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Yersinia pestis—Etiologic Agent of Plague

ROBERT D. PERRY* AND JACQUELINE D. FETHERSTON
Department of Microbiology and Immunology, University of Kentucky, Lexington, Kentucky 40536

"The effects of this natural and human disaster changed Europe profoundly, perhaps more so than any other series of events. For this reason, alone, the Black Death should be ranked as the greatest biological-environmental event in history, and one of the major turning points of Western Civilization." (108)

"Outbreaks of plague in India remind us once again of the need to maintain a core of skills in infectious diseases and the public health infrastructure to detect, monitor, and combat a wide range of disease agents, some new, some revisiting. Plague may have retreated over the past decades, but it has not gone away." (75)

* Corresponding author. Mailing address: Department of Microbiology and Immunology, MS415 Medical Center, University of Kentucky, Lexington, KY 40536-0084. Phone: (606) 323-6341. Fax: (606) 257-8994. E-mail: rperry@pop.uky.edu.

INTRODUCTION

The September to October 1994 outbreak of two plague epidemics in western India was the latest demonstration that plague is not an eradicated disease, a curiosity with importance only to medieval history. Indeed, the official reactions of several countries and the flight of up to 600,000 residents of Surat,

India, show that plague remains one of the most feared of the infectious diseases. This zoonotic disease, with reservoirs on nearly every major continent, exhibits an impressive ability to overcome mammalian host defenses.

Both recent and earlier reviews and minireviews describing different aspects of virulence determinants, bacteriological and epidemiological characteristics, disease characteristics, and clinical treatment of the three human pathogenic species of *Yersinia*—*Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica*—are available (32, 33, 40, 145, 156, 199, 262, 263, 264). This review will focus on the plague bacillus, *Y. pestis*, and attempt to compile and update all aspects of the organism, its epidemiology, the disease it causes, disease treatment and control, and our current understanding of the virulence mechanisms essential for this obligate pathogen.

The literature review for this article ended in June 1996.

HISTORICAL BACKGROUND

> "The European, in the face of the Black Death [1347–1351 epidemic], was in general overwhelmed by a sense of inevitable doom. . . . The doctor might prescribe remedies, but with the tepid enthusiasm of a civil-defence expert advising those threatened by imminent nuclear attack to adopt a crouching posture and clasp their hands behind their necks." (314)
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> "In the autumn of 1665 the spread of the Great Plague caused the closing of the University until the spring of 1667. During those 18 months Newton (sent home from Cambridge) laid the foundations for his famous discoveries in mathematics and physical science." (*Encyclopedia Britannica*, 1970 ed., cited in reference 32)

Although 200 million has been suggested as a credible number for the plague death toll throughout recorded history (81), such absolute numbers can be disputed. However, it is clear that the devastation caused by bubonic and pneumonic plague dwarfs that of most other infectious diseases. Although some of the numerous references to plagues in ancient texts may be attributable to *Yersinia pestis*, the cycles of plague epidemics between 541 and 750 A.D. are called the first pandemic.

First Pandemic

The Justinian plague (A.D. 541 to 544) began in Pelusium, Egypt, after arriving from Ethiopia. It quickly spread through the Middle East and Mediterranean basin with a limited foray into Mediterranean Europe. After that, the 2nd through 11th epidemics (A.D. 558 to 654) occurred in 8- to 12-year cycles (26, 81, 235). Constantinople, for example, experienced epidemics in A.D. 556 to 558, 561, 567 to 568, 573, 577, 586, 618, and 622. This pandemic eventually affected all the "known world"—North Africa, Europe, central and southern Asia (eastern Asia was largely unaffected), and Arabia. The paucity of records and a tendency of contemporary chroniclers to exaggerate death tolls (some reaching a fatality rate of over 130% of the currently accepted population levels) led most historians to avoid assigning numbers. However, death rates between 15 and 40% for specific locales and epidemics have been ventured, and it is estimated that from 541 to 700 A.D. there was a population loss of 50 to 60% (26, 81, 108, 235). Clearly, this depopulation is not solely due to plague, since other epidemics such as smallpox probably occurred during this period. However, a number of economic, religious, and political consequences, including the weakening of the Byzantine Empire, have been attributed in part to this first pandemic (26, 235).

Second Pandemic

From the 8th to 14th centuries, Europe seems to have escaped most epidemic diseases, and it experienced a 300% population increase between the 10th and mid-14th centuries. Plague spread from 1330 to 1346, probably from the steppes of central Asia westward along trade routes, and its introduction into Sicily in 1347 heralded the start of the second pandemic, which again encompassed the "known world." The first epidemic (A.D. 1347 to 1351), which later became known as the Black Death, killed an estimated 17 million to 28 million Europeans, representing ~30 to 40% of that population (108, 314). Despite the high mortality of the Black Death epidemic, the most important effects resulted from the relentless epidemic cycles. In England, national, regional, or London plague epidemics occurred in 2- to 5-year cycles from 1361 to 1480. Most of the epidemics are estimated to have killed 10 to 15% of the population in affected areas, with a few epidemics having mortalities "as low as 5%" (108, 314). Although not as frequent, epidemics continued late into the 17th century, and chronic depopulation was an important characteristic of this period. In addition to plague, smallpox, malaria, dysentery, influenza, typhus, and syphilis made significant contributions to depopulation during this period. The reason(s) for the decline of the first and second pandemics remains unresolved. Various theories, involving changes in weather, rodent populations, public health, and *Y. pestis* itself, are all flawed to some extent (152, 174, 245).

The second pandemic is believed to have accelerated and in some instances directed great changes in economic, societal, political, religious, and medical systems and convictions. In the area of medicine, the Black Death and subsequent epidemics initiated a crisis that stimulated numerous new policies in medical education and practice. These included the advent of clinical research, inclusion of surgeons and surgery in medical education, public health regulations and regulatory boards with enforcement authority, and the development of hospitals that attempted to cure patients, not just isolate them (108, 314).

Third Pandemic and Alexandre Yersin

The third pandemic probably started in 1855 in the Chinese province of Yünnan; troop traffic from the war in that area caused rapid spread of the disease to the southern coast of China. It reached Hong Kong and Canton in 1894 and Bombay in 1898; by 1899 to 1900 steamships had disseminated the disease to Africa, Australia, Europe, Hawaii, India, Japan, the Middle East, the Philippines, North America (the United States), and South America (14, 43, 81, 215). By 1903, in India alone, plague was killing a million people per year, and a total of 12.5 million Indians are estimated to have died of plague between 1898 and 1918. The mortality rate and dissemination of sporadic plague outbreaks that have continued into this century are greatly reduced compared to previous pandemics, largely due to the advent of effective public health measures and, since about 1950, antibiotics. However, this current pandemic, now on the decline, has established stable enzootic foci on every major inhabited continent except Australia (14, 43, 81, 164, 215, 304).

During the Hong Kong epidemic in June 1894, both Alexandre Yersin and Shibasaburo Kitasato independently announced, within days of one another, the isolation of the plague organism. Although Kitasato was initially credited with the discovery, the Yersin bacillus fits the current description of *Y. pestis* and Yersin used antiserum he developed against the organism to cure a plague patient in 1896 (19, 43). Kitasato's

description of his isolate, which he insisted was different from Yersin's organism, included features that were probably due to a contaminating pneumococcus. While Yersin made the connection between rats and plague, Masanori Ogata and Paul-Louis Simond, during the Indian epidemic in 1897, independently discovered the role of the flea in plague transmission (19, 43, 47). In 1897, Haffkine demonstrated the efficacy of his vaccine during an outbreak in Bombay, India (113). During the 1910 to 1911 Manchurian outbreak, L.-T. Wu recognized that the epidemic was the pneumonic form of plague and instituted the use of protective measures against aerosal spread of the disease. Much of our information on the epidemiology and pathology of pneumonic plague stems from the work of L.-T. Wu, R. P. Strong, and others during this epidemic (64, 307). The works of K. F. Meyer and associates advanced our understanding of vaccine and antibiotic efficacy, animal models, and pathology of the disease (43, 51, 176, 177, 178, 179, 180, 181). The studies of M. Baltazard provided early descriptions of the role of resistant or silent enzootic reservoirs in the maintenance and epidemic outbreaks of plague (7, 8, 9, 11).

Y. pestis has undergone several nomenclature changes—*Bacterium pestis* until 1900, *Bacillus pestis* until 1923, *Pasteurella pestis* (after Yersin's mentor), and, finally, *Yersinia pestis* in 1970 (43). A proposal to reclassify *Y. pestis* as a subspecies of *Y. pseudotuberculosis* has not been implemented due to historical as well as unique laboratory and public health safety considerations associated with plague (22, 285).

ETIOLOGY

Bacteriological Characteristics

Biochemical and physiological traits. The genus *Yersinia*, a member of the family *Enterobacteriaceae*, consists of 11 species, of which 3 are human pathogens (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*). The type species, *Y. pestis*, is a gram-negative, nonmotile, non-spore-forming coccobacillus (0.5 to 0.8 μm in diameter and 1 to 3 μm long) that exhibits bipolar staining with Giemsa, Wright's, or Wayson staining. The organism grows at temperatures from 4 up to ~40°C (optimum at 28 to 30°C); the optimum pH for growth ranges between 7.2 to 7.6; however, extremes of pH 5 to 9.6 are tolerated (32, 125, 214, 215). *Y. pestis* has typical cell wall and whole-cell lipid compositions and an enterobacterial antigen, in common with other enteric bacteria. Its lipopolysaccharide is characterized as rough, possessing core components but lacking extended O-group side chains; while there is no true capsule, a carbohydrate-protein envelope, termed capsular antigen or fraction 1 (F1), forms during growth above 33°C (14, 32, 215). This facultative anaerobe possesses a constitutive glyoxylate bypass and unregulated L-serine deaminase expression but lacks detectable adenine deaminase, aspartase, glucose 6-phosphate dehydrogenase, ornithine decarboxylase, and urease activities, as well as a possible lesion in α-ketoglutarate dehydrogenase (32, 33, 125). At all temperatures, *Y. pestis* has nutritional requirements for L-isoleucine, L-valine, L-methionine, L-phenylalanine, and glycine (or L-threonine); these auxotrophies, some of which are capable of reversion, are due to cryptic genes. At 37°C, the organism has additional requirements for biotin, thiamine, pantothenate, and glutamic acid. These metabolic requirements preclude a saprophytic existence; *Y. pestis* is an obligate parasite (32, 33). Diagnostic tests characterize *Y. pestis* as positive by an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) test without acid production from lactose. The Vitek identification system identifies derivatives of strain KIM (Kurdistan Iran man) as ONPG positive, although classical β-galactosidase assays are negative (14, 106, 207). Sulfur requirements are met by cysteine, S^{2-}, $S_2O_3^{2-}$, or SO_3^{2-} but not by L-methionine or SO_4^{2-}. Growth is somewhat slow, generally requiring over 24 to 48 h for colony formation on most enriched media; growth in 5% CO_2 can decrease this incubation period. However, generation times in defined media can be as short as 1.25 h (33, 215, 253).

Wild-type *Y. pestis* organisms can be safely handled in laboratories by standard microbiological methods (215). Processing of potentially infectious clinical specimens and cultures should use biosafety level 2 precautions and containment. Work with a high potential for generating aerosols or with large amounts of the organism, as well as studies using antibiotic-resistant strains, should adhere to biosafety level 3 conditions (276).

Three biotypes (or biovars) of *Y. pestis* are recognized on the basis of conversion of nitrate to nitrite and fermentation of glycerol. Biotype antiqua is positive for both characteristics, orientalis forms nitrite but does not ferment glycerol, and mediaevalis ferments glycerol but does not form nitrite from nitrate. Strains of the three biotypes exhibit no difference in their virulence or pathology in animals or humans (32, 214). DNA macrorestriction patterns of the three biotypes determined by pulsed-field gel electrophoresis support this division (166). However, individual isolates within a strain demonstrate pulsed-field gel electrophoresis heterogeneity, suggesting that relatively frequent spontaneous DNA rearrangements are occurring (110), possibly due to insertion sequences within the genome (see below). Ribotyping identified 16 patterns that can be organized into the three classical biotypes. Two ribotypes (B and O) comprise the majority of strains examined and may be responsible for all three plague pandemics. Guiyoule et al. (110) suggested that antiqua ribotype O caused the first pandemic while the second pandemic was caused by a mutant of this clone unable to reduce nitrate (mediaevalis ribotype O). Geographical locations of strains suggest that orientalis ribotype B initiated the third pandemic (110).

Genome structure, gene transfer systems, and global regulation. Chromosomal DNAs from wild-type *Y. pestis* and *Y. pseudotuberculosis* show a very high degree of relatedness; *Y. enterocolitica* is not as closely related (22, 33). A genome size of ~4,380 ± 135 kb with a 46 to 47 mol% G+C content has been estimated for *Y. pestis* (166, 188). Although there are bacteriophages specifically lytic for *Y. pestis* (6, 10, 14, 32, 214), endogenous bacteriophages and plasmids capable of conjugation and transduction have not been found (33). Most molecular biology techniques are adaptable for use in *Y. pestis*. Genetic transfer has been accomplished with conjugative plasmids (33, 207, 224, 273) and some bacteriophages (P1, Mu hP1, and λ). Lytic growth of these bacteriophages in *Y. pestis* has not been observed (33, 106, 206). Plasmid isolation, as well as transposon and bacteriophage insertional and fusion mutagenesis, is accomplished by standard techniques (106, 206). Although standard transformation of *Y. pestis* is very inefficient, plasmid transfer via electroporation is highly successful (68, 85). Both bacteriophage λ and plasmids with *oriR6K* origins that require the π replication protein (*pir*) for replication have been used as suicide delivery vehicles for allelic exchange of constructed mutations (85, 162, 242, 252).

There are a number of regulatory systems controlling gene expression common to many bacterial species. These include the stringent response to essential nutrient starvation, the SOS response to DNA damage, and the cAMP receptor protein (CRP), which responds to carbohydrate sources. *Y. pestis* demonstrates a normal stringent response from essential amino acid starvation, although recovery from temperature or nutri-

tional shifts is sluggish (33). UV irradiation increases the synthesis of the bacteriocin pesticin (130), suggesting an intact SOS regulatory system. The structural genes for pesticin activity (*pst*) and immunity protein (*pim*) have been sequenced; the promoter region for *pst* exhibits a potential binding site for the LexA repressor of the SOS system (209). A protein cross-reacting with antibody against the *Salmonella typhimurium* flagellar regulatory protein FlgM (provided to Scott Minnich by Kelly Hughes) is highly expressed at 25 and 37°C. Thus, expression of the cryptic flagellar class I, II, and III genes (139, 140) of nonmotile *Y. pestis* may be constitutively repressed. Finally, a putative CRP-binding sequence has been identified in at least one *Y. pestis* gene, indicating that a catabolite repression system is probably present (254). Most of the research on *Y. pestis* regulatory systems has focused on virulence genes (262) and will be discussed below in the section on virulence determinants.

The majority of *Y. pestis* strains regardless of biotype or origin contain three plasmids of 9.5, 70 to 75, and 100 to 110 kb (21, 83, 92, 156, 164, 207). A cryptic fourth plasmid (~33 kb) was present in 8% of strains in one survey (92), and strains with a variety of different plasmid sizes and content have been identified (83, 225, 273). Some of these plasmids are probably deletion, multimeric, or recombination products of the three prototypical plasmids. In strain KIM derivatives, these three plasmids are termed pPCP1 (pesticin, coagulase, plasminogen activator), pCD1 (calcium dependence), and pMT1 (murine toxin). Altered plasmid profiles in KIM derivatives were obtained by selection on magnesium oxalate plates to cure pCD1 (33, 106) or growth at 4°C to isolate pPCP1- and pMT1-negative derivatives (33, 86) (Fig. 1A). Given the differences in these three plasmids among the different strains surveyed (see below), we have numbered plasmids to identify the strain of origin (e.g., plasmids in strain Kuma were designated pMT2 and pPCP2, while those in strain M23 were designated pMT12, pCD12, and pPCP12) (206). Other researchers use alternative names for each of the plasmids (see below).

Plasminogen activator (*pla*), the bacteriocin pesticin (*pst*), and a pesticin immunity protein (*pim*) are encoded on the 9.5-kb pPCP1 (Fig. 1A and B). Both the size and genetic organization of this plasmid appear remarkably consistent in other strains (where it is often termed pPst) (83, 92, 172, 173, 207, 247, 248); however, one restriction enzyme site difference has been observed between the plasmid in KIM derivatives and that in strain 106 Otten (212) (Fig. 1B). Some natural isolates lack this plasmid, and one pesticin-overproducing strain possesses a multimer of the plasmid (83, 92, 207).

The low-calcium response stimulon (LCRS) is encoded on pCD1 (Fig. 1A and C) (termed pCad or pYV in some other strains and in the enteropathogenic yersiniae, *Y. pseudotuberculosis* and *Y. enterocolitica*) and includes regulatory genes controlling the expression of secreted virulence proteins and a dedicated multiprotein secretory system. Different strains have plasmids that range in size from 68 to 75 kb; however, the LCRS region is highly conserved (92, 214, 264). LCRS plasmids are necessary for virulence in all three human pathogenic *Yersinia* species (33, 214, 246, 264). Natural LCRS-negative mutants occur by plasmid loss, plasmid deletions that can encompass 70 kb, disruption by an insertion sequence (IS), or mutations causing no alteration detectable by restriction endonuclease analysis (90, 206, 207, 219). Transformation with F-*lac* has caused integration of an LCRS plasmid into the chromosome with loss of LCRS functions (315); integration has also been observed without F-*lac* (225).

Murine toxin (Ymt) and the structural gene for fraction 1 (F1) protein capsule or envelope and its putative regulatory and assembly genes are encoded on the largest plasmid (Fig. 1A and D; pMT1 in KIM strains, and pFra in other strains) (63, 141, 156, 224). The size of this plasmid can vary dramatically, ranging from a 60-kb deleted version in strain Dodson (206) to ~90 to 280 kb in other strains (92). In a number of strains, the plasmid has integrated into the chromosome (95, 207, 225, 287). *Y. pestis* KIM11 and KIM13 (Fig. 1A) both have at least a portion of pMT1 integrated into their chromosome but still express murine toxin and F1 (207). In other strains, synthesis of these two factors from the integrated state ranges from unaffected to complete loss of expression (95, 207, 225, 287). Integration can occur at different sites, and excision of the integrated plasmid has been noted (95, 207, 225, 287).

Portnoy and Falkow (219) discovered an active IS element they termed IS*100*. In KIM derivatives, up to 30 copies of IS*100* reside in the genome. One copy is present in pPCP1 (Fig. 1B) and in pCD1 (in the *ypkA-yopJ-yopH* region encoding some of the genes for secreted virulence proteins—*Yersinia* outer proteins [Fig. 1C]), and 2 copies are found in pMT1 (88, 207). The DNA sequence of IS*100* in several strains has been determined by examining various insertion sites (91, 212, 271). In *Y. pestis* 358, a second active IS element, IS*285*, has been identified and sequenced (91, 225); a similar element resides in KIM strain derivatives (16). Finally, an IS*200*-like element disrupting the invasin (*inv*) gene of *Y. pestis* 6/69M has been sequenced (240).

In KIM6+, a 102-kb region of the chromosome termed the pigmentation (*pgm*) locus (Fig. 2A) is bounded by directly repeated copies of IS*100*, which are probably causing deletion of this region via homologous recombination (86, 88) with an observed frequency of 10^{-5} (30). This deletable region, representing ~2.3% of the genome, is not a giant mobile genetic element, since an open reading frame (ORF) showing homology to *Escherichia coli phoE* (structural gene encoding a porin) has been disrupted by one of the *pgm*-associated IS*100* elements (Fig. 2A) (cited in reference 86). Surveys involving 18 different strains found the deletion to be consistent in 16 strains (86, 88, 166). A separate survey of entirely different strains found a significant percentage with alternate and possibly sequential deletions (133). Thus, the deletion event appears to be highly consistent in some strains but variable in others. The variability could arise in the distribution of IS*100* or other IS elements, allowing alternative deletions. These deletions and the presence of active IS elements suggest that insertions, inversions, and other deletions may occur during the propagation of strains.

Proven and Putative Virulence Determinants

The life cycle of *Y. pestis* (see below) suggests that virulence determinants necessary for survival in mammals and in fleas are likely. We would define virulence determinants as factors that promote bacterial survival, growth, and/or transmission by directly affecting the host through adherence, thwarting host defense responses, disrupting cellular metabolism, or acquiring essential nutrients from the host. Thus, we would not classify purine biosynthetic enzymes, although required for virulence of *Y. pestis* (32, 33, 40) and other bacteria, or other internal metabolic enzymes as virulence determinants. While a number of virulence systems are common to all three human pathogenic yersiniae, *Y. pestis* and the enteropathogenic yersiniae each possess unique factors (33, 199, 262, 264). The enteropathogenic virulence determinants YadA (*Yersinia* adherence protein, formerly YopA) and Inv are not functional in the *Y. pestis* strains examined (190, 234, 236, 240, 244). Although *Y. pestis* possesses a possible accessory invasion locus (*ail*) ho-

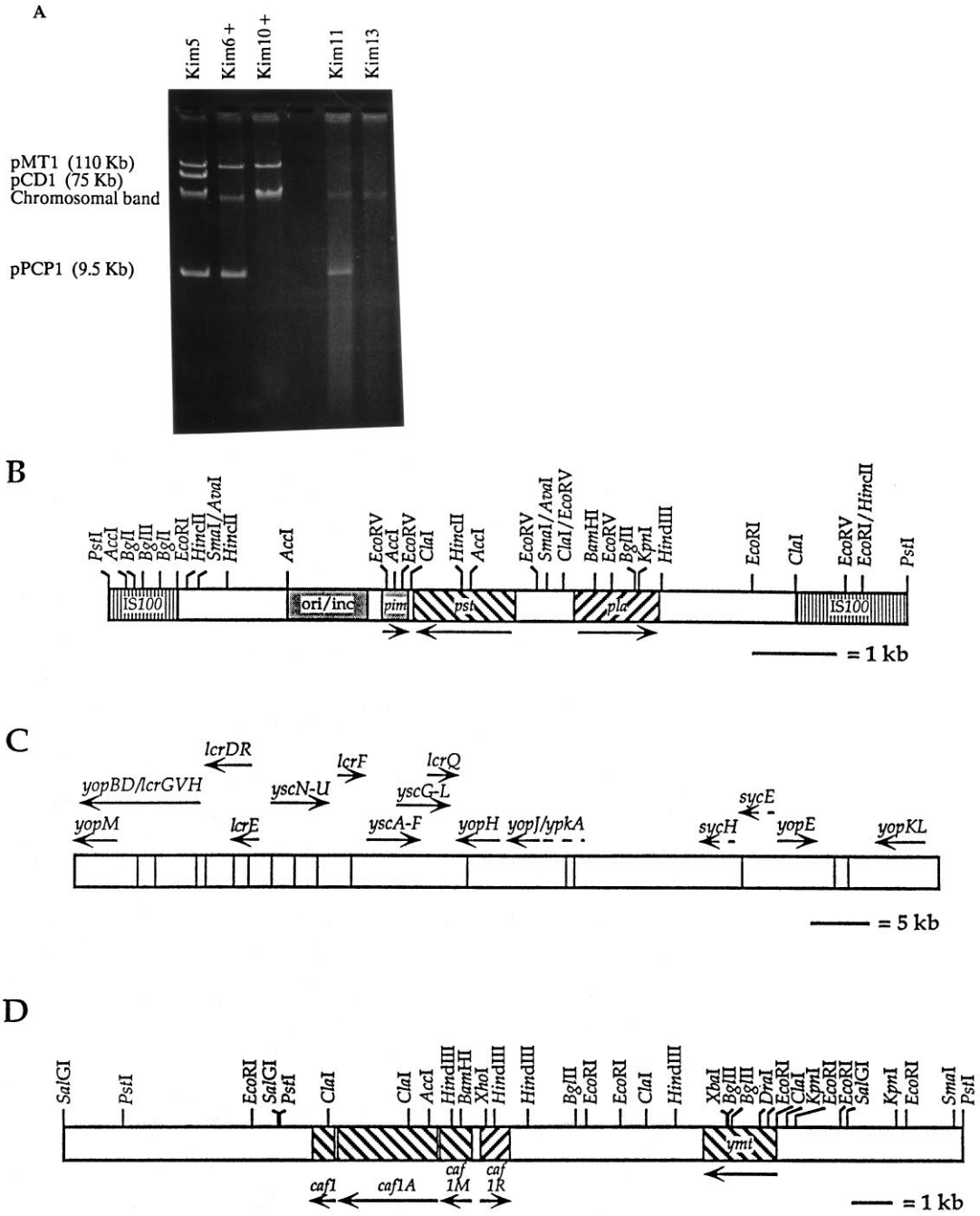

FIG. 1. Plasmids in *Y. pestis* KIM strains. (A) Plasmid profiles of *Y. pestis* KIM5 and derivatives cured of pMT1, pCD1, and/or pPCP1. Plasmids were isolated, separated by electrophoresis through 0.7% agarose, and visualized by staining with ethidium bromide as previously described (206, 253). Although KIM11 and KIM13 derivatives are apparently missing pMT1, at least part of the plasmid DNA is integrated into the chromosome (207). Selected restriction endonuclease sites and genes of plasmids pPCP1 (panel B), pCD1 (panel C), and pFra (panel D) are shown, with arrows indicating the direction of transcription. The largest plasmid is called pMT by some investigators and pFra by others. Dashed lines with arrows indicate that the corresponding pCD1 genes and/or direction of transcription has been determined in *Y. pseudotuberculosis* but not *Y. pestis*. For pCD1, vertical lines denote *Hin*dIII restriction sites. Due to space limitations, pCD1 operon sizes are not proportional to the space occupied. Since no genetic mapping has been performed with pMT1, a 22-kb region of pFra plasmids from several *Y. pestis* strains is depicted to localize murine toxin (*ymt*) and fraction 1 capsule (*caf*) genes. Maps were compiled from references cited in the text.

molog (185), it is disrupted by IS*285* (272). In *Y. pestis* KIM6+, a potential homolog of *ymoA* (*Yersinia* modulator), which modulates the expression of several temperature-regulated genes in enteropathogenic yersiniae (262), is disrupted by the IS*100* element bordering the right-hand end of the *pgm* locus (Fig. 2A) (87). Whether functional *ymoA* homologs also exist in this or other strains of *Y. pestis* has not been examined.

Routes of *Y. pestis* infection other than the aerosol route in mice do not significantly alter 50% lethal doses (LD_{50}s) (33, 34, 287, Table 1). However, the increased LD_{50}s of cells grown at 25°C compared to those of cells grown at 37°C highlight the temperature regulation of a number of virulence determinants (54) (Table 1). Although in vitro conditions for the expression of a number of *Y. pestis* virulence determinants are defined

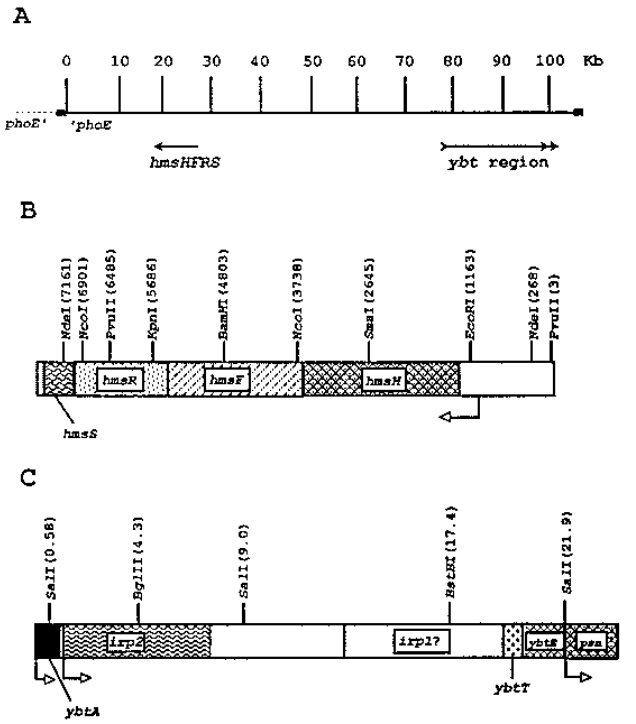

FIG. 2. The *pgm* locus of *Y. pestis* KIM6+. (A) The entire locus is shown, with the location and direction of transcription of *hmsHFRS* and iron-regulated genes (*ybt* region) indicated by lines with arrows. Solid boxes indicate the two IS*100* elements, while the dashed line represents DNA outside the locus that remains in *Y. pestis* KIM6 after the deletion event. The disrupted *phoE* homolog is depicted as *phoE'* and *'phoE* around the left-hand IS*100*. (B and C) The expanded *hmsHFRS* operon (B) and *ybt* region (C) are depicted with ORFs in boxes. Selected restriction endonuclease site locations are indicated in base pairs (B) or kilobases (C). Open arrows signify identified promoters. The designated *irp1* ORF is tentative. Although not clearly depicted, the *hmsF* and *hmsR* ORFs overlap slightly.

(262), additional in vivo parameters may allow the expression of some determinants under unexpected circumstances.

Y. pestis is a facultative intracellular pathogen that is now thought to maintain intracellular residence only during the early stages of infection, with extracellular growth being predominant at later stages (33, 257, 264). Nevertheless, virulence determinants to combat intracellular as well as extracellular defenses would be essential. Some determinants have not been directly tested for their role in virulence, while results with others are ambiguous due to the use of undefined mutants or double mutants. In addition, a mutant strain may demonstrate widely different degrees of virulence loss depending upon the type of animal infected (32, 34, 40, 181). Finally, studies on virulence determinants generally use laboratory-passaged strains that may have accumulated undetected alterations. However, these putative differences have not, to date, adversely affected virulence in the mouse model. Table 2 shows the results of LD_{50} testing in mice with the most definitive mutations available in various determinants.

Pigmentation and iron assimilation. Iron is well established as an essential nutrient that is chelated by mammalian proteins, making it less available to invading pathogens (36, 184, 199). Early studies by Jackson and Burrows (134, 135) defined exogenous iron-independent virulence as part of the pigmentation phenotype. Pigmented (Pgm^+) cells of *Y. pestis* strains do not produce a pigment but adsorb large amounts of exogenous hemin that cause formation of "pigmented" colonies at

TABLE 1. Effect of growth temperature and route of infection on LD_{50}s for mice[a]

Strain	LD_{50} of cells incubated for 18 h at:		Route	Fold increase in LD_{50} (25°C/37°C or i.v., i.p., or aerosol/s.c.)
	25°C[b]	37°C[b]		
195/P	383	18		21.3
BAC001884[c]	157	3		52.3
751207[d]	390	9		43.3
752675[d]	166	19		8.7
Mean of above	274	12.3		22.3
Alexander		6.1	s.c.	
Alexander		9.9	i.p.	1.6
Alexander		8.1	i.v.	1.3
C092		1.9	s.c.	
C092		4×10^4	Aerosol	2.1×10^4

[a] Compiled and adapted from references 34, 54, and 287.
[b] Incubation temperatures simulate flea (25°C) versus pneumonic (37°C) route.
[c] Isolates from human pneumonic plague patients.

26 but not 37°C and are virulent in mice. Spontaneous nonpigmented (Pgm^-) mutants are avirulent unless hemin, ferrous sulfate, or ferric chloride is injected into the mouse with the bacterial challenge. This was the first demonstration that in vivo iron availability and the ability of the pathogen to acquire host iron affects the severity of infection and disease. Spontaneous Pgm^- mutants, which occur with a frequency of $\sim 10^{-5}$ (30), lose not only hemin adsorption mediated by several unique proteins (197, 198, 239, 260) but also the ability to grow in iron-chelated media at 37°C (85, 238, 239), sensitivity to the bacteriocin pesticin (Pst^s) (30, 274), and expression of a number of iron-repressible polypeptides (85, 86, 239). A greatly increased *pgm* locus deletion frequency is observed in iron-surplus cultures of a *Y. pestis fur* mutant. In the presence of excess iron, Fur (ferric uptake regulation) generally represses the expression of iron-regulated genes. The increased frequency of Pgm^- mutants in cultures of iron-replete *Y. pestis fur* mutants may reflect the toxic consequences of overexpression of an iron transport system encoded within the *pgm* locus (252, see below).

In *Y. pestis* KIM6+ (a + indicates a Pgm^+ strain), conversion to Pgm^- strain KIM6 involves the deletion of ~102 kb of chromosomal DNA. For a number of different strains and independent mutants within individual strains, this deletion appears to be constant in size and location (88, 166, 206) (Fig. 2A). The deletion event may be mediated by recombination between two IS*100* elements in the same orientation bordering the 102-kb *pgm* locus (86, 88) (Fig. 2A). The locus necessary for hemin adsorption within the *pgm* locus has been cloned and given the designation *hms* (hemin storage) (197, 198, 206) (Fig. 2A and B) to differentiate this trait from the other characteristics of the Pgm^+ phenotype. In two strains that we have examined (86, 88) and in a different set of strains analyzed by Iteman et al. (133), different deletion events were detected; Hms^-/*irp2*$^+$ (iron-regulated protein 2 gene [Fig. 2]) mutants accounted for 27% of Hms^- strains examined by Iteman et al. (133). These results suggest that the majority of deletions involve homologous recombination between IS*100* elements flanking the 102-kb *pgm* locus. However, different deletion events or successive deletions can occur possibly due to additional IS*100*s or other repetitive sequences (88, 133). Given this complexity, we use the term Pgm^+ to indicate strains that

TABLE 2. Mouse LD_{50} studies with *Y. pestis* harboring mutations in putative virulence genes

Strain[a]	Virulence characteristics[b]						Route	LD_{50}	Reference
	Lcr	Pgm	Pla	F1	Ymt	pH 6			
CO92-C11	+	(+)	?	+	?	?	s.c.	2.8	306
CO92-C12 (*caf1*)	+	(+)	?	−	?	?	s.c.	9.2	306
EV76	+	Δ*pgm*	(+)	+	+	?	i.p.[c]	<10	80
EV76(*caf1*::*luxAB*)	+	Δ*pgm*	(+)	−	+	?	i.p.[c]	<10	80
EV76(*ymt*::*luxAB*)	+	Δ*pgm*	(+)	+	−	?	i.p.[c]	~50–100	80
231(708)	+	Hms^+	+	+	+	?	s.c.	4.6	79
231pFra/pFS23 (*caf1A*::*kan*)	+	Hms^+	+	−	+	?	s.c.	6.4	79
231pFra$^-$	+	Hms^+	+	−	−	?	s.c.	7.9	79
231pPst$^-$	+	Hms^+	−	+	+	?	s.c.	~1	79
231Psb$^-$	+	Hms^-	+	+	+	?	s.c.	6.5	79
358	+	Hms^+	+	+	+	?	s.c.	2	155
358/12	+	Hms^+	+	−	−	?	s.c.	1	155
358/15	+	Hms^+	−	−	−	?	s.c.	4	155
358/24	+	Hms^-	−	−	−	?	s.c.	10^8	155
M-231	+	Hms^+	Pst^+	?	?	?	s.c.	10	154
KM130	+	Hms^-/Pst^s	Pst^+	?	?	?	s.c.	10	154
KM130Pst^r	+	Hms^-/Pst^r	Pst^+	?	?	?	s.c.	$>10^8$	154
KIM5+	+	(+)	(+)	(+)	(+)	(+)	s.c.	<10	274
KIM6+ (pCD1−)	−	(+)	(+)	(+)	(+)	(+)	s.c.	$>10^7$	274
KIM5	+	(Δ*pgm*)	(+)	(+)	(+)	(+)	s.c.	$>10^7$	274
KIM5	+	(Δ*pgm*)	(+)	(+)	(+)	(+)	i.v.	15	274
KIM1001	+	(+)	+	(+)	(+)	(+)	s.c.	42	250
KIM1002 (pPCP1$^-$)	+	(+)	−	(+)	(+)	(+)	s.c.	8.8×10^6	250
KIM1008 (Δ*pla*)	+	(+)	−	(+)	(+)	(+)	s.c.	6.0×10^7	250
KIM1003 (*pst*::Tn*5*)	+	(+)	+	(+)	(+)	(+)	s.c.	33	250
KIM5-3001 (Sm^r)	+	(Δ*pgm*)	(+)	(+)	(+)	+	r.o.[d]	42	162
KIM5-3001.1 (Sm^r, (*psaA3*::*m*-Tn*3*)	+	(Δ*pgm*)	(+)	(+)	(+)	−	r.o.	9×10^3	162
KIM5-3233 (Sm^r, *yopM*::*lacZYA*)	YopM$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	3.4×10^5	158
KIM5	+	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	80	258
KIM5 (pCD1$^-$)	−	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	$>3.8 \times 10^6$	258
KIM5-3022 (*yopE*::Mu dI1*b*::Tn*9*)	YopE$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	$>8.5 \times 10^5$	258, 261
KIM5 (*yopH*::Mu dI1*b*::Tn*9*)	YopH$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	$>1.0 \times 10^6$	258
KIM5-3031 (*yopK*::Mu dI1*b*::Tn*9*)	YopKL$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	2.5×10^5	258, 261
KIM5-3073 (*yopJ*::Mu dI1*b*::Tn*9*)	YopJ$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	120	258, 261
KIM5-3173 (*yopJ*::MudI1734)	YopJ$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	420	220
KIM5-3241.2 (*yopJ*::MudI1734 Δ*lcrV*)	YopJ$^-$ LcrV$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	5×10^6	220
KIM5-3173 (*yopJ*::MudI1734 *lcrH*::*cat*)	YopJ$^-$ LcrH$^-$	(Δ*pgm*)	(+)	(+)	(+)	(+)	r.o.	420	220

[a] Where identified, relevant mutations are noted in parentheses.
[b] ( ) indicates characteristics determined in a separate publication or in the parental strain. For Lcr, a plus sign indicates LCRS plasmid detected and positive for Ca^{2+}-deficient growth restriction and/or synthesis of LcrV or Yops. For Pgm, a plus sign indicates that the 102-kb *pgm* locus was present and function tests for hemin storage and iron-repressible proteins were performed; Δ*pgm* indicates deletion of the entire 102-kb *pgm* locus; Pst^s and Pst^r indicate sensitive and resistant, respectively, to the bacteriocin pesticin, a presumptive test for the functional pesticin/yersiniabactin receptor, Psn; Hms^+ and Hms^- indicate positive and negative, respectively, for hemin or Congo red adsorption; iron-regulated traits of *pgm* locus were not tested. For Pla, a plus sign denotes detection of plasmid and Pla activity; Pst^+ indicates production of pesticin, presumptive evidence for presence of Pla-encoding plasmid. For Fl, Ymt, and pH6, a plus sign indicates that fraction 1, murine toxin, and pH6 antigens, respectively, were detected.
[c] i.p. injection of bacterial cells plus 0.4 mg of iron.
[d] r.o., retro-orbital injection (equivalent to i.v. injection).

are pesticin sensitive (Pst^s) and Hms^+ and express the iron-regulated proteins defined as part of this phenotype. Pgm^- refers specifically to strains with the characteristic 102-kb deletion.

(i) Hemin storage. An Hms^+ phenotype is manifested by the formation of dark greenish brown or red colonies on hemin agar or Congo red agar, respectively (134, 265). Congo red has a three-dimensional configuration resembling hemin (149). The cloned *hmsHFRS* locus, which contains four structural genes encoding mature proteins of 89.5 kDa (HmsH), 72.2 kDa (HmsF), 52 kDa (HmsR), and 17.5 kDa (HmsS) (Fig. 2B), restores an Hms^+ phenotype to Pgm^- deletion mutants (160, 197, 198, 206). HmsH and HmsF are acidic surface proteins (pIs of 5.18 and 5.25) with precursor weights of 93.4 and 74.6 kDa (160, 197, 198, 206) (GenBank accession number U22837) (Fig. 3A). No significant homologies to any of the four Hms proteins have been identified in the database. Thus, the Hms^+ phenotype of *Y. pestis* is apparently distinct from Congo red and hemin binding reported in a number of other organisms. The *hmsHFRS* operon appears to be transcribed from a single promoter, with levels of HmsH and HmsF being higher at 26°C than 37°C. Either HmsR (pI 10.83), HmsS (pI 6.68), or both may be regulatory proteins since polar insertion into *hmsR* reduces the levels of HmsH and HmsF proteins expressed.

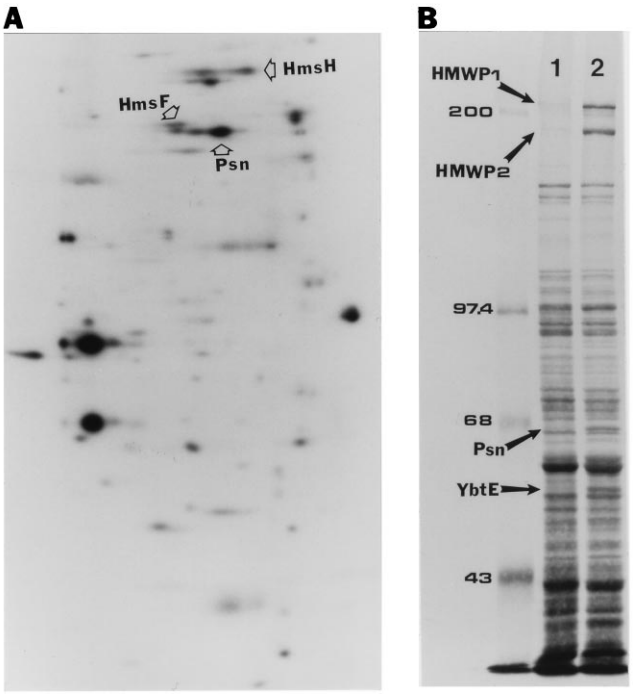

FIG. 3. Surface-exposed and/or iron-regulated proteins encoded within the *pgm* locus of *Y. pestis*. Cells were grown and labeled and proteins were separated as previously described (86). (A) Fluorograph of ^{125}I surface-labeled proteins separated by two-dimensional gel electrophoresis (isoelectric focusing followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] [12% polyacrylamide]). *Y. pestis* KIM6+ cells were grown in a chemically defined, iron-deficient medium (PMH) at 26°C. (B) Fluorograph of ^{35}S-labeled proteins electrophoresed on a 9% polyacrylamide gel containing SDS. In the unmarked lane, molecular masses of protein standards are indicated in kilodaltons. Iron-regulated polypeptides apparent in this gel are labelled. Although the *irp2* sequence predicts a 228-kDa HMWP2 gene product (109), this protein consistently migrates on SDS-PAGE as a 190-kDa product (48, 49, 85, 86). *Y. pestis* KIM10+ cells were grown at 37°C in PMH in the presence (lane 1) or absence (lane 2) of 10 μM ferric chloride.

While *hmsHFRS* is essential for an Hms$^+$ phenotype, it is not sufficient; *Escherichia coli* strains carrying the cloned *hmsHFRS* locus remain Hms$^-$. Genes involved in temperature regulation of the Hms$^+$ phenotype appear to lie outside of the *pgm* locus (160, 197, 198).

Nearly all the hemin adsorbed by Pgm$^+$ cells at 26°C is stored intact in the outer membrane. KIM6+ cells cultivated at 26°C with hemin adsorbed 317- and 51-fold more hemin in their outer membranes than did KIM6+ grown at 37°C or Pgm$^-$ KIM6 cells, respectively. Outer membrane concentrations of inorganic iron exhibited similar patterns with respect to the cultivation temperature (25.5-fold for 26°C compared to 37°C) and phenotype (5-fold for Pgm$^+$ compared to Pgm$^-$) (205). However, the physiological relevance of increased hemin or iron adsorption has not been established.

It is clear that expression of the Hms$^+$ phenotype is essential in fleas. By using defined mutations (197, 198), it has been established that a functional *hms* locus is necessary for blockage and death in fleas (118). Other researchers using undefined Hms mutations have also found an effect on blockage in fleas (25, 154). Flea blockage (see below) is essential for efficient transmission of plague from fleas to mammals (37, 50, 54, 143). Thus, these studies have demonstrated the importance of an Hms$^+$ phenotype in disease transmission. However, the physiological mechanism causing blockage of fleas remains undetermined.

While Hms is essential in the flea, its role in mammalian infection is less certain. Virulence testing of undefined Hms mutations in mice have yielded results ranging from little or no attenuation to complete avirulence (79, 118, 133, 154, 155) (Table 2). This variation probably reflects the range of undefined mutants used, from point mutations in *hms* genes to 102-kb deletions. Mutants showing negligible loss of virulence may be caused by mutations in *hms* genes and not large deletions. While this indicates that the Hms$^+$ phenotype has no role in the mammalian disease process, there are two caveats to this conclusion. First, undefined mutants were used, and second, cells were grown either without hemin for adsorption or under conditions that would reduce expression of the Hms proteins.

If there is a role for Hms in virulence in mammals, possible functions in the initial stages of infection include (i) increased uptake into eukaryotic cells, (ii) increased survival in phagocytic cells by inactivation of killing mechanisms, and/or (iii) use of stored hemin as a nutritional source of iron. Recent in vitro experiments seem to eliminate the nutritional use of hemin stored by the Hms system during subsequent periods of iron starvation. However, Hms$^+$ cells with stored hemin or inorganic iron displayed a modest increase in resistance to in vitro killing by superoxide, nitric oxide, and hydrogen peroxide; whether this is physiologically relevant in vivo is undetermined (161). Further studies are required to determine if Hms plays a role in uptake into eukaryotic cells or has some other function in mammals.

(ii) Yersiniabactin transport and biosynthesis. Wake et al. (280) first detected putative siderophore activity in *Y. pestis*. A siderophore is defined as a small, nonproteinaceous compound (usually <1,000 Da) that is synthesized and secreted by the bacterium to acquire iron from the environment. Siderophores have extremely high affinities for ferric iron and are usually capable of removing iron from transferrin, lactoferrin, and ferritin (184). Although the existence of a *Y. pestis* siderophore (280) was not confirmed initially (200), it is now clear that this organism synthesizes at least one siderophore (85, 207, 281).

A number of studies suggest that *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* have a nearly identical siderophore-dependent iron transport system (48, 49, 85, 109, 165, 229, 239), and we will use results from all three organisms to draw a picture of the *Y. pestis* system. The siderophore yersiniabactin (Ybt), purified from *Y. enterocolitica*, has a molecular mass of 482 Da and contains aromatic and nonaromatic iron-chelating groups (111). Under some in vitro culture conditions, it displays relatively weak iron binding, since the growth of *Y. pestis* is readily inhibited by the iron chelator ethylenediamine-di-*o*-hydroxyphenylacetic acid (EDDA) (128, 253). The synthesis of yersiniabactin requires *aroA* (*Y. enterocolitica* [116]); *irp2* (*Y. pestis* [85]), which encodes a 228-kDa protein with homology to the siderophore biosynthetic enzymes AngR and EntF (*Y. enterocolitica* [109]); and probably at least three other genes—*irp1*, *ybtT*, and *ybtE* (15) (Fig. 3B). *irp2*, *irp1*, *ybtE*, *ybtT*, and pesticin receptor genes (*psn*) all lie within the *pgm* locus (15, 85, 86) (Fig. 2A and C). The function of *irp1*, which encodes another large iron-regulated protein, is unknown (15, 48, 85). *ybtT* and *ybtE* show strong homologies to siderophore biosynthetic genes for anguibactin and enterobactin, respectively (15) (GenBank accession number U50364). Transport of the iron or iron-yersiniabactin complex back into the bacterial cell requires TonB (*Y. enterocolitica* [111, 116]) and a surface receptor, termed *psn* in *Y. pestis* and *fyuA* in *Y. enterocolitica*, that also confers Pst^s (85, 111, 154, 229, 239) (Fig. 3). Putative inner membrane, periplasmic, and cytoplas-

mic components of this transport system have not been identified and may lie outside of the *pgm* locus (165).

The Hms and yersiniabactin biosynthetic/transport systems together encompass all of the identified characteristics unique to Pgm^+ cells of *Y. pestis*. The drastic loss of virulence in Δ*pgm* mutants (Table 2) is now ascribed primarily to loss of the yersiniabactin system. Strains with mutations in *irp2*, *psn*, and *ybtE* are unable to synthesize or transport yersiniabactin and unable to grow in iron-chelated media at 37°C (15, 85, 165, 238, 239). The yersiniabactin system probably functions at 26°C as well as 37°C, since several of the proteins are expressed at both temperatures; however, *Y. pestis* cells cultivated at 26 to 30°C are insensitive to moderately sensitive to pesticin compared to cells grown at 37°C (35, 252). As expected, expression of *irp2*, *irp1*, and *psn* is Fur regulated (see below), such that repression occurs under iron-surplus conditions (252) (Fig. 3B). In addition, yersiniabactin and a transcriptional activator appear to be required for maximal expression of some components of the biosynthetic and transport systems even under iron starvation conditions (15, 84, 85; see below).

(iii) Other iron and hemin transport systems. In addition to the yersiniabactin system, *Y. pestis* seems to possess other 26 and 37°C inorganic iron transport systems and a system for utilizing hemin and heme-containing compounds (128, 165, 199, 238, 239). Pst^r and Δ*pgm* mutants still possess a reduced ability to acquire iron at 37°C from iron chelators such as citrate, pyrophosphate, and nitrilotriacetic acid, suggesting the presence of a transport system independent of yersiniabactin. At 26°C, the ability of Pgm^+ and Pgm^- cells to acquire iron from conalbumin, desferal, citrate, pyrophosphate, and nitrilotriacetic acid is enhanced compared to cells at 37°C; thus, an iron transport system functioning at 26 but not 37°C probably exists. While components of these two putative iron transport systems have not been elucidated, several temperature- and iron-regulated proteins are likely candidates (166) (Table 3). We have cloned and sequenced a *Yersinia* Fe uptake operon (*yfeABCD*) that restores growth to an *E. coli* strain unable to grow in the presence of iron chelators. Genes in the *yfe* operon are members of the ABC transporter family and are highly homologous to a *Haemophilus influenzae* operon that encodes at least one iron-repressible protein (16). Whether this system is separate from or part of either of the two putative inorganic iron transport systems remains to be determined. Finally, a *Y. pestis* hemin utilization system (*hmu*) that appears to transport the entire hemin moiety into the cell before removing the central iron atom is being characterized (128). *Y. pestis* KIM6+ and KIM6 utilize hemin, hemoglobin, and myoglobin, as well as complexes of heme-albumin, heme-hemopexin, and hemoglobin-haptoglobin, as sole iron sources (128, 200, 239, 253). Deletion of the ~7-kb *hmu* region results in a mutant unable to utilize any of the above heme-compounds. Sequencing of the *hmu* region (129) (GenBank accession number U50364) indicates a high degree of homology to a *Y. enterocolitica* system for hemin uptake (*hemPRSTUV*) (255, 256).

(iv) Iron regulation. In common with numerous other gram-negative bacteria, *Y. pestis* possesses a ferric uptake regulation (Fur) system. Upon chelating ferrous iron in its C-terminal portion, Fur binds to a characteristic sequence in the promoter region of affected genes and prevents transcription. Under iron starvation conditions, cytoplasmic iron is unavailable for binding to Fur, and Fur-regulated operons are expressed at high levels. *Y. pestis* Fur is a ~16-kDa protein that exhibits >84% homology to *E. coli* Fur. *Y. pestis fur* is expressed from a monocystronic operon that is autoregulated and possesses a CRP-binding site (253, 254). By repressing the synthesis of iron transport systems, this regulator prevents intracellular accumulation of excessive levels of iron. A *Y. pestis* 620-kDa putative bacterioferritin, with a 19-kDa polypeptide as the likely subunit, would store excess intracellular iron under these conditions (205).

A *fur*::*kan-9* mutation causes constitutive maximum expression of at least 17 normally iron-repressible proteins including the gene products of *psn*, *irp1* (HMWP1), and *irp2* (HMWP2) (252). The promoters for *fur*, *psn*, *ybtA*, *irp2*, *yfeA-D*, and *hmu* genes all contain Fur-binding sequences (15, 16, 84, 85, 129, 254). In addition, Fur is required for full expression of four proteins under iron-surplus conditions (252) (Table 3). Whether Fur functions as an activator or represses a repressor of the operons encoding the structural genes for these four proteins is undetermined.

The *fur*::*kan-9* mutant exhibited two other interesting traits. The first is loss of temperature regulation of the Hms^+ phenotype; Fur^- cells are Hms^+ at 37°C (252). The mechanism for this is unexplained since the *hmsHFRS* promoter region contains no characteristic Fur-binding sequence (160). Second, growth of the Pgm^+ Fur^- mutant with surplus iron caused dramatic growth inhibition and nearly 100% conversion of the culture population by late log phase to Pgm^- deletion mutants. The current hypothesis for this phenomenon is that overexpression of the yersiniabactin system, partially encoded within the 102-kb *pgm* locus, causes accumulation of iron to toxic levels; spontaneous Δ*pgm* mutants survive and overgrow the inhibited or dead Pgm^+ population (252).

While Fur is an important global regulator, other iron-responsive regulators are probably present in *Y. pestis*. At least nine iron-repressible (Arps; auxiliary iron repressible proteins) and two iron-inducible (Aips; auxiliary iron inducible proteins) proteins appear to be regulated in a Fur-independent manner in *Y. pestis*; a hypothetical auxiliary iron-responsive regulator of these proteins has been tentatively termed AirX (252, 262) (Table 3). Similar to the pyochelin siderophore-dependent transport system in *Pseudomonas aeruginosa*, which requires PchR, a transcriptional regulator, and the pyochelin siderophore for maximal expression of pyochelin biosynthetic and receptor genes, the *Y. pestis* yersiniabactin biosynthetic system and *psn* appear to require the presence of yersiniabactin and YbtA (yersiniabactin activator) for maximal expression. YbtA is a member of the AraC family of transcriptional activators and has high homology to PchR (15, 84, 85) (GenBank accession number U50452). The complexity of unraveling iron-regulatory systems and iron-responsive phenotypes in *Y. pestis* is increased not only by the number of different systems but also by ongoing spontaneous Δ*pgm* and possibly other deletions or rearrangements.

Low-calcium response stimulon. In vitro, the expression of V antigen (LcrV) and Yops (*Yersinia* outer proteins) virulence determinants are maximal at 37°C in the absence of millimolar concentrations of exogenous calcium or nucleotides. Paradoxically, the expression of these components in vitro is associated with growth cessation (termed growth restriction). LcrV, Yops, and most elements that control their expression (Lcr proteins) and secretion (Ysc [Yop secretion] and Syc [specific Yop chaperones]) comprise the LCRS; all these factors are encoded on a 70- to 75-kb plasmid (pCD1 in *Y. pestis* KIM strains [Fig. 1A and C]) common to the three human pathogenic yersiniae. While differences exist among the Lcr plasmids of *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*, LCRS members appear to be highly conserved and homologous. This is the most intensively studied area in *Yersinia* research, making complete literature citation too voluminous; consequently, we will cite current reviews, recent advances, and seminal papers primarily examining *Y. pestis*. Results unique, to date, to *Y. pseudotuber-*

TABLE 3. Iron-regulated and Hms polypeptides expressed by *Y. pestis*[a]

Polypeptide designation	Mol mass (kDa) and location[b]	Iron and temp regulation[c]	Function or activity
IrpA	80, OM	R	?
IrpF[d]	113, OM	R	?
IrpG	36, OM	R	?
IrpH	34, OM	R, T-26	?
IrpI	29, OM	R, T-26	?
IrpJ	23, OM	R, T-37	?
IrpK	20, OM	R, T-37	?
IrpL	19, OM	R	?
IrpM	80–85, OM	R	?
HmsF	72, OM	T-26, HmsR	Hemin storage
HmsH	90, OM	T-26, HmsR	Hemin storage
Psn[e]	72, OM	R, Fur, YbtA	Yersiniabactin/pesticin receptor
HMWP1[e]	240, OM, P, IM, C	R, Fur, YbtA	Yersiniabactin biosynthesis?
HMWP2[e]	228, OM, P, IM, C	R, Fur, YbtA	Yersiniabactin biosynthesis
PirpA	75, P	R	?
PirpB	40, P	R	?
PirpC	39, P	R	?
PirpD	38, P	R	?
PirpE	37, P	R	?
PirpF	37, P	R	?
PirpG	34, P	R, T-26	?
PirpH	32, P	R	?
PirpI	31, P	R	?
PirpJ	30, P	R, T-26	?
PirpK	25, P	R	?
PirpL[e]	19, P	R	?
YfeA[f]	36, P	R[g], Fur[g]	Periplasmic binding protein[g]
MirpA	127, IM	R	?
MirpB	115, IM	R	?
MirpC	94, IM	R	?
MirpD	56, IM	R	?
MirpE	36, IM	R	?
MirpF	35, IM	R	?
MirpG	33, IM	R	?
MirpH	30, IM	R	?
YfeB[h]	32, IM[g]	R[g], Fur[g]	ATP-binding protein[g]
YfeC[h]	32, IM[g]	R[g], Fur[g]	Permease[g]
YfeD[h]	32, IM[g]	R[g], Fur[g]	Permease[g]
Fur	16, C	R[g], Fur[g], CRP[g]	Iron-responsive regulator
HmsR	52[g]	HmsR[g]	*hmsHFRS* regulator?
HmsS	18[g]	HmsR[g]	*hmsHFRS* regulator?
YbtA[e]	36[g]	R[g], Fur[g]	Yersiniabactin system regulator
YbtE[e]	56	R, Fur?[g], YbtA	Yersiniabactin biosynthesis
YbtT[e]	30[g]	R?[g], Fur?[g], YbtA?[g]	Yersiniabactin biosynthesis?[g]
≥14 Irps	<18 to >100	R, Fur	?
4 Iips[i]	ND	I, Fur	?
9 Arps[i]	ND	R, AirX	?
2 Aips[i]	ND	I, AirX	?

[a] Compiled from references 15, 16, 48, 84, 85, 109, 128, 129, 162, 239, 252, 253, and 312.
[b] Abbreviations: OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm; ND, not determined.
[c] R, repressed during iron surplus; I, induced during iron surplus; Fur, HmsR, YbtA, and AirX, regulated by Fur, HmsR (and/or HmsS), YbtA, or hypothetical iron-responsive regulator AirX, respectively; T-26 and T-37, temperature regulation with maximal expression at 26 and 37°C, respectively; YbtE and YbtT regulation is proposed from the *irp2* promoter sequence and is valid only if no intervening promoters exist.
[d] Expression is down-regulated or absent in Pgm^- and Hms^+ Pst^r mutants.
[e] Expression is absent in Δ*pgm* mutants; on two-dimensional gels, Psn is detected as three or four spots (originally termed IrpB to IrpE) with somewhat different pIs but similar molecular masses.
[f] YfeA may correspond to PirpD, PirpE, PirpF, or PirpG.
[g] Proposed from the DNA sequence.
[h] YfeB, YfeC, and YfeD may correspond to MirpE, MirpF, MirpG, or MirpH.
[i] Iips, Fur-regulated iron-surplus induced proteins; Arps, auxiliary iron-repressible proteins not regulated by Fur; Aips, iron-surplus induced proteins not regulated by Fur.

culosis and *Y. enterocolitica* will be included to provide a more complete picture of the LCRS operons and functions that will likely apply to *Y. pestis*. Table 4 summarizes relevant properties of LCRS components (21, 33, 70, 83, 93, 219, 263, 264).

(i) LCRS virulence roles and physiological effects. Growth media used in studies of LCRS were devised to maximize growth restriction as well as LcrV and Yop expression, and they contain 20 mM Mg^{2+} with no calcium. Although not used nutritionally, 2.5 mM Ca^{2+} relieves growth restriction and represses LcrV and Yop expression (117, 202, 258, 310, 311). In vitro, growth restriction results from an ordered metabolic step-down characterized by adenylate energy charge reduction, inhibition of stable RNA synthesis, and inhibition of protein synthesis, excluding LCRS members and most virulence deter-

TABLE 4. Genes involved in LCRS function, expression, and secretion[a]

Gene	Operon	Function or activity	Mol mass (kDa) and location[b]	Nonpolar mutant phenotype[c]
Virulence loci				
lcrV	*lcrGVHyopBD*	Immunosuppression	37, C, S	CD or CI, AV
yopB	*lcrGVHyopBD*	YopE deployment, immunosuppression?	42, S	
yopC	?	?	40	?
yopD	*lcrGVHyopBD*	YopE deployment	33, S	Intermediate phenotype
yopE	*yopE*	Cytotoxin, actin depolymerization, antiphagocytic	23, S	CD, AV
yopF	May be YpkA?	?	76	?
yopH	*yopH*	Protein tyrosine phosphatase, antiphagocytic	51, S	CD, AV
yopJ	*ORF1ypkAyopJ*	?	31, S	CD, AV
yopK[d]	*yopKL*	?	21, S	CD, AV
yopL	*yopKL*	?	15, S	CD
yopM	*yopM*	Anti-inflammatory?, dissemination?, thrombin binding	42, S	CD, AV
ypkA[e]	*ORF1ypkAyopJ*	Similarity to Ser/Thr kinases	82, S	CD, AV
Up-regulatory loci				
lcrF[f]	*lcrF*	AraC-like activator, thermal activation	31, C	CI
lcrV	*lcrGVHyopBD*	Modulator of Lcr induction	37, C, S	CD or CI, AV
Down-regulatory loci				
lcrE[g]	*lcrEORF1-2*	Ca^{2+} sensing?, blocks secretion?	33, S, OM?	CB
lcrG	*lcrGVHyopBD*	Ca^{2+} sensing?, blocks secretion?	11, S	CB
lcrQ/yscM	*lcrQ*	Ca^{2+} sensing?, blocks secretion of LcrV and YopD	12, S	CB[h]
Secretory loci				
lcrH	*lcrGVHyopBD*	Indirect negative regulation, YopB and YopD chaperone	19, C	CB
lcrD[i]	*lcrDR*	Yop/LcrV transport through IM?	77, IM	CI
lcrR	*lcrDR*	?	17	
yscB	*yscA–F*[j]	?	15	
yscC	*yscA–F*	?	67/64, OM	CI
yscD	*yscA–F*	?	47, IM	CI
yscE	*yscA–F*	?	7	CI
yscF	*yscA–F*	?	9	CI
yscG	*yscG–L*[j]	?	13, IM?	CI
yscH	*yscG–L*	Encodes YopR	18	CD
yscI	*yscG–L*	?	13	CI
yscJ	*yscG–L*	Yop/LcrV transport through OM?	27/24, OM	CI
yscK	*yscG–L*	?	24	CI
yscL	*yscG–L*	?	25	?
yscN	*yscN–U*	ATP-binding protein	48, SO?	CI
yscO	*yscN–U*	Yop/LcrV transport?	19	?
yscP	*yscN–U*	?	50	?
yscQ	*yscN–U*	Yop/LcrV transport through IM?	34, SO	CI
yscR	*yscN–U*	Yop/LcrV transport through IM?	24, IM	CI
yscS	*yscN–U*	Yop/LcrV transport through IM?	9, IM	?
yscT	*yscN–U*	?	28	?
yscU	*yscN–U*	?	40, IM	CI
sycE	*sycE*	YopE chaperone	15, C	CD, YopE stability and Sec^{-}[k]
sycH	*sycH*	YopH chaperone	16, C	CD, YopH Sec^{-}
lcrH/sycD	*lcrGVHyopBD*	YopD chaperone	19, C	CB, YopBD stability and Sec^{-}

[a] Compiled from a multitude of references cited in the text.
[b] Locations are indicated by C for cytoplasm, IM for inner membrane, OM for outer membrane, S for secreted, and SO for soluble proteins likely to be found in the periplasm or cytoplasm; unprocessed and processed molecular masses of YscC and YscJ are indicated.
[c] CD, Ca^{2+}-dependent growth, normal Lcr regulation; CI, Ca^{2+}-independent growth, constitutively repressed LCRS operon transcription, constitutively blocked LCRS protein secretion; CB, Ca^{2+}-blind growth restriction, constitutively induced LCRS operon transcription and LCRS protein secretion; AV, attenuated virulence.
[d] May correspond to *yopQ* of *Y. enterocolitica.*
[e] Also termed *yopO.*
[f] Termed *virF* in *Y. enterocolitica.*
[g] Also termed *yopN.*
[h] A *Y. enterocolitica* YscM^{-} mutant was CD.
[i] Termed *virA* in *Y. enterocolitica.*
[j] In *Y. enterocolitica*, *yscA–M* has been reported as a single operon.
[k] Sec^{-}, secretion negative.

minants (see below) (61, 175, 311). A recent study found that a defined medium lacking Na^{+} and Cl^{-} and having a decreased L-glutamate concentration eliminated growth restriction while permitting the expression of LcrV during cultivation at 37°C in the absence of Ca^{2+}. Consequently, growth restriction is probably an in vitro artifact caused by an artificial growth environment not encountered in vivo (94).

The distinction between the secreted virulence factors, LcrV

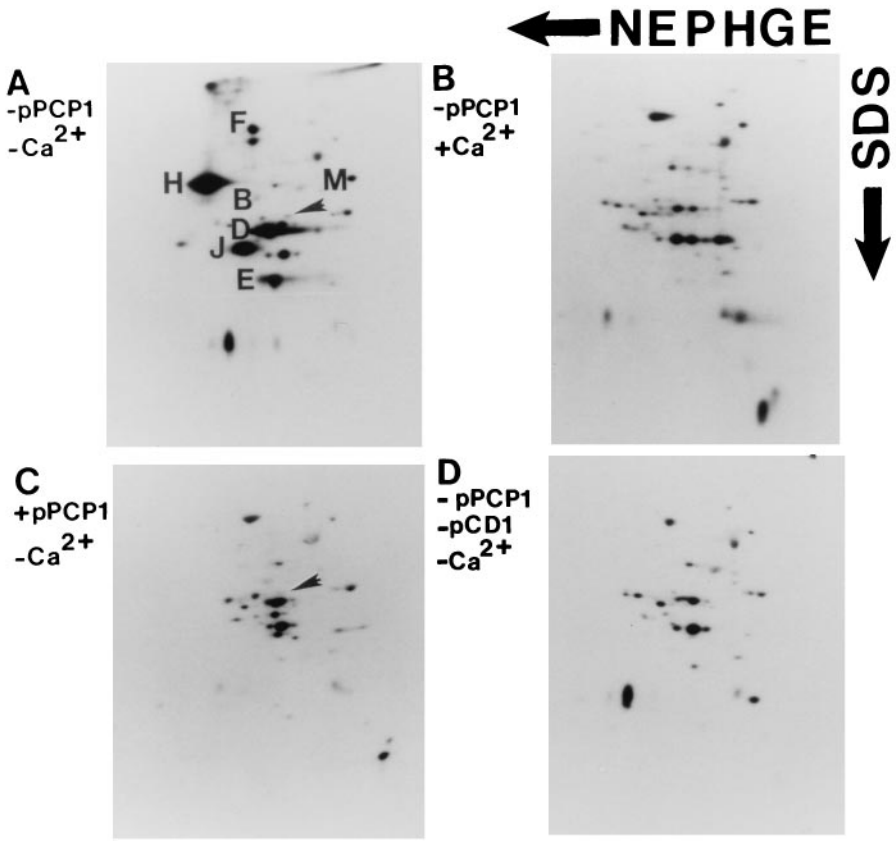

FIG. 4. Surface-expression of Yops in *Y. pestis*, and the effect of the Pla protease. The figure shows fluorographs of ^{125}I-labeled proteins separated by two-dimensional gel electrophoresis in which the first dimension was a nonequilibrium pH gradient (NEPHGE) and the second was a 12% polyacrylamide gel containing SDS. Cultures were labeled after 4 h at 37°C in the chemically defined medium TMH containing or lacking 2.5 mM Ca^{2+} (204, 211, 258). The presence or absence of Ca^{2+} and plasmid pPCP1 is indicated in each panel. The arrowhead identifies V antigen (LcrV), and letters indicate individual Yop proteins. (A) In the absence of Ca^{2+}, *Y. pestis* cells lacking Pla-encoding pPCP1 accumulate surface-associated Yops but not LcrV. (B) Ca^{2+} prevents Yop and LcrV expression and secretion. (C) Yops do not accumulate on the bacterial surface due to degradation by the Pla protease. (D) The surface profile of *Y. pestis* cells lacking both pPCP1 and the Yop- and LcrV-encoding plasmid pCD1 is shown for reference. The experimental data and figure were provided by S. C. Straley.

and Yops, including YpkA, may be largely historical; LcrV was discovered 25 years before Yops (38, 259, 264). Unlike most Yops, LcrV is not degraded by the Pla protease (see below). Furthermore, it is secreted with only trace amounts detected in the outer membrane (Fig. 4) and plays a role in the regulation of LCRS (33, 203, 258, 259). A second virulence-associated antigen, W antigen, is now thought to be a complex of LcrV and GroEL (33). LcrV is a protective antigen that has been historically associated with resistance to phagocytosis (33, 38, 157, 189, 275). The role of LcrV in virulence has been disputed since *lcrV* mutations also repressed the expression of LCRS components. However, a study of three nonpolar, in-frame *lcrV* deletion mutants revealed that all three were avirulent, with two mutants unaffected in LCRS regulation (220, 243). Recent studies (191, 192) suggest that LcrV may play an immunosuppressive role, muting the normal inflammatory response by inhibiting cytokine production. Consequently, the data supporting a virulence role for LcrV are now overwhelming (33, 191, 192, 220, 243, 264, 275).

Although they are true secreted proteins, Yops can be isolated from outer membrane fractions as well as culture supernatants in vitro. Their discovery in *Y. pestis* was delayed due to degradation of most membrane-associated Yops by Pla protease (illustrated in Fig. 4). Most (but not all) Yops are clearly demonstrated virulence determinants (33, 70, 93, 115, 203, 258, 259, 262, 264). YopE indirectly depolymerizes actin microfilaments, has demonstrated cytotoxic and antiphagocytic effects, and is required for virulence and growth in the liver and spleen. The cytotoxic effects of YopE are manifested upon its delivery into the host target cell (93, 232, 233, 251, 258, 261, 264). YopB and YopD are thought to play an active role in translocating YopE into the host target cell. However, they are not required for secretion to the bacterial surface (see below). YopB has significant similarity to the contact hemolysin IpaB of *Shigella flexneri* and the RTX family of hemolysins and toxins and may help form a pore in the eukaryotic membrane through which YopE is translocated (70, 93, 251, 264). Although not as rigorously demonstrated as LcrV activity (191, 192), a cytokine suppressive activity has also been attributed to YopB (24).

YopH and YpkA (*Yersinia* protein kinase) appear to subvert signal transduction. YopH dephosphorylates several eukaryotic proteins and has significant homology to the active domain of eukaryotic protein tyrosine phosphatases. Like YopE, YopH must be delivered, probably by YopB and YopD, into the host target cell to effectively block phagocytosis and inhibit the oxidative burst. This inhibition can apply to Fc-receptor-mediated signal transduction. Mutations in *Y. pseudotuberculosis yopH* that cause loss of enzymatic activity cause the bacteria to be avirulent and do not prevent signal transduction (27, 93, 264). YpkA is an autophosphorylating enzyme that shares homology with Ser/Thr protein kinases and is required for

virulence (of *Y. pseudotuberculosis*). YpkA is delivered from the surface-located bacterium in a YopB-dependent manner to the inner surface of the eukaryotic cell membrane (114). Thus, both YopH and YpkA may modulate signalling important for the immune response (70, 93, 99, 251, 264).

YopM is required for virulence and has been shown to prevent thrombin-platelet aggregation and to bind thrombin but not prothrombin. It shares significant homology with a family of leucine-rich glycoproteins, including the α chain of human platelet glycoprotein Ib (GPIb). GPIb is required for platelet-platelet aggregation and for platelet adhesion to exposed subepithelium, while thrombin activates platelets and converts fibrinogen to fibrin. Consequently, YopM is thought to mute the inflammatory response by sequestering thrombin. Alternative roles could include promoting dissemination by preventing clot formation, preventing thrombin interaction with other unidentified host cells, or mediating as yet unidentified interactions via its leucine-rich architecture (158, 159, 230, 264).

The remaining Yops are less thoroughly characterized. In *Y. pseudotuberculosis*, YopK is essential for virulence, but YopK mutants still retain cytotoxic and antiphagocytic properties (93, 124). A polar $YopK^-$ $YopL^-$ mutant of *Y. pestis* was avirulent and unable to sustain growth in the spleen and liver (258, 261). Whether these effects are due to loss of YopK, YopL, or both has not been clarified. YopJ is not essential for virulence in *Y. pseudotuberculosis* or *Y. pestis*, at least via an intravenous (i.v.) route of infection (93, 99, 258, 264). Expression of YopG and YopI has not been demonstrated in *Y. pestis*. While the synthesis of *Y. pestis* YopC and YopF has been noted (33, 203, 258), the function and genetic organization of these Yops in all three pathogenic *Yersinia* species remains undefined. However, based on molecular weight comparisons, YopF may be YpkA. Overall, the demonstrated or proposed antihost activities of LcrV and the Yops are to prevent phagocytosis, poison phagocytic cells, moderate the inflammatory response by sequestering thrombin, and delay development of a cell-mediated immune response by subverting signal transduction and inhibiting cytokine synthesis (70, 93, 191, 192, 262, 264).

(ii) LCRS regulation and secretion. Intensive scrutiny of the pathways regulating the expression of LCRS components has identified many of the regulators and yielded a general outline of the mechanisms employed; however, our understanding of this complex system is far from complete. Upon temperature shift from 26 to 37°C, transcription of LCRS operons is induced 20- to 100-fold and secretion of Yops, LcrV, LcrE, LcrG, and LcrQ/YscM occurs (up-regulatory pathway). Increased transcription has been observed within 45 min of the temperature shift. At 37°C, millimolar concentrations of Ca^{2+} or nucleotides repress the transcription of LCRS operons 3- to 20-fold and block secretion (down-regulatory pathway). Nucleotides and Ca^{2+} serve as external signals of the surrounding environment, since these molecules are not transported into the yersinial cell. Although the nucleotide effect has not been extensively analyzed, it has been suggested that Mg^{2+} chelation caused the observed regulatory effects. However, various nucleotides have differing Mg^{2+}-binding affinities which do not completely correlate with repression. Furthermore, a mutation in the *lcrG* regulatory gene (see below) causes loss of regulation by Ca^{2+} without affecting nucleotide regulation. This raises the specter of a nucleotide signaling pathway independent of the Ca^{2+} signaling pathway. Secretory mutants also affect LCRS operon transcription, possibly by feedback inhibition or secretion of a repressor; a functional secretory mechanism is required for full expression of LCRS members (33, 70, 93, 202, 203, 211, 242, 243, 262, 263, 310).

Thermal induction of LCRS operons requires the transcriptional activator LcrF (termed VirF in *Y. enterocolitica*). A consensus binding site for VirF in *yop* operon promoters has been proposed in *Y. enterocolitica*. LcrF expression is probably thermally regulated at both the transcriptional and translational levels. It has been shown to be translationally regulated in *Y. pestis* and transcriptionally regulated in *Y. enterocolitica*. Its expression is not Ca^{2+} regulated; consequently, it is not a member of LCRS. In addition, DNA topology may play a role in thermal induction of LCRS (70, 93, 120, 121, 262, 263, 283, 308). YmoA (*Yersinia* modulator) has been proposed as a histone-like protein that moderates LCRS operon thermal induction in *Y. enterocolitica* (70). Whether a functional *ymoA* exists in *Y. pestis* is undetermined. Although it does not contain any recognized DNA binding motifs, LcrV is required for full induction of LCRS operons in *Y. pestis* and is hypothesized to indirectly regulate genes through an antirepressive action on components of the downregulatory pathway (220, 222, 243). Mutations in *lcrF* cause a Ca^{2+}-independent phenotype, which is defined as loss of growth restriction with constitutively repressed LCRS operon transcription and constitutively blocked LCRS protein secretion at 37°C. A null mutation in *lcrV* also causes a Ca^{2+}-independent phenotype with little induction and secretion of Yops in the absence of Ca^{2+} (243, 262, 263).

Essential components of the Ca^{2+}-dependent downregulatory pathway include *lcrE*, *lcrG*, *lcrH*, and *lcrQ/yscM*. Mutations in these genes cause a Ca^{2+}-blind phenotype where 37°C incubation causes constitutive growth restriction, LCRS operon transcription induction, and LCRS protein secretion (23, 70, 93, 211, 242, 262, 263, 309). LcrE, LcrG, and LcrQ/YscM are all secreted proteins that are proposed to function as Ca^{2+} sensors which participate in preventing secretion in the presence of Ca^{2+}. None of these proteins contains obvious DNA- or Ca^{2+}-binding domains (93, 211, 233, 242, 262, 263). LcrH exerts its regulatory effects after LcrE and LcrG and acts at or close to the level of transcriptional repression. Since LcrH possesses no recognized DNA-binding motif, it has been proposed to act in concert with a corepressor to inhibit LCRS operon transcription. LcrQ/YscM is a candidate for this corepressor (23, 93, 211, 262, 263). In another model, LcrH acts only as a Yop chaperone (70) (see below). The Yop/LcrV secretory machinery (Ysc [see below]) could affect transcription by secretion of this putative co-repressor or by posttranslational feedback inhibition (23, 70, 93, 211, 262). A combined but still incomplete model of LCRS regulation and secretion is shown in Fig. 5.

Since blood and extracellular spaces in mammals should contain sufficient Ca^{2+} to repress LCRS, it was originally thought that LCRS components might be expressed during intracellular residence (33). Although some studies suggest that *yop* genes may be expressed inside cells, other studies failed to detect significant Yop protein expression (93, 208, 216, 233). Since the antihost activities of Yops are either anti-inflammatory or antiphagocytic, extracellular expression of these virulence determinants would appear to be most beneficial. Studies in cell culture medium with 1 mM Ca^{2+} showed that bacterial cell-eukaryotic cell contact initiated direct, polarized transfer of YopE and YopH from the bacterial cell into the eukaryotic cell. From these studies, it has been suggested that the in vivo signal for full expression of LCRS components is contact with a target cell and that in vitro absence of Ca^{2+} in a nonphysiological environment somehow mimics this signal (93, 114, 208, 232, 233). There are several caveats to the cell-contact-signal hypothesis. The Mg^{2+}/Ca^{2+} ratio in the cell culture medium used may not have been sufficient to completely repress LCRS. Cell contact may generate a local Ca^{2+}-

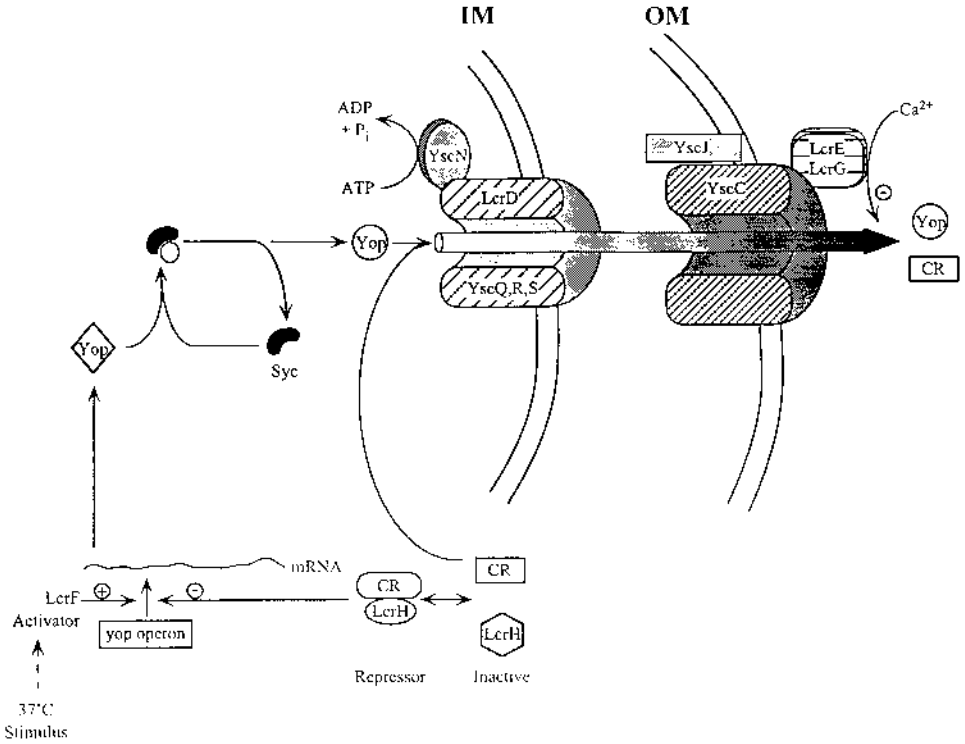

FIG. 5. Model for the regulation of Yop and LcrV expression and secretion. A generic Yop, which requires a chaperone (Syc), is depicted. Circled + and − symbols indicate activation and repression of transcription or blockage of secretion. The hypothetical corepressor is indicated by CR; IM indicates the inner membrane, and OM indicates the outer membrane. This model is based on those described in references 70, 93, and 262.

deficient environment or override the Ca^{2+} signal (262, 264). Finally, *Y. pestis* cells growing in necrotic lesions may express LCRS if the cytosol of lysed eukaryotic cells provides a Ca^{2+}-deficient environment (94). Thus, the mechanism of the in vivo signal for LCRS expression has not been conclusively demonstrated.

The secretion of Yops, LcrV, LcrE, LcrG, and LcrQ/YscM may involve up to 22 proteins (YscA-L, YscN-U, LcrD, LcrQ/YscM). Homologous proteins have been identified as components of export systems for virulence determinants and/or flagellar components in other bacteria. These functionally diverse systems, which do not utilize signal peptide sequences, comprise a new secretory pathway termed type III (1, 70, 183). The reason for this degree of complexity in a secretory pathway is unknown but may involve the regulatory effects on LCRS gene transcription observed in secretory mutants (1, 70, 112, 183, 210, 211) (Table 4). In *Y. pestis*, Yop/LcrV secretion requires at least functional LcrD, YscC, YscD, YscG, YscQ, and YscR products. In *Y. pseudotuberculosis* or *Y. enterocolitica*, LcrD, YscC to YscG, YscI to YscL, YscN, YscR, and YscU are proven essential secretory pathway components (1, 70, 89, 93, 210, 211). The products of *yscA* and *yscH* may not be components of the secretory pathway. Translation of the 32-amino-acid ORF of *yscA* has not been demonstrated (112). A *Y. enterocolitica* strain with a mutation in *yscH*, which encodes YopR, was not defective in Yop secretion (1). *lcrR* has been placed with secretory loci (Table 4) solely because it resides in the *lcrDR* operon; earlier regulatory effects of an *lcrR* mutation were due to polar effects on *lcrG* expression (242). Models of the secretion pathway have many common components (70, 93, 211, 262) (Fig. 5). It has been proposed that LcrD, YscQ, YscR, and YscS form a complex that transports secreted LCRS components through the inner membrane; YscN may serve to energize this transport. YscC and YscJ components are seen as facilitating transport through the outer membrane. LcrE and LcrG may serve as surface calcium sensors that not only function in signal transduction but also block secretion in the presence of calcium (70, 89, 211, 262, 298). Roles for the remaining Ysc proteins are currently undetermined.

In *Y. enterocolitica* and *Y. pseudotuberculosis*, secretion of three Yops also requires specific Yop chaperone proteins, termed Syc (Table 4; Fig. 5). The common properties of this new family of chaperones (284) are low molecular mass (15 to 20 kDa), an acidic pI, and probably interaction with the first 100 N-terminal amino acids of the escorted protein. Studies with *Y. enterocolitica* YopE and YopH indicate that the first 15 and 17 amino acids, respectively, are sufficient for secretion from the bacterial cell. A total of 50 and 71 N-terminal amino acid residues are required for translocation of YopE and YopH, respectively, into a macrophage cell line. SycH is required for secretion of YopH while SycE (also termed YerA in *Y. pseudotuberculosis*) is necessary for secretion of YopE. A cross-reactive protein with the molecular mass of SycH has been detected in *Y. pestis*. LcrH (also termed SycD) copurifies with YopD by immunoprecipitation. An $LcrH^{-}$ mutant of *Y. enterocolitica* was defective in YopB and YopD secretion; the mechanism for the defect in YopB secretion has not been elucidated (93, 96, 208, 251, 282, 284). A *Y. pestis* LcrH mutant has a Ca^{2+}-blind phenotype, while SycH and SycE mutants of *Y. enterocolitica* remain Ca^{2+} dependent (208, 223, 282). This suggests that LcrH serves additional functions. It remains to be determined whether specific chaperones are required for each of the Yops and LcrV.

Plasminogen activator (Pla protease). Brubaker et al. (34) first suggested that pesticin, fibrinolytic, and coagulase activities were linked on an extrachromosomal element. Subsequent

studies showed that the structural genes for these activities reside on a 9.5-kb plasmid designated pPCP1 in *Y. pestis* KIM strains (21, 83, 151, 247) (Fig. 1A and B). The fibrinolytic and coagulase activities have been cloned, sequenced, and found to reside in a single gene encoding a 34.6-kDa outer membrane protein called plasminogen activator (Pla protease) (172, 247, 248). While temperature-dependent transcription and translation of *pla* has not been observed (172), Pla synthesis continues during 37°C Ca^{2+}-deficient growth restriction when expression of most other proteins is inhibited (175). The Pla protein shares homology with other proteolytic plasminogen activators. In addition to plasminogen, the Pla protease cleaves complement component C3 and degrades YopB through YopF, YopH, YopJ, and YopK, as well as a ~24-kDa polypeptide (237, 249, 250) (Fig. 4). While Pla also binds type IV collagen, it shows relatively weak binding affinity, and negligible loss of collagen binding occurs in Pla^- cells (150). The fibrinolytic activity of Pla is temperature dependent, being higher at 37 than 28°C, possibly due to temperature-induced modifications or conformational changes in the protein (50, 172). Some strains with Pla^- mutations show greatly reduced virulence from peripheral routes of infection. Pla^- mutants injected subcutaneously (s.c.) produced a localized infection with increased numbers of inflammatory cells in lesions compared with Pla^+ cells (34, 105, 250) (Table 2). Various virulence roles for the Pla protease have been postulated, including (i) cleaving fibrin deposits that trap the organism, (ii) producing excess active plasmin that causes poorly organized or ineffective structures between inflammatory cells and fibrin, (iii) degrading mechanical constraints to spreading such as extracellular and basement membrane proteins, and (iv) reducing chemoattractants at the infection site possibly via inhibition of interleukin-8 production (105, 250). An invasive role for Pla has been hypothesized, since Pla^- mutants injected i.v. are fully virulent. Virulence after injection via the intraperitoneal (i.p.) route is enhanced if iron is injected with the mutant (34). Rather than serving as a nutrient, iron probably inactivates a host defense factor that compensates for the mutation (33). It should be noted that there are other strains lacking Pla activity whose virulence in mice is unaffected or minimally reduced (79, 155) (Table 2). Perhaps some other factor serves the function of Pla in these Pla-independent strains.

Pla protease also exhibits temperature-dependent coagulase activity (higher at 28 than 37°C) against rabbit but not human, mouse, or rat plasma, and it has been suggested that this activity may be important in the pathogenesis of *Y. pestis* (50, 54, 172, 250). Fleas (*Oropsylla montanus*) artificially fed a blood meal infected with Pla^- derivatives of *Y. pestis* EV76-6 had a lower death rate at 4 days than did fleas fed Pla^+ derivatives. However, the Pla phenotype did not affect bacterial growth in fleas (171). At present the physiological role of Pla in fleas is undetermined.

While pesticin has long been genetically linked to Pla activity and thus to virulence (34), this bacteriocin has now been clearly shown to lack virulence-enhancing effects in organisms after s.c. injection (250) (Table 2). Pesticin synthesis continues during Ca-deficient growth restriction, when synthesis of many other proteins has ceased (175).

pH 6 antigen. *Y. pestis* pH 6 antigen (which may correspond to classical antigen 4 [33]) was named for its regulated expression. Cells grown between pH 5 and 6.7 at temperatures of 35 to 41°C expressed this antigen, while cells grown outside these parameters did not. The antigen is expressed in vivo in the liver and spleen of mice and in vitro during Ca^{2+}-deficient growth restriction (20, 175). Genes encoding the pH 6 antigen have been cloned and sequenced; they appear to encode a fibrillar structure. A 15-kDa mature protein with limited homology to *E. coli* PapG (pyelonephritis-associated pili) is encoded by *psaA* (pH 6 antigen), while *psaB* encodes a 30.6-kDa protein with similarities to several pilus component proteins and *Y. pestis* F1 chaperone protein. A partial ORF (ORF4′) immediately downstream of *psaB* shows amino acid similarities to several pilus accessory proteins. A transposon insertion in *psaA* caused an increased LD_{50} via i.v. injection (Table 2). Transcriptional regulation of the monocistronic *psaA* operon by pH, temperature, and *psaE*, a gene upstream of *psaA* and in a separate transcriptional unit, has been demonstrated. A transposon insertion into *psaE* lowered the expression of *psaA* and caused loss of virulence, although pH and temperature regulation was retained. Thus, the initial environmental stimulus appears to be pH, with elevated temperature and PsaE required for maximal expression (20, 162, 163, 221). Intracellular synthesis of pH 6 antigen is dependent upon acidification of the phagolysosome and has been demonstrated in macrophages. Possible extracellular sites where pH 6 antigen could be expressed include abscesses such as buboes and lesions in the liver and spleen (162, 163). It has been suggested that the pH 6 fibrillar structure may provide entry into naive macrophages and participate in the delivery of Yops into phagocytic cells (257).

Serum resistance. *Y. pestis* must survive and/or grow in blood to be transmitted between its insect and mammalian hosts. Thus, resistance to complement-mediated lysis (serum resistance) is probably required for survival in both hosts. *Y. pestis* displays Lcr-independent serum resistance after growth at 26 or 37°C (201, 218, 274). In these studies, complement was activated by the alternate pathway in the absence of specific antibody. In experiments with 20% serum and antibody against fraction 1 antigen (F1), Pgm^+ and Pgm^- KIM derivatives that lacked pPCP1 and pCD1 but contained an integrated pMT1 were completely resistant to in vitro complement lysis. In addition, *Y. pestis* strains which appear to completely lack pMT DNA were resistant to complement killing. This suggests that *Y. pestis* is serum resistant even in the presence of antibodies and that this resistance is encoded chromosomally but outside of the *pgm* locus (207). The rough or short lipopolysaccharide of *Y. pestis* appears to mediate this resistance, possibly by causing aberrant attachment of the terminal membrane attack complex as in *Neisseria gonorrhoeae* (218).

Fraction 1 capsule. At 37 but not 26°C, the F1 protein (15.5 kDa) forms a large gel-like capsule or envelope (32, 279). Consequently, cells cease F1 synthesis during growth in the flea (50). As with Pla, pH 6 antigen, and pesticin, in vitro expression of F1 continues during Ca^{2+}-deficient growth restriction (175). The capsular material is readily soluble and dissociates during in vitro cultivation. F1 has been called a glycoprotein because an early purification protocol indicated that it possessed a galactolipid moiety containing galactose and fucose (104). However, a more recent purified preparation of F1 contained less than 0.12% lipids and carbohydrates. F1 proteins can form polymers that are several megadaltons in size (32, 40, 279). In one model F1 forms a bilayer, with an internal hydrophobic region, around the cell by the organization of dimers (tens to hundreds) bound through hydrogen bonds (279).

The structural genes for F1 (*caf1* [capsular antigen F1]) and the associated genes *caf1M*, *caf1A*, and *caf1R*) are encoded on the 110-kb plasmid (63, 156, 224) (Fig. 1A and D) and have been cloned and sequenced (100, 101, 141, 142, 241). After removal of a 21-amino-acid signal sequence, mature F1 with 149 amino acids has a calculated pI of 4.3. The 28.7-kDa Caf1M protein shares homology with PapD, a chaperone pro-

FIG. 6. Alignment of the predicted amino acid sequence of *Y. pestis* murine toxin (Ymt) with that of *Streptomyces antibioticus* phospholipase D precursor (Pdp). Alignment was performed with BestFit version 8.1 from the Genetics Computer Group software package from the University of Wisconsin. A vertical line indicates amino acid identity. A colon identifies conservative changes with a comparison value of ≥0.5. A period identifies conservative changes with a comparison value of ≤0.5.

tein, and is proposed as an F1 chaperone that is hypothesized to play a role in posttranslational folding and secretion of F1. Caf1M$^-$ mutants are defective in F1 capsule (100, 101). Molecular modeling of F1 and Caf1M predicts structures that are consistent with other chaperone systems (312, 313). Caf1A is a 93.2-kDa protein with homology to PapC and other proteins involved in the assembly of *E. coli* pilus structures. Caf1R appears to be a 30-kDa positive activator with homology to the AraC family of transcriptional activators (80, 141, 142). The region between *caf1M* and *caf1R* contains two divergent promoter elements, multiple direct repeats, and two indirect repeats which have been proposed to affect the expression of F1 (101, 141). Strain K25 (31) contains a spontaneous deletion encompassing two of the direct repeats in the *caf1*/*caf1R* intergenic region and does not express F1 (95, 287). F1$^-$ mutants can be isolated from animals vaccinated with F1 and challenged with an F1$^+$ strain; one such isolate had a deletion identical to that of K25 (95). Whether similar deletions occur in fresh *Y. pestis* isolates at a significant frequency is undetermined.

Resistance to phagocytosis by monocytes coincides with development of the F1 capsule (5, 53, 54). Despite this correlation, numerous virulence tests of undefined and defined F1$^-$ mutants indicate that this factor is of little importance in disease in mice, causing at most a slight alteration in time to death. Aerosol infection of African green monkeys with a defined F1$^-$ mutant did not significantly decrease virulence (74). However, a moderate loss of virulence in other F1 mutants in rats and guinea pigs has been observed, suggesting a possible role in other animal infections (2, 34, 39, 74, 79, 80, 95, 287, 293, 306, Table 2). While F1 is a protective antigen, fully virulent F1$^-$ mutants have been isolated from vaccinated mice challenged with F1$^+$ strains (95, 293). Nevertheless, fresh *Y. pestis* isolates with a F1$^-$ phenotype appear to be exceedingly rare.

It is unclear whether putative virulence factors in addition to F1 and murine toxin (see below) are encoded on the 110-kb plasmid family. Attempts to cure strains of these plasmids have generally resulted in plasmid integration into the chromosome rather than loss (95, 207, 225, 287). One strain with an integrated pFra that weakly expressed F1 did demonstrate slightly reduced virulence in mice, suggesting that a plasmid or chromosomally encoded virulence gene was affected by the integration. However, others found no loss of virulence in strains lacking pFra plasmids (74, 79, 287) (Table 2).

Murine toxin. Murine toxin refers to two forms of a protein (native polymers of 240 and 120 kDa) toxic for mice and rats but relatively ineffective against guinea pigs, rabbits, dogs, monkeys, and chimpanzees. Although some toxin is released during in vitro growth, it is associated primarily with the cell envelope or membrane until cell death and lysis. The LD_{50} for mice ranges from 0.2 to 3.7 μg depending upon the degree and type of toxin purification (32, 40, 186). Murine toxin has been proposed as a β-adrenergic antagonist that may directly block the β-adrenergic receptor after in vivo activation (186) and cause circulatory collapse (32, 40).

The structural gene for murine toxin (*ymt*) resides on the 110-kb plasmid and has been cloned and sequenced; *ymt* appears to be transcribed as a monocistronic message and probably encodes a 61-kDa protein (63, 156) (GenBank accession number X92727) (Fig. 1D). Consequently, the 12-kDa polypeptide described by Montie (186) may be a degradation product (80). A BLAST search identified strong homology between the deduced amino acid sequences of *ymt* and a phospholipase D precursor encoded by *Streptomyces antibioticus* (Fig. 6). How this homology correlates with the murine toxin activities described by Montie (186) is unclear.

Expression of *ymt* may be positively regulated by a region immediately upstream of the structural gene (63). A *ymt*::*luxAB* fusion mutant (where luciferase activity is expressed only from transcription from the *ymt* promoter) indicates that *ymt* transcription is ~threefold higher at 26 than 37°C. Expression also occurs in vitro in Lcr$^+$ cells undergoing Ca^{2+}-deficient growth restriction (175). The *ymt*::*luxAB* mu-

tant was 5 to 10 fold less virulent after i.p. inoculation in mice than was the parent strain. However, the LD_{50} in mice of other *Y. pestis* strains lacking pFra plasmid and murine toxin activity was relatively unaffected (79, 80, 155, Table 2). The effects of defined *ymt* mutations have not been tested in other animals or in an otherwise wild-type strain.

Catalase activity. Reviews cite catalase activity (antigen 5) as a possible virulence determinant (32, 33, 40). "Catalase activity" in *Y. pestis* as measured by decomposition of hydrogen peroxide is high compared to that in a number of other bacteria (41). Rockenmacher (231) found a correlation between high "catalase activity" and virulence while Burrows et al. (41) did not. Mehigh and Brubaker identified a ~70-kDa protein that bound hemin and had a modest catalase but no peroxidase or superoxide dismutase activities (175). Although this protein cross-reacted with antibody against antigen 5, it represented less than 10% of the catalase activity in cell extracts. Intriguingly, this protein was expressed during in vitro Ca^{2+}-deficient growth restriction, when protein synthesis is limited primarily to known or putative virulence determinants (175). Specific mutants will be required to determine whether antigen 5 and/or catalase activity are important in the virulence of plague.

EPIDEMIOLOGY

Plague Life Cycle

Plague is a zoonotic disease primarily affecting rodents; humans play no role in the long-term survival of *Y. pestis*. Transmission between rodents is accomplished by their associated fleas. While infection can occur by direct contact or ingestion, these routes do not normally play a role in the maintenance of *Y. pestis* in animal reservoirs. Fleas acquire *Y. pestis* from an infected blood meal. Infection in the flea is restricted to the alimentary canal with other organs and tissues including salivary glands, reproductive organs, and the hemocele being unaffected. The organism is not transmitted transovarially, and artificially infected larvae clear the organism within 24 h. Consequently, maintenance of plague in nature is absolutely dependent upon cyclic transmission between fleas and mammals (Fig. 7) (5, 6, 25, 37, 54, 126, 214, 215).

The oriental rat flea (*Xenopsylla cheopis*), the classic vector for plague, will ingest from 0.03 to 0.5 μl of blood (119). A bacteremia of 10^4 CFU/ml would ensure ingestion of at least 300 *Y. pestis* organisms. Although no detailed dose studies have been performed, the level of bacteremia in the infected rodent correlates with the percentage of fleas that become infected (78, 146, 217). The organisms are cleared from some fleas but multiply in the midgut (stomach) of others. A lysozyme-like enzyme secreted into the slightly acidic flea stomach (pH 6 to 7) may be responsible for antibacterial activity (50, 290). Two days after an infected blood meal, the stomach exhibits clusters of brown specks containing *Y. pestis*. These develop into cohesive dark brown masses, containing bacilli, a fibrinoid-like material, and probably hemin (4, 5, 6, 50, 290), which extend throughout the stomach and into the proventriculus and esophagus. The proventriculus, which separates the stomach and esophagus, is a sphincter-like organ with needle-like teeth directed back toward the stomach; it aids in the rupture of blood cells and normally prevents regurgitation of a blood meal (5, 6, 50). Between days 3 and 9 after the infected blood meal, the bacterial masses may completely block the proventriculus, extend into the esophagus, and prevent ingested blood from reaching the stomach (4, 5, 6, 25, 50, 289). As the hungry flea repeatedly attempts to feed, the blood sucked from the mammalian host distends the esophagus, mixes with bacilli, and is regurgitated into the mammalian host when the feeding attempt is terminated (5, 50). At higher environmental temperatures (>28 to 30°C), blockage of fleas decreases and clearance of the infection increases, possibly due to the temperature regulation of hemin storage and/or Pla protease. While not all blocked fleas transmit plague, blockage is an important process in ensuring transmission; transmission from unblocked fleas is exceedingly rare (37, 50, 54, 143). The fate of the blocked flea is death from starvation and dehydration (5, 25). The digestive process of fleas involves maintaining the blood meal as a liquid, which is degraded primarily by proteolytic enzymes (290). It has been suggested that formation of the solid bacterial mass may hasten starvation by preventing proper digestion (171).

Although possibly an overestimate, it has been suggested that as many as 11,000 to 24,000 bacilli are regurgitated by the flea into the mammalian host (37). While *X. cheopis* and *Xenopsylla astia* appear to be capillary feeders, the feeding method, i.e., capillary or wound (pool feeding), for the vast majority of fleas is undetermined (76). One study suggested that bacilli injected by the flea are rapidly removed from the wound site, presumably by capillary spread (37). However, poor recovery of control organisms from wound sites reduces the reliability of this conclusion. Other reports assume that s.c. or intradermal (i.d.) routes simulate the flea regurgitation route (33, 123, 214, 215, 226, 236, 296). This uncertainty needs to be resolved before experimental plague infections can mimic flea bite infections.

Due to the lower temperature, *Y. pestis* cells released from fleas are not expressing F1 and LCRS components and are readily phagocytized by polymorphonuclear leukocytes (PMNs) and monocytes. Cells phagocytized by PMNs are largely destroyed, while those engulfed by monocytes grow intracellularly and develop resistance to further phagocytosis. By 3 h of growth in vivo at 37°C (prior to F1 capsule expression), cells are resistant to phagocytosis by PMNs but not monocytes. After 3 to 5 h, cells are resistant to uptake by both types of phagocytes (5, 53, 54). The alteration in expression of virulence factors that occurs during this transition period from growth in the flea to growth in mammals probably accounts for observed growth temperature-dependent LD_{50} values (54) (Table 1).

The current scenario suggests that *Y. pestis* spreads from the site of the flea bite to the regional lymph nodes and grows to high numbers, causing formation of a bubo (swollen lymph node [Fig. 8]). Now the infection spreads into the bloodstream, where bacilli are preferentially removed in the spleen and liver. Growth of the organisms continues in the blood, liver, and spleen and spreads to other organs. This progression is similar to that of other bacterial infections but is supported by only limited experimental evidence. The few studies done that indicate that *Y. pestis* spreads from the lymph nodes to the blood followed by colonization of internal organs have not been conclusive (137, 286). However, it is clear that susceptible infected animals develop primary and sometimes secondary buboes and bacteremia (217). It is also clear that organisms injected i.v. are preferentially removed by the liver and spleen. Lungs and other internal organs may also become colonized (136, 274). Development of a bacteremia of sufficient degree and duration is essential for effective transmission in nature (214, 215). Significant bacteremias are required to achieve ~30% transmission to fleas experimentally (217). Infection of the flea via the blood of an bacteremic or septicemic rodent completes the cycle.

An important axiom of host-parasite relationships is that the parasite will adapt to minimize disease symptoms in essential hosts to help ensure the survival of the parasite's required

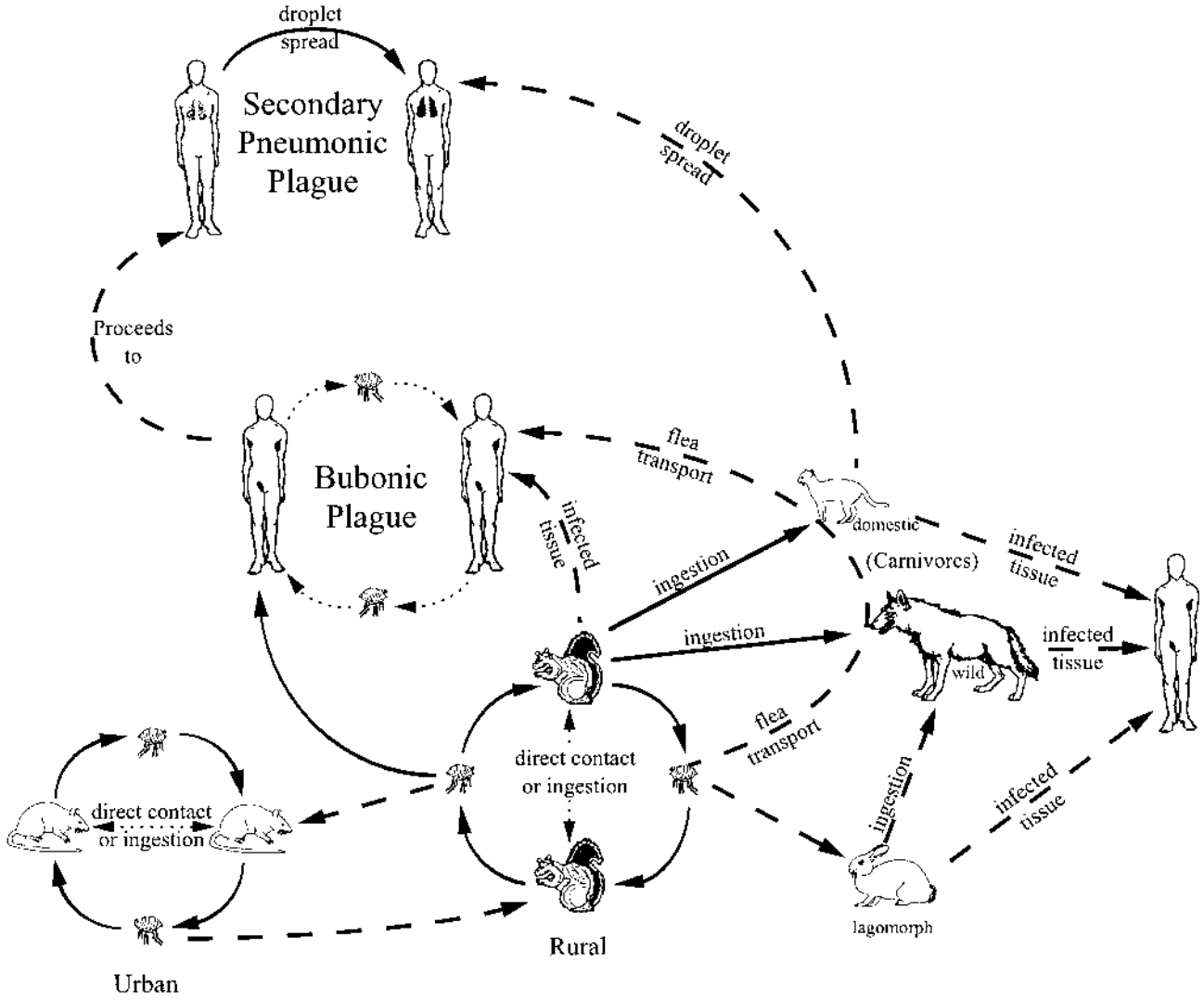

FIG. 7. Transmission routes of plague. Solid lines with arrows show usual transmission routes, while dashed lines with arrows indicate occasional routes. Theoretical, rare, and/or controversial routes are shown as dotted lines with arrows. To reduce the complexity, transmission routes to humans are shown only for the rural cycle and the bubonic/septicemic cycle is labeled bubonic. Urban cycles have the same transmission routes to humans. This figure was compiled from references in the text, modified from reference 213, and used with the permission of the copyright holder (Lippincott-Raven Publishers).

niche for multiplication and dissemination. Clearly, *Y. pestis* must cause a mammalian bacteremia sufficient to infect fleas and fleas must become blocked to enhance transmission to mammals. While many mammals have high susceptibility and mortality to plague, others are more resistant, with milder disease symptoms. These mammals are thought to be the maintenance (or enzootic) hosts essential for the long-term survival of plague (7, 8, 9, 11, 54, 107, 123, 145, 214, 215). Other, less widely accepted scenarios for long-term survival of *Y. pestis* include alternate insect hosts and revertible mutation of *Y. pestis* to attenuated, chronic disease forms (54, 103, 126, 217, 234, 268).

Y. pestis has been isolated from lice and ticks (54, 103, 126, 217, 268). Thomas et al. (268) were able to experimentally infect ticks and detect plague bacilli for up to 1 year. However, they were unable to demonstrate transmission of plague by ticks to mammals. The existence of attenuated or chronic plague strains in nature (103, 217, 234) led to the suggestion that such strains are responsible for the persistence of plague between epizootics (disease outbreaks in susceptible animals characterized by high death rates). Epizootics would occur when these attenuated strains revert to high virulence (234, 268). Mutations in *inv* and *yadA* have been proposed as a possible mechanism for generating attenuated *Y. pestis* strains (234). *Y. pestis* strains examined have been YadA$^-$ and Inv$^-$ (190, 234, 244), and a *Y. pseudotuberculosis* mutated to this phenotype increased in virulence. A constructed YadA-expressing *Y. pestis* strain lost virulence (60- to 600-fold increase in the LD_{50}) but retained an impressive LD_{50} of 6×10^2, with no indication of a switch to chronic symptomology. Chronic and attenuated strains cited in the literature were not characterized or tested for their ability to revert (103, 217). At present, the concept of enzootic mammalian hosts demonstrating relatively minor plague symptomology remains the most prevalent theory for persistence between epizootics (215).

Flea Vectors

Over 1,500 species of fleas have been identified. While only a small number (over 31) of these are proven vectors of plague,

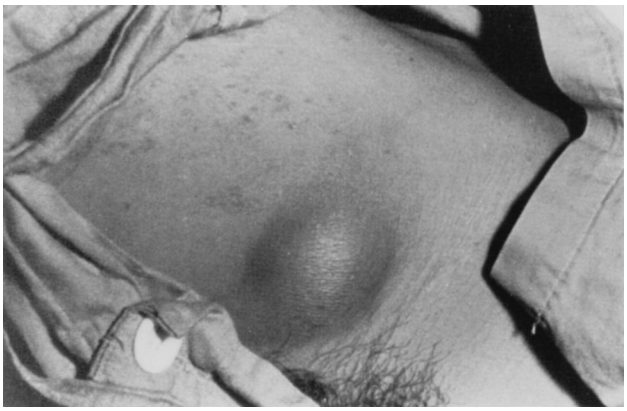

FIG. 8. The enlarged, exquisitely tender inguinal bubo of bubonic plague. Reprinted from reference 54 with the permission of the publisher.

any flea species may be biologically capable of transmission under the appropriate conditions. Due to its role in previous pandemics, *X. cheopis* (the Oriental rat flea) is considered the classic vector and is the standard against which all other fleas are measured (6, 66, 214, 215). Douglas and Wheeler defined the ability of fleas to transmit plague (vector efficiency) as the product of three potentials—infection potential, vector or infective potential, and transmission potential (78, 288). The infection potential is the percentage of fleas taking an infected blood meal that become infected; the infective potential is the percentage of infected fleas that become capable of transmitting; and the transmission potential is the observed ability of each flea to transmit plague prior to its death. Differences in the size and structure of the proventriculus (affecting blockage potential), feeding frequencies, and survival time after infection affect the last two numbers (37, 78, 122, 144, 214, 288). A vector index is derived by multiplying the vector efficiency by the blocking-survival potential, which is defined as the mean day of death divided by the mean day of blocking and accounts for the average time a flea may be infective (145). Vector efficiencies and indices have been determined for a number of flea species. *X. cheopis* remains one of the most effective vectors of plague, while the vector efficiency of the rock squirrel flea *Oropsylla montanus* (formerly *Diamanus montanus*) varies considerably in different tests. The cat flea (*Ctenocephalides felis*), which fails to become blocked, and the so-called human flea (*Pulex irritans*) were found to be very poor vectors (37, 78, 122, 144, 145, 288).

While these experimental determinations have proven useful, poor vectors cannot be dismissed as unimportant in the ecology of plague. Some of these fleas are capable of transmitting the disease in nature or en masse in the laboratory. For example, *Malaraeus telchinum* has a low vector efficiency but appears to be critical in maintaining plague in California meadow mice. The effects of extrinsic environmental factors such as temperature, humidity, mammalian host specificity, and flea and mammalian host abundances and densities, as well as characteristics of *Y. pestis* strains are poorly understood and may drastically affect the vector efficiency in nature (6, 37, 145).

Although the frequency of feeding may determine the amount of time that fleas remain on their mammalian hosts, they are essentially nest-dwelling insects. The degree of shelter provided by burrows and nests moderates the temperature and relative humidity and may determine the species of infesting flea. Moderately warm, moist climates are preferred by *X. cheopis*, while hot weather adversely affects *Xenopsylla brasiliensis*; *Nosopsyllus fasciatus* (a flea of the domestic rat) has adapted to damp, cool conditions. *X. cheopis* generally infests burrow-dwelling rats in urban areas, while *X. brasiliensis* prefers rural rats living above ground (in roofs and walls) (6, 145, 217). Flea infestations can be seasonal. *Hoplopsyllus anomalus* (with a poor vector efficiency) displaces *O. montanus* on squirrels during the summer and fall months, which correlates with the subsidence of squirrel epizootics (288). Important flea vectors include *X. cheopis* (nearly worldwide in moderate climates), *X. brasiliensis* (Africa, India, and South America), *Xenopsylla astia* (Indonesia and Southeast Asia), *Xenopsylla vexabilis* (Pacific islands), and *N. fasciatus* (nearly worldwide in cool, temperate climates) (6). *O. montanus* is the most important flea vector in the United States (97, 171). In the former USSR, *Ctenophilus tesquorum*, *Oropsylla silantiewi*, *Rhadinopsylla ventricosa*, as well as species of *Xenopsylla*, *Nosopsyllus*, *Neopsylla*, and *Citellophilus*, are considered important plague vectors (126, 277).

Animal Hosts and Infections

Since the work of Baltazard et al. (7–9, 11), investigation of plague infections in rodents that could serve as enzootic reservoirs has been generally limited to determining LD_{50}s, antibody titers, and the distribution of *Y. pestis* in survivors. In these rodents, there is a heterogeneous response to infection—some animals are highly sensitive, but most are at least moderately resistant. Survivors generally have significant antibody titers against plague (3, 107, 123, 145, 214, 215, 226).

Carnivores such as domestic dogs, domestic ferrets, Siberian polecats, black bears, badgers, coyotes, raccoons, and skunks appear to be highly resistant to plague. In most of these animals, ingestion of plague-infected rodents causes inapparent to mild disease and seroconversion. Although black-footed ferrets have often been listed as resistant to plague, recent evidence suggests that this may not be the case. In contrast to other carnivores, orally infected domestic cats became acutely ill and generally developed buboes and bacteremia. Over one-third died within 10 days, while 44% became ill but recovered. Of the infected cats, 75% had blood cultures positive for *Y. pestis*, and the organism was isolated from the oral cavity of 92% of the cats that became ill. This might account for the high rate of pneumonic plague transmission from cats to humans (see below). Similar susceptibilities in wild felines is suspected but not proven (102, 168, 214, 215, 236, 291).

Early work used guinea pigs as experimental models for epizootic plague due to their high susceptibility to the disease. Guinea pigs naturally infected via flea bites exhibited an almost immediate red areola around the bite wound with development of a red papule within 2 to 3 days. Similar papules formed in guinea pigs infected i.d. but not s.c. Following development of the papule, lymph nodes draining the bite area became enlarged, followed by septicemia and subsequently death at approximately 2 weeks (217, 286). In a separate study of i.d.-infected guinea pigs, development of a local lesion, regional buboes, and involvement of internal organs (spleen and liver) appeared to occur sequentially (137). s.c.-infected animals developed necrotic lesions in buboes, liver, spleen, and occasionally lungs; no gross pathology was observed in the heart, kidneys, or brain (214).

Similar pathological findings have been noted in naturally infected commensal rats, tarabagans, susliks, and ground squirrels. Most (but not all) animals develop a bubonic form of the disease with different degrees of internal organ involvement and bacteremia. However, a significant number of animals that

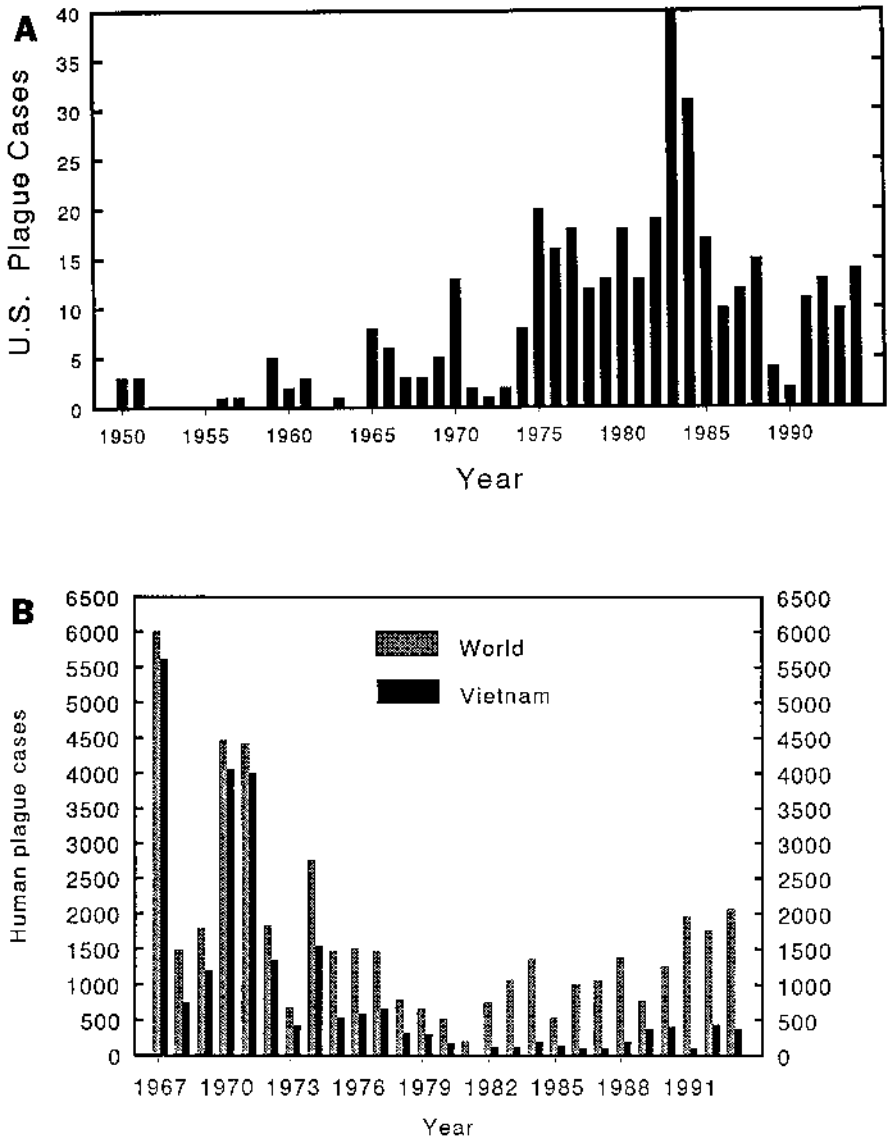

FIG. 9. Yearly incidence of human plague in the United States (A) and the world (B). This figure was compiled from references in the text.

rapidly succumb to plague have internal organs colonized by *Y. pestis* without macroscopic pathological changes, presumably due to the rapidity of death. While some researchers have suggested that susceptibility varies with seasonality, this has not been substantiated by other observations and studies. However, animals infected just prior to hibernation survive the winter and may initiate epizootics the following spring. This phenomenon may allow overwintering of plague epizootics (145, 147, 214, 215, 217, 226, 296).

Spread of Plague to and among Humans

Through the start of the third pandemic, transmission from urban rodents (especially rats) was the norm. Currently, most human plague cases in the world and all cases in the United States are classified as sylvatic plague (contracted from rural wild animals such as squirrels, chipmunks, marmots, voles, gerbils, mice, and rabbits) (66, 72, 97, 214, 215, 217, 277) (Fig. 7). The peak years of human plague in the United States coincide with widespread epizootics in the southwest (56, 72, 97). For example, a vast epizootic from 1982 to 1984 that wiped out prairie dogs and other plague-prone rodents in vast areas of New Mexico, Arizona, and Colorado correlates with the large number of human plague cases in 1983 and 1984 (72, 97) (Fig. 9A).

In the United States, transmission to humans occurs primarily via the bites of fleas from infected rodents (Fig. 7 and 10A). Although prominently affected, prairie dogs are not a common source of human plague since their fleas are not prone to feed on humans even in the absence of their normal host. Squirrels carrying *O. montanus*, which will bite humans, constitute the most common source of human plague (58, 97, 98, Fig. 10B). While cat and dog fleas (*C. felis* and *Ctenocephalides canis*, respectively) will also feed on humans, they are poor vectors and do not cause significant human disease (217). Thus, the infesting flea often determines the potential of an epizootic to cause human disease. Isolated cases also occur as a result of direct contact with infected rodents, their predators, and other animals, including exceedingly rare cases of transmission from

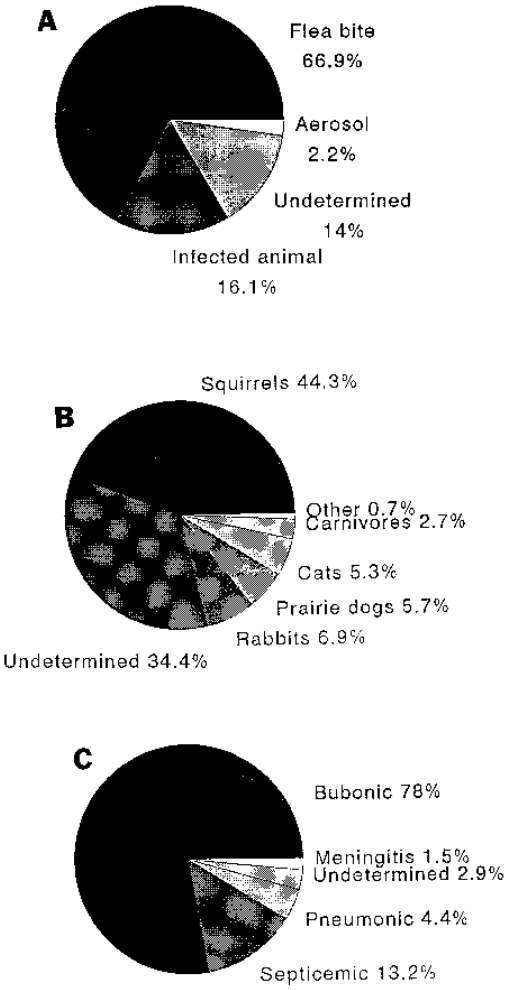

FIG. 10. Relative incidence of U.S. human plague transmission routes, animal sources, and clinical forms. (A) Transmission routes, 1970 to 1994. (B) Animal sources of human plague, 1970 to 1994. (C) Relative prevalence of human plague clinical forms, 1988 to 1994. This figure was compiled from references in the text.

goats and camels. As discussed below, domestic cats in the United States recently have become significant sources of human plague (102, 214, 215).

Human epidemics generally start as bubonic plague from bites of fleas that have left their dead or dying rodent hosts (214, 215, 217) (Fig. 7). Experiments indicate that *X. cheopis* fleas infected with *Y. pestis* prefer a slightly lower temperature (1.6°C) than do uninfected specimens (269). Since rats have a normal core temperature 1.5°C higher than humans, it has been suggested that this temperature preference may have accentuated the spread to humans (29). Plague epidemics subside under conditions of high temperatures and low humidity, possibly due to adverse effects on the flea life cycle (5, 50, 144) and/or the failure of fleas to become blocked at higher temperatures (50, 54, 143). The role of *P. irritans* (erroneously called the human flea despite its wide host range) in sustaining epidemics is controversial (Fig. 7). While the majority opinion is that this flea is rarely involved in human-to-human transmission, others assign it an essential role in human epidemics (11, 103, 127, 217, Fig. 7).

When a bubonic plague victim develops secondary pneumonic plague, the potential for respiratory droplet spread and a primary pneumonic plague epidemic occurs (214, 215) (Fig. 7). This type of epidemic is currently uncommon due to the advent of effective antibiotics and modern public health measures.

Geographical Distribution and Incidence

Plague, cholera, and yellow fever are the only three internationally quarantinable infectious diseases. Figure 11 identifies countries that reported human plague cases to the World Health Organization (WHO) between 1970 and 1994 and indicates the locations of probable endemic foci (14, 43, 215). Permanent plague foci occur in the semiarid but not hot-desert areas of most of the continents, where plague has become established in maintenance rodent populations (Fig. 11). These foci are not clearly defined, since field studies have generally not attempted to distinguish enzootic and epizootic hosts. In addition, some epizootics can last for years by contiguous or saltatorial spread to new or repopulated colonies (e.g., prairie dogs and rock squirrels) causing them to be classified mistakenly as permanent foci. Finally, disease in enzootic hosts is relatively inapparent compared to dramatic die-offs of highly susceptible hosts (145, 214, 215). The former USSR with the second largest enzootic plague area in the world, has 10 separate foci, some of which are isolated from each other by unfavorable terrain and environments (214, 277). Enzootic North American foci, the largest in the world, are primarily in the southwestern United States and Pacific Coastal region, with plague-infected animals detected as far north as Alberta and British Columbia, Canada, as far south as the state of Coahuila, Mexico, and as far east as Dallas, Tex. (13, 97). Multiple stable foci occur in Africa, Asia, and South America but not in western Europe (214, Fig. 11). Humanity's primary contribution to the maintenance of plague in nature was to spread the disease around the world via steamship at the turn of the century. In some regions (Australia and Hawaii), plague caused epizootics and human epidemics but eventually disappeared, possibly due to a failure to become established in a suitable enzootic host (14, 81, 215).

Animal reservoirs. While over 200 mammalian species in 73 genera have been reported to be naturally infected with *Y. pestis*, rodents are the important hosts for plague. Other than potentially spreading the disease from one rodent population to another, infections in other animals are unimportant in the long-term survival of *Y. pestis*. Rodents characterized as enzootic hosts have not been conclusively identified but should have a heterogenic response to *Y. pestis*, with most individuals showing moderate resistance to infection (as a result of previous exposure or possibly an inherent characteristic of the species or subspecies), relatively mild signs, and low mortality rate (214, 215, 226). A relatively short lifespan with high replacement rate from multiple litters during a long breeding season would also be characteristic of enzootic hosts. Significant flea activity should be present with these hosts during all seasons. Depending upon previous exposure, few to nearly all individuals will have antibodies against plague antigens. Some species of *Microtus* and *Peromyscus* (voles and mice) have been suggested as maintenance hosts in western North America (3, 107, 144, 145, 214, 215, 226, 227). Some types of mice (in Africa and the former USSR), gerbils (in the former USSR, India, Iran, South Africa, Syria, and Turkey), and voles (in the former USSR and Mongolia) are relatively resistant to plague and are suspected enzootic hosts (8, 9, 66, 145, 168, 214, 215, 217, 277). One type of highly resistant rat (*Dipodomys* spp.) seroconverts, with few animals becoming ill and rarely dying. Die-offs in nature are not observed despite evidence of plague activity in the population (214).

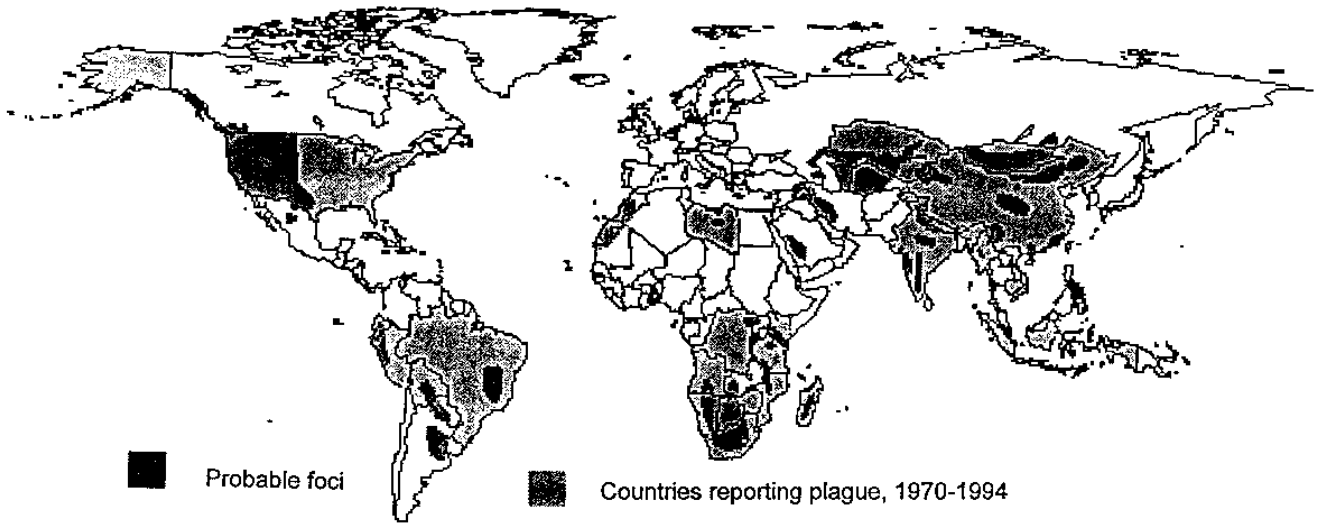

FIG. 11. Global distribution of plague. Figure provided by K. L. Gage.

While enzootic hosts serve to maintain plague, epizootic hosts amplify and may allow the spread of disease into new areas, where new enzootic hosts may become infected. Epizootic rodents are associated with high to moderate susceptibility and uniformly high mortality. Outbreaks in these hosts periodically decimate rodent populations in Africa, the Americas, Asia, and the former USSR (6, 97, 214, 215). Such epizootics and consequent human disease are favored by sufficient populations of fleas and susceptible rodents as well as cooler temperatures with above average rainfall (54, 58, 97). Highly susceptible or epizootic plague hosts include various species of mice, rats, voles, gerbils, ground squirrels (including susliks), marmots, tarabagans, and prairie dogs (66, 168, 214, 215, 217, 277). The apparent overlap in enzootic and epizootic hosts is due to widely varying susceptibilities among highly related species in different regions. In the former USSR and the Kurdistan plague focus (parts of Iran, Iraq, Syria, and Turkey), species of gerbils show either high-level resistance (*Meriones meridianus*), moderate resistance (*Meriones libycus*), or susceptibility (*Meriones tamariscinus*) (11, 277). Although rats have historically been a primary carrier of plague, their current role in the ecology of the disease appears to be limited to Vietnam, Burma, China, Indonesia, Madagascar, and some areas of Africa and South America. Interestingly these areas tend to have the highest incidence of human plague (215, 217, Fig. 11).

Worldwide human disease. The third plague pandemic, although declining, is ongoing. From 1967 to 1993, WHO reported an average of 1,666 plague cases per year worldwide, with a 1967 high of 6,004 cases and a 1981 low of 200 cases (72, 97, 214, 215, 301, 302, 304) (Fig. 9B). Between 1967 and 1993, the average fatality rate was ~10%. Since 1979, 10 countries have reported over 200 plague cases: Tanzania, Vietnam, Zaire, Peru, Madagascar, Burma, Brazil, Uganda, China, and the United States, in decreasing order of frequency (304). The worldwide numbers are probably an underestimate due to undetected or unconfirmed cases and the routine failure of some countries to report plague cases. For example, although large natural and active foci are present, no human plague cases were reported to WHO until 1979 by China or until 1989 by Mongolia and the former USSR (81, 277, 304). The high level of plague cases in Vietnam during the war highlights the consequences of disrupting rodent and human populations as well as health and public services in a plague endemic area (43) (Fig. 9B). While several reporting countries have constant and relatively low numbers of plague cases each year, epidemics continue to occur. A 1992 Zaire epidemic had 191 cases and 78 deaths (40.8% mortality rate), and an epidemic in Peru from October 1992 to May 1994 recorded 1,151 cases with 54 deaths (4.7% mortality rate) (300, 303). The two outbreaks of plague in India from August to October of 1994 are the most recent and most publicized examples.

While it is estimated that plague was responsible for 12.5 million deaths in India between 1889 and 1950, the last laboratory-confirmed case prior to 1994 was in 1966 (46, 75). Although the 1994 outbreaks in the Beed District of the Maharashtra State and in the city of Surat, Gujarat State, could be independent epidemics, the most likely scenario, described below, links the two. The Latur earthquake and subsequent storage of grains in crumbled houses may have caused an influx of wild, plague-infected rodents into the area, leading to infection of urban rodents. By August 1994, villages in Maharashtra State began reporting flea nuisances and "rat fall" (large numbers of dead rats, originally named because of sick or dead rats falling from rafters of buildings). This area had 70 to 80% of the presumptive human plague cases, and all were the bubonic form of the disease. A person with secondary pneumonic plague may have traveled from this area to Surat, causing the pneumonic plague epidemic in that city (where nearly all the fatalities occurred). After early pneumonic cases were reported, over 600,000 of Surat's 2 million population fled the city and 110 patients with suspected plague cases escaped from the hospital before completing treatment. In addition, floods in Surat had disrupted municipal services, leading to an increased rodent population due to the accumulation of garbage and dead animals. Both the potential for active rodent transmission and the massive exodus of a potentially infected populace raised the possibility of spreading plague throughout India and beyond (75, 153, 193, 305); fortunately, this did not occur. The last plague case was reported on 11 October 1994. Of the over 6,300 patients with suspected cases of plague, 876 were positive for plague by serological testing (presumptive cases) and there were 54 fatalities. None

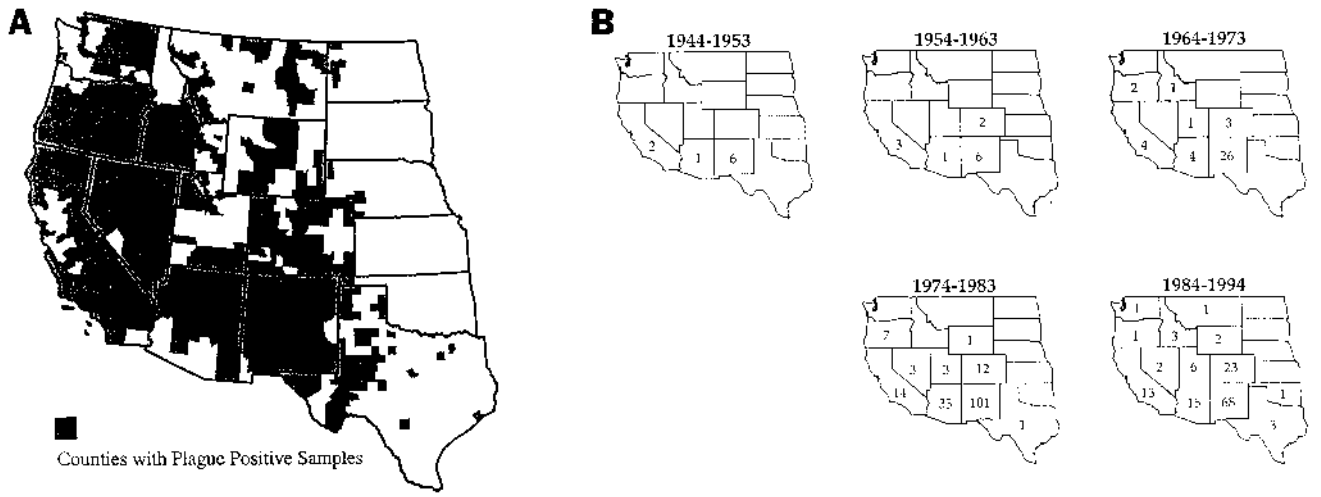

FIG. 12. U.S. distribution of plague. (A) Counties with plague-positive animal or flea samples, 1970 to 1994. Modified from a figure provided by K. L. Gage. (B) Human plague cases by state and decade. Numbers in states indicate plague cases in that state over the years indicated. Compiled from references 58, 97, and 98.

of the suspected cases in Bengal, Bihar, Punjab, Rajasthan, and West Bengal were positive by serological testing (46, 305).

The response to the 1994 epidemics in India provides examples of intensive and effective efforts as well as illustrating some major deficiencies. Within days of reports of the outbreak, nearly a million people received tetracycline prophylactically. Vast areas were sprayed with flea-killing pesticides, and a house-to-house search for suspected plague patients was instituted (42, 193). However, officials from the various Indian states failed to heed warnings from India Plague Surveillance Units of a potential for epidemics from a combination of natural disasters and a rise in both rodent populations and plague seropositive rats. Many patients with other common infections were misdiagnosed as having plague by physicians with no experience or educational training in dealing with plague. Although laboratory diagnosis of *Y. pestis* is reliable and specimens were collected appropriately, subsequent poor handling and a lack of experienced laboratory personnel and reagents for identifying the organism prevented proper epidemiological evaluation (42, 138, 153, 193). For these reasons, issues concerning the number of plague cases and the number of cases of disease caused by other infectious agents producing similar symptoms cannot be resolved.

The level of panic in India and the international community far exceeded the public health threat. Even if all suspected cases were confirmed and resulted in death, these numbers would be dwarfed by those due to other infectious diseases occurring yearly in India. It would appear that the reputation of pneumonic plague for contagious spread is vastly greater than the reality. Certainly, reports in the press contributed to the overreaction and panic in both the Indian populace and the international community (46, 71, 170, 305).

Human disease in the United States. At the turn of the century, plague was introduced into San Francisco and caused hundreds of cases during the next 25 years. The last urban plague epidemic with pneumonic spread among humans occurred in 1924 to 1925 in Los Angeles; since then, human plague cases have been primarily rural (sylvatic) and isolated (13, 97). American Indians have accounted for up to one-third of human plague cases due to their residence in areas of endemic foci and lifestyle (living in natural areas, shepherding, and hunting). Since 1977, the U.S. fatality rate has been 14.3%; this rate would probably be greater if more cases occurred outside of areas of endemic foci. Although the average of eight human plague cases a year since 1947 is low, the incidence has increased since 1975 (Fig. 9A) (58, 60, 97, 98). Peak years of human plague cases correspond to preceding or ongoing animal epizootics. By 1991, prairie dog repopulation from the previous epizootic in the southwestern United States was nearly complete; thus, conditions are suitable for another major epizootic, and there is some preliminary evidence that 1992 may mark its start (54, 58, 97). While the affected regions have been limited generally to the southwest and Pacific west coast, over the years the number of states reporting plague has increased to include states farther east and north (Fig. 12) (58, 97, 98). Since 1926, only one confirmed case of imported plague has occurred (57).

Two important new trends in plague epidemiology include increased peridomestic transmission as residential areas encroach upon formerly rural enzootic foci and significant transmission by domestic cats. No cat-related cases were reported prior to 1977. Since then, 18 human cases that were probably due to contact with infected cats have occurred, with 28% of these patients developing primary pneumonic plague. Over 25% of these cat-associated cases occurred in veterinarians or their assistants. Consequently, plague has become a potential but rare occupational risk factor for veterinary workers in areas of endemic foci. However, bubonic plague remains the most common form of the disease, with a flea bite being the primary form of transmission to humans (Fig. 10A and C). Progression to secondary pneumonic plague is rare, representing <6% of cases since 1988 (58, 97, 98, 102). Although transmission by cats is often emphasized, ground and rock squirrels remain the most common animal source of human infections (Fig. 10B). Rabbit involvement appears to be declining—nearly all cases occurred prior to 1987 (58, 97, 98).

CLINICAL DISEASE FEATURES

In the United States, most human plague cases occur from May to September (148) and usually present as one of three primary forms—bubonic, septicemic, or pneumonic. Over the past 24 years, the majority of patients in the United States have had either bubonic or septicemic plague (77) (Fig. 10C). Complications can arise, generally from delayed treatment; they include a number of clinical states, but secondary plague sep-

ticemia, pneumonia, and meningitis are the most common complications. Case fatalities for untreated bubonic plague range from 40 to 60%, while untreated septicemic and pneumonic forms of the disease are invariably fatal (73, 214). The average fatality rate for plague cases in the United States is about 14% (72). In most instances, fatal cases involve patients who do not seek treatment soon enough after becoming sick or are incorrectly diagnosed when they do see a physician. Death usually results from an overwhelming septic shock (44).

Bubonic Plague

Bubonic plague is the classic form of the disease. Patients usually develop symptoms of fever, headache, chills, and swollen, extremely tender lymph nodes (buboes) (Fig. 8) within 2 to 6 days of contact with the organism either by flea bite or by exposure of open wounds to infected materials. In addition, gastrointestinal complaints such as nausea, vomiting, and diarrhea are common (131, 278). Skin lesions infrequently develop at the initial site of an infection. Soreness in the affected lymph nodes will sometimes precede swelling (214), and any of the lymph node areas can be involved, depending upon the site of the initial infection. Buboes are typically found in the inguinal and femoral regions but also occur in other nodes (44, 69) (Fig. 8). Bacteremia or secondary plague septicemia is frequently seen in patients with bubonic plague (97). In one study, blood culture colony counts ranged from <10 to 4×10^7/ml. Not surprisingly, patients with colony counts greater than 100/ml had a higher fatality rate, although at least one patient reported to have 10^7 bacteria/ml of blood did survive (45).

Septicemic Plague

Primary septicemic plague is generally defined as occurring in a patient with positive blood cultures but no palpable lymphadenopathy. In the 30-year period between 1947 and 1977, about 10% of U.S. plague patients were diagnosed with primary septicemic plague (214). However, in the early 1980s in New Mexico, 25% of plague patients had primary septicemic plague (131). Clinically, plague septicemia resembles septicemias caused by other gram-negative bacteria. Patients are febrile, and most have chills, headache, malaise, and gastrointestinal disturbances. There is some evidence that patients with septicemic plague have a higher incidence of abdominal pain than do bubonic plague patients (131). The mortality rate for people with septicemic plague is fairly high, ranging from 30 to 50%, probably because the antibiotics generally used to treat undifferentiated sepsis are not effective against *Y. pestis* (73, 131, 214).

Pneumonic Plague

Primary pneumonic plague is a rare but deadly form of the disease that is spread via respiratory droplets through close contact (2 to 5 ft) with an infected individual. It progresses rapidly from a febrile flu-like illness to an overwhelming pneumonia with coughing and the production of bloody sputum. The incubation period for primary pneumonic plague is between 1 and 3 days. From 1970 to 1993, about 2% of the plague cases in the United States were diagnosed as primary pneumonic plague. The vast majority of these were contracted from infected cats (72, 77, 97, 102). This tendency and the danger posed to veterinarians are reasons why feline transmission of plague has received a great deal of attention in recent years (102, 266). The last case of pneumonic plague in the United States that was the result of person-to-person spread occurred during the 1924 to 1925 Los Angeles epidemic (177). Between 1970 and 1993, 12% of the U.S. plague patients developed pneumonia secondary to either the bubonic or septicemic form of the disease (77). In general, patients who develop secondary plague pneumonia have a high fatality rate.

DIAGNOSIS AND TREATMENT

Patient Diagnosis

A clinical diagnosis of plague is generally based on the patient symptoms and exposure history. Thus, bubonic plague is suspected in a patient with a painful, swollen lymph node, fever, and prostration who has been exposed to fleas, rodents, or other animals in the western United States. Septicemic plague is harder to diagnose on clinical grounds since the symptoms resemble those of other gram-negative septicemias. Likewise, pneumonic plague has been mistaken for other pulmonary syndromes (58). Recent data indicate that pneumonic plague should be suspected in persons exposed to infected pets, especially cats (72, 77, 97). In any case, if possible, samples should be obtained for epidemiological and laboratory diagnostic purposes before treatment is begun. However, treatment should not be delayed by waiting for the laboratory results. All suspected cases of plague are reported to the local and state health departments, and the diagnosis is confirmed by the Centers for Disease Control and Prevention (CDC). Only presumptive or confirmed cases are officially reported by the CDC to the WHO.

Laboratory Diagnosis

A laboratory diagnosis of plague is based on bacteriological and/or serological evidence (10, 214). Samples for analysis can include blood (if possible, four samples taken at 30-min intervals), bubo aspirates, sputum, cerebrospinal fluid in patients with plague meningitis, and scrapings from skin lesions, if present. Staining techniques such as the Gram, Giemsa, Wright, or Wayson stain can provide supportive but not presumptive or confirmatory evidence of a plague infection (214). A positive fluorescent-antibody test can be used as presumptive evidence of a *Y. pestis* infection (214). The antibody, available at many western state health department laboratories as well as the CDC, is directed against purified F1, a capsular antigen expressed predominantly at 37°C (80, 241). Samples that have been refrigerated for more than 30 h, from cultures that were incubated at temperatures less than 35°C, or from fleas will be negative (228). To confirm a diagnosis of plague by bacteriological means, it is necessary to isolate the organism. *Y. pestis* grows readily on most routine laboratory culture media but takes 2 days to achieve visible colonies. The colonies are opaque and smooth with irregular edges that have a "hammered-metal" appearance when magnified (214). A presumptive identification of *Y. pestis* can be made on the basis of biochemical tests. However, not all of the automated microbiological test systems are programmed to identify *Y. pestis* (77). In addition, rapid biochemical identification systems may not be reliable for identification of *Y. pestis* due to its lower growth rate. Lysis by a specific bacteriophage is used by the CDC to conclusively identify *Y. pestis* (10, 77).

Although not a rapid diagnostic technique, a serological response is often used retrospectively to confirm cases of plague. Paired serum samples, either acute and convalescent phases or convalescent and post-convalescent phases, are best, but a single serum sample can be used to provide presumptive evidence of plague. The samples are analyzed at the CDC for the presence of anti-F1 antibodies by a passive hemagglutina-

tion test (62). A fourfold rise or fall in the titer of paired serum samples is considered confirmatory for plague. A single serum sample with a titer greater than 10 in a person not previously infected or vaccinated against plague is presumptive evidence of recent infection. There have been reports in the literature on the isolation of unusual *Y. pestis* strains from patients, including one with a fatal case, where the organism apparently expressed reduced amounts of F1 (18, 132, 295, 297). While such isolates are rare, strains lacking F1 would not be identified by the present serological methods. Alternative methods for diagnosing plague have been developed, including enzyme-linked immunosorbent assays (52, 294), PCR analysis (195), and DNA hybridization studies (173). Enzyme-linked immunosorbent assays have been used to measure levels of either F1 antigen or antibodies to F1 in serum. Both antigen and antibody need to be assayed because patients who are positive for one are negative for the other (294). This technique, which is simple and reproducible, could be applied to other unique *Y. pestis* antigens to identify strains that are missing F1. McDonough et al. (173) used a fragment from the *pla* (structural gene for the plasminogen activator) region of the pesticin plasmid to specifically detect *Y. pestis* in ground flea suspensions or squashed whole fleas blotted onto nitrocellulose. While this method might be applicable in field studies, the limits of detection (a minimum of 10^5 bacteria) and the length of time required for DNA hybridization analysis make it unlikely that this approach will be useful as a clinical diagnostic tool. PCR has the advantage of being both rapid and sensitive; with primers to *pla* and *caf1* (structural gene for F1 antigen) regions, as few as 10 to 50 bacteria have been detected (195). However, none of these techniques have been sufficiently developed and evaluated to be routinely used by clinical laboratories.

Treatment

All patients suspected of having bubonic plague should be placed in isolation until 2 days after starting antibiotic treatment to prevent the potential spread of the disease should the patient develop secondary plague pneumonia. The antibiotics and regimes used to treat *Y. pestis* infections and as prophylactic measures are listed in Table 5. Streptomycin has been used to treat plague for over 45 years (176) and still remains the drug of choice. Because streptomycin is bacteriolytic, it should be administered with care to prevent the development of endotoxic shock. Due to its toxicity, patients are not usually maintained on streptomycin for the full 10-day treatment regimen but are gradually switched to one of the other antibiotics, usually tetracycline. The tetracyclines are also commonly used for prophylactic therapy, while chloramphenicol is recommended for the treatment of plague meningitis (17). While *Y. pestis* is susceptible to penicillin in vitro, this antibiotic is considered ineffective against human disease (73, 215). Other, newer antibiotics have been used to successfully treat experimental plague infections in mice but are not generally used to treat human cases (28). Antibiotic-resistant strains are rare and are not increasing in frequency.

TABLE 5. Recommended plague chemotherapies[a]

Antibiotic	Daily dose	No. of times taken/day	Dose interval (h)	Route
Streptomycin				
Adult	2 g	2	12	i.m.
Child	30 mg/kg	2–3	12 or 8	i.m.
Gentamicin				
Adult	3 mg/kg	3	8	i.m. or i.v.
Maximum dose	5 mg/kg	3	8	i.m. or i.v.
Child	6–7.5 mg/kg	3	8	i.m. or i.v.
Infant/neonate	7.5 mg/kg	3	8	i.m. or i.v.
Oxytetracycline				
Adult	300 mg	2–3	12 or 8	p.o.[b] or i.m.[c]
Child ≥9 yr	15–25 mg/kg (250 mg maximum)	2–3	12 or 8	p.o. or i.m.[c]
Tetracycline				
Adult	2 gm	4	6	p.o.
Child ≥9 yr	25–50 mg/kg	4	6	p.o.
Chloramphenicol	25 mg/kg as loading dose 50 mg/kg	4	6	p.o. or i.v.[c]
Doxycycline				
Adult	200 mg (day 1)	2–4	12 or 6	p.o.
	100 mg	1–2	24 or 12	p.o.
Child ≥9 yr	2 mg/lb (day 1)	2	12	p.o.
	1 mg/lb	1–2	24 or 12	p.o.

[a] Compiled from *Physician's Desk Reference*, 49th ed., Medical Economics Data Production Co., Montvale, N.J., 1995, and *CDC Plague Treatment Guidelines*, Document 351513, Centers for Disease Control and Prevention, Atlanta, Ga.
[b] p.o., per os (oral).
[c] Until oral medication tolerated.

PREVENTION AND CONTROL

Prevention of Disease

Both antibiotics and vaccines have been used to prevent *Y. pestis* infections from occurring in the first place. The tetracyclines are popular antibiotics for plague prophylaxis. Usually, antibiotics are given as prophylactic measures only to close contacts of pneumonic plague patients.

There are two types of plague vaccine currently used in various parts of the world. The live vaccine is derived from a Pgm^- attenuated strain, usually related to EV76, while the killed vaccine uses a formalin-fixed virulent strain of *Y. pestis* (178). A killed plague vaccine was developed in the United States in the early 1940s to immunize military personnel (292). Aside from manufacturers, not much has changed in the vaccine production or vaccination protocol since 1967 (55, 169). The current vaccine is manufactured by Greer Laboratories (Lenoir, N.C.) from *Y. pestis* 195/P and is administered intramuscularly as a series of three primary shots. The initial dose is 1.0 ml of a suspension containing 1.8×10^9 to 2.2×10^9 fixed bacteria/ml; this dose is followed 1 to 3 months later by a 0.2-ml dose. A third primary injection of 0.2 ml is given 5 to 6 months after the second. Two booster doses of 0.2 ml are administered at 6-month intervals, and additional booster shots are administered every 1 to 2 years. Only people at high risk for *Y. pestis* infection take the vaccine. These include individuals who work with or are potentially exposed to fully virulent strains and military personnel serving in areas where plague is endemic. Evidence that the vaccine is effective against plague in humans is indirectly based on the number of confirmed plague cases in U.S. military personnel during World War II and in Vietnam. Of the vaccinated individuals, none contracted plague during World War II and there were only three cases in Vietnam (178, 179).

There is interest in developing a new plague vaccine for a

number of reasons. First, the current vaccine causes an adverse reaction in a significant percentage of vaccinees. Although the reactions are generally mild, they can be severe (169). In addition, the antibodies directed against the vaccine wane relatively quickly, requiring booster inoculations every 1 to 2 years (299). Finally, experimental evidence indicates that the plague vaccine does not provide protection against the pneumonic form of the disease (67). One protein which has been examined as a potential candidate for a subunit vaccine is F1 (180). The gene encoding F1 (*caf1*) has been cloned and expressed in *E. coli*. Purified *Y. pestis* F1 protein and/or recombinant F1 preparations provided a degree of protection to mice lethally challenged with *Y. pestis* via aerosol, i.p., or s.c. routes (2, 241). F1 given to mice intragastrically does not protect against *Y. pestis* infection (270). However, mice force-fed a *Salmonella typhimurium* strain producing F1 were protected against a lethal s.c. challenge with *Y. pestis* (196). Although F1 is a protective antigen, studies have shown that it is not required for virulence in mice, raising the possibility that it is not an ideal vaccine candidate (39, 79, 80). Other factors that are essential for virulence, such as LcrV (V antigen) and Yops, are also being considered as potential vaccine candidates (157, 182, 189, 192, 275).

Control of Plague

Given the complexity of its life cycle, including the number and variety of potential animal and vector hosts involved, it is unlikely that plague will ever be completely eradicated by human endeavors. At one time, both the United States and the former USSR attempted to eliminate plague in a defined geographical area by exterminating the major plague carriers in that region (12, 82). This approach did not work in the United States, and while the Soviets were apparently successful in reducing the size of the focus, the effort was extremely labor-intensive and expensive (82) and the results are probably transitory.

In the United States, other approaches have been taken to reduce the number of human plague cases. Extensive surveys of rodents and fleas have been used to identify sources of plague and detect plague activity. Serological surveys of wild and domestic carnivores have also been used to monitor sylvatic plague. Finally, surveillance networks of people who report suspected plague activity, such as the sudden disappearance of rodents or the appearance of sick and/or dying animals, have been established (12, 13, 65, 187, 194, 267). States with active rodent and carnivore surveillance programs include Arizona, California, Colorado, New Mexico, and Texas (59).

In a study (187) done in Bernalillo County, N.M., the news media also participated by keeping the public informed of plague-positive areas. An extensive grass-roots campaign was used to recruit the help of citizens in reporting possible plague activity. Credible reports were investigated by screening the immediate and surrounding areas for rodents. Animals and fleas were tested for signs of *Y. pestis* infection. Wide areas were surveyed to determine the extent of the activity and the need for control measures. If evidence of plague was found, the rodent burrows were treated with 5% carbaryl dust or bait stations were set up to kill fleas. Plague-positive areas were posted, and announcements were carried by the news media. In some cases, recreational areas were closed. Finally, measures were taken to reduce the appeal of residential areas to rodents. Although plague activity in rodents and fleas did not decline, the program was successful in significantly decreasing the number of human cases (187). However, this approach was again very labor-intensive and probably not cost-effective on a wide-scale basis.

All human cases of plague in the United States are investigated by local and state health authorities as well as the CDC to confirm that the disease is due to *Y. pestis* and to determine the likely source of the infection and where contact with the organism occurred (12). Close contacts of plague patients are identified and monitored for any sign of the disease. Epizoological studies are conducted by local and state health authorities, with help from the CDC, to identify plague-infected animal and flea species and define the geographical extent of the epizootic (12). This information is used to determine if there is further risk of infection to humans and what control measures, if any, need to be taken. Insecticides such as carbaryl, Diazinon, and bendiocarb are fast, short-term measures to eliminate fleas, while permethrin is more effective for long-term control (13, 215). Rodenticides such as cholecalciferol can be used against rock squirrels (13, 215). However, flea control measures should be done concurrently with or precede the use of any rodenticide. In the United States, most human plague cases result from peridomestic exposure (59, 187). Residents of areas of endemic foci need to be aware of the disease and how to reduce their risk of exposure. The best control measures are to eliminate habitats for plague-susceptible rodents and treat domestic pets for fleas (167).

CONCLUDING REMARKS

Although plague is not currently a prevalent human disease, it is thoroughly entrenched in widespread zoonotic loci that are unlikely to be eliminated. The recent epidemics in India remind us that its resurgence merely requires favorable environmental and public health conditions.

Research on the disease mechanisms of *Y. pestis* has historically opened or contributed greatly to new areas of investigation of the host-parasite relationship. These include extrachromosomal inheritance of virulence determinants, bacterial iron acquisition, regulation of expression of virulence genes, and prevention of host immune responses via paralysis of phagocytic cells, as well as suppression and disruption of signal transduction.

Over the past several decades, research in these areas and on the epidemiology of plague has exponentially advanced our understanding of this organism and the disease it causes. However, many areas of uncertainty and ignorance remain. Does the long-term survival of *Y. pestis* depend upon enzootic hosts or modulation of bacterial virulence? What route of artificial infection mimics the flea bite? Are disparate results on the importance of some virulence factors due to undetermined bacterial defects, differences in animal models, or bacterial strains with differing virulence mechanisms? Also, our understanding of how some of these virulence factors promote virulence is rudimentary. A new vaccine that induces long-lived immunity against bubonic and pneumonic plague awaits development. The rapid progress made in recent years suggests that the answers to many of these questions and completion of some of these goals can be accomplished in the not too distant future.

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