (*caf1M-caf1A-caf1* and *caf1R*) with opposite directions. Caf1R is a positive transcriptional activator responsible for the regulation of capsule formation (29). Thus, the newly acquired *caf* genes constitute a self-controlling regulatory cascade. According to our microarray expression data, CRP activated *caf1R* whereas it repressed the *caf1M-caf1A-caf1* operon (79), and the nucleoid-associated protein Fis stimulates all the *caf* genes (unpublished data). Whether these two *caf* operons are controlled by the host's regulators still needs to be elucidated.

DECAY OF REDUNDANT OR DELETERIOUS REGULATORS/TARGETS

As mentioned above, *Y. pestis* has evolved the genetic inactivation of inhibitory functions (RcsA or NghA) on biofilm formation, which greatly contributes to efficient flea-borne transmission. RovA and VirF positively control *inv* and *yadA*, respectively, while reductive inactivation of these two targets has occurred in *Y. pestis* (see above). However, the upstream regulators RovA and VirF still function to regulate other virulence genes in *Y. pestis*, and both of them are required for virulence of *Y. pestis* (8, 19). The established target of the activator RovA is PsaABC (pH 6 antigen) (8), while VirF stimulates several components (YpkA-YopJ, LcrG/V/D-YopB/D, VirC, YopH, and YopE) of T3SS (77), which appears to be conserved between *Y. pestis* and *Y. pseudotuberculosis*.

PROBABLE DARWINIAN EMERGENCE OF PLAGUE

Y. pseudotuberculosis harbors a set of functional or structural determinants including adhesion/invasin, T3SS, pH 6 antigen, Tc proteins, iron uptake systems, and enzymes for biofilm formation. Thereby, it has the potential to attack mammals to cause systemic infection and to be transmitted by fleas. At a certain stage of history, the change of natural environment might have led to the dramatic increase of population size or to behaviors of a certain rodent, probably the woodchuck (70). This change in animal reservoir might trigger the speciation of *Y. pestis* from *Y. pseudotuberculosis* as a directional positive selection (Darwinian selection). *Y. pseudotuberculosis* is found

widely in the environment and is a common cause of animal infections. The bacteria can invade individual rodents suffering from cold, hunger, or illness due to drastic in-species competition or a harsh environment and then are sucked into the bodies of fleas through flea biting.

Y. pseudotuberculosis or ancestral *Y. pestis* shares a niche with other organisms in rodents and fleas, which might allow the random occurrence of lateral gene transfer. At the same time, gene loss or inactivation occurs randomly as well. Beneficial events of genetic variations would be stabilized by vertical inheritance under Darwinian selection. The positive and directional selection promotes the acquisition of novel virulence determinants as well as the decay of redundant or deleterious functions, which would stimulate the emergence of *Y. pestis*. In addition, remodeling of gene regulation enables the coordinated regulation of existent and newly acquired virulence markers. The Darwinian selective advantages contribute to the demonstrated differences between *Y. pestis* and *Y. pseudotuberculosis* and favor an ordered buildup of specific combinations of virulence determinants enabling the establishment and transmission of *Y. pestis* as a new clone from *Y. pseudotuberculosis*.

ADAPTIVE INTRASPECIFIC MICROEVOLUTION AND DIVERSIFICATION OF *Y. PESTIS*

Y. pestis has been historically divided into three biovars, namely, Antiqua, Medievalis, and Orientalis, and they are thought to be linked with the first to third plague pandemics, respectively. Each biovar has unique genes, a different profile of inactivated genes, and a distinct genome structure according to the relevant sequenced genomes. In addition to the above three classical biovars, another distinct group of strains, called biovar Microtus (83), are avirulent in primates and some large rodent species and are thought to be the intermediate evolutionary clade between *Y. pseudotuberculosis* and *Y. pestis* (36, 72, 81). Compared to other types of *Y. pestis*, biovar Microtus strains have a unique genomic profile of gene loss and pseudogene distribution (36, 72, 81, 83). The specific loss of genes or gene functions documented for this group of strains is thought to be responsible for the human attenuation of these strains, providing candidates for further hypothesis-driven investigations of virulence microevolution of *Y. pestis*.

Y. pestis strains in North and South America are clonally derived from a biovar Orientalis strain due to the third pandemic of human plague in the early 20th century and thus genetically restricted (2). In China, Mongolia, and the former Soviet Union, there are large areas of enzootic plague foci containing genetically diverse strains (2). *Y. pestis* isolates from these plague foci can be classified into many different genotypes (also known as genomovars) (2, 10, 36, 72, 81). Accumulation of genetic variations promotes the diversification of *Y. pestis*, while distribution of *Y. pestis* genotypes is plague focus specific (36, 81). The parallel expansion of plague foci as well as the directional diversification of *Y. pestis* within these foci is likely subject to the action of the complex of interactions between the environment, the hosts, and the pathogen (81). For a specific plague focus, *Y. pestis* genotypes can be assigned into major and minor genomovars (36). Strains of major genomovars represent the dominant populations in a specific plague

focus, and they are generally isolated from the main reservoirs/vectors and distributed throughout the focus. In contrast, the strains of minor ones account for a very small portion of the tested stains from the specific plague focus and are distributed sporadically in a focus or along the border of neighboring foci. Notably, major and minor genomovars make sense in combination with the concept of a specific plague focus. The major genomovar in one plague focus might be the minor one in the others.

In summary, adaptive microevolution likely promotes diversification of *Y. pestis* (different major and minor genomovars) within enzootic foci; major genotypes would play a crucial role in the maintenance of plague in enzootic foci, whereas minor ones (likely representing evolutionary dead ends) would make little contribution to the well-balanced interactions between environment, hosts, and *Y. pestis* (36, 81). This speculation still needs further evidence to show phenotypic effects of gene loss/gain during microevolution of *Y. pestis*.

DARWINIAN ADAPTIVE EVOLUTION VERSUS NEUTRAL GENETIC DRIFT

The driving forces of molecular evolution involve two aspects, namely, “genetic drift” and “natural [or Darwinian] selection.” Genetic drift promotes the accumulation of neutral, random changes in a gene pool, and thus it affects only genotypic frequencies within a population and has no phenotypic causes. Darwinian selection makes alleles more or less widespread in a population due to their effects on fitness advantages; therefore, it influences both phenotype and genotype components in a population. Darwinian selection impels the creation of adaptations, while genetic drift does not. Classical Darwinian evolution refers to the specific selection and fixation of small allelic variants (point mutations) that confer positive evolutionary advantage. It is becoming more and more evident that accumulation of large genomic changes (gene loss/acquisition) can alter the phenotypes in “quantum leaps” during the evolution of bacterial pathogenesis (43). Herein, selection and fixation of inactivation, loss, and acquisition of functional genes are all thought to be under the framework of Darwinian adaptive evolution.

Although evolved from the mildly pathogenic bacterium *Y. pseudotuberculosis*, *Y. pestis* is a highly virulent pathogen and has switched from an enteric lifestyle to a mammalian blood-borne one. For it to become a more virulent pathogen, at least three adaptive evolution mechanisms were involved to gain more pathogenic phenotypes: (i) the horizontal acquisition of genes encoding specific virulence determinants (“gain-of-function” mechanism), (ii) an appropriate functional inactivation or loss of preexisting genetic materials (“loss-of-function” mechanism), and (iii) laterally acquired virulence genes either with or without their own specific regulators, which evolve to being integrated into the host’s regulatory cascades to coordinate expression of virulence factors within global gene regulatory networks for maintaining homeostasis through the infectious life cycle (“regulation-remodeling” mechanism). Genetic variations occur randomly, yet only those beneficial to mammalian blood-borne infection or vector-borne transmission of *Y. pestis* would be stabilized by vertical inheritance under Darwinian selection. Darwinian adaptive evolution (rather than

neutral genetic drift) would induce *Y. pestis* to evolve from *Y. pseudotuberculosis* to a newly emerged pathogen that is not only able to parasitize insects in part of its life cycle but also highly virulent to rodents and humans, causing pandemics of a systemic and often fatal disease.

PERSPECTIVES

Comparison of available genome sequences of *Y. pestis* and *Y. pseudotuberculosis* enables us to find genetic differences in minute detail. Subsequent hypothesis-directed studies have presented a wealth of experimental evidence that promotes our understanding of the positive evolution of virulence during speciation of *Y. pestis*, whereas phenotypic characterization of intraspecific microevolution of *Y. pestis* and its link to expansion of enzootic plague foci is lacking.

Tightly regulated virulence genes are important components of the global gene regulation network, which is a three-dimensional architecture involving various regulators and structural genes. *Y. pestis* has evolved extremely complex signaling and regulatory pathways that are activated during different stages of infection. Global comparative analysis of gene regulation (84) in *Y. pestis* and *Y. pseudotuberculosis* would bring a dynamic and complete picture of virulence and host adaption in *Y. pestis*.

The insertion sequence (IS) elements constitute about 3% of the whole genome in *Y. pestis* (67). Due to IS-mediated homologous recombination, the genome structures of different *Y. pestis* strains are markedly different, although their genome contents and sequences show high similarity (67). There are no reported data for the IS-mediated recombination event that is experimentally linked to the virulence evolution of *Y. pestis*.

An evolutionary “source-sink” model was recently proposed to detect genetic adaptation of bacterial pathogens, with which the evolution of bacterial pathogens can be seen from the angle of continuous switching between permanent (source) and transient (sink) habitats (66). Virulence habitats are marginal sink habitats for some pathogens, and virulence-enhancing genetic adaptation is mostly transient in nature. The adaptation to harsh, or “sink,” environments is the supply of beneficial mutations via migration from a “source” population. Experimental evidence to support the source-sink model for *Y. pestis* needs to be characterized and would provide a conceptual framework for understanding the population dynamics and molecular mechanisms of virulence evolution.

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