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Review

Current Trends in Plague Research: From Genomics to Virulence

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Yersinia pestis is the causative agent of plague, which diverged from *Yersinia pseudotuberculosis* within the past 20,000 years. Although these two species share a high degree of homology at the DNA level (>90%), they differ radically in their pathogenicity and transmission. In this review, we briefly outline the known virulence factors that differentiate these two species and emphasize genetic studies that have been conducted comparing *Y. pestis* and *Y. pseudotuberculosis*. These comparisons have led to a better understanding of the genetic contributions to the differences in the virulence and pathogenicity between these two organisms and have generated information that can be applied in future diagnostic and vaccine development. Comparison of the genetic differences between *Y. pestis* and *Y. pseudotuberculosis* has also lent insight into the emergence of acute pathogens from organisms causing milder diseases.

Keywords: Genomic comparison; Virulence; *Yersinia* spp.

The genus *Yersinia* within the family Enterobacteriaceae consists of 11 species and includes three human pathogenic species: *Yersinia pestis, Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. *Y. pestis*, the causative agent of plague, is a close relative of and evolved from *Y. pseudotuberculosis*.[1,2] *Y. pestis* has caused approximately 200 million human deaths historically,[3] with at least 2000 cases of plague reported annually by the World Health Organization (WHO).[4] *Y. pestis* is also a potential agent of biowarfare or bioterrorism.[5] In contrast, *Y. pseudotuberculosis*, and to a lesser degree *Y. enterocolitica*, share a high degree of similarity with *Y. pestis* at the genomic level, but cause self-limiting, food-borne, enteric diseases that rarely lead to death. The current, available published genomic information includes whole genome sequences of fully virulent *Y. pestis* strains, CO92 and KIM;[6,7] human-avirulent *Y. pestis* strain, 91001;[8] and *Y. pseudotuberculosis* strain, IP32953.[9] Recently, several draft *Yersinia* spp. genomic sequences have been added to the National Center for Bioinformatics (NCBI) microbial genomes in progress web site (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). The generation of these complete genome sequences provides a new opportunity to make comparisons of these genomes in order to better understand genetic contributions to the stark difference in the virulence and pathogenicity of *Y. pestis* and *Y. pseudotuberculosis*. This information can also be compared with published DNA microarray analyses[10-12] to identify genes unique to or diverged in *Y. pestis* versus *Y. pseudotuberculosis* for more focused genetic analysis. Studies of gene expression during temperature transition in *Y. pestis*[13-15] are also very useful for further genetic dissection of *Yersinia* pathogenicity.

PATHOGENESIS OF *Y. pestis* AND OTHER YERSINIAE

Y. pestis causes a fatal infection in healthy humans called plague. Plague can take three forms: bubonic plague, septicemic plague and primary pneumonic plague. The first, Bubonic plague, occurs following the bite of an infected flea. Inflammation is suppressed initially and then induced by the uncontrolled growth of the pathogen in the host. The microorganism gains access to the circulatory system via the lymphatic system and reaches high numbers in the liver and spleen. *Y. pestis* can be recovered from the blood shortly before death. Septicemic plague, the second form, is characterized by recovery of *Y. pestis* from the blood of a patient in the absence of a bubo. Death in this case is caused

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by multiple organ failure that is thought to be a consequence of lipopolysaccharide (LPS) toxicity.[16] The third form of this disease, primary pneumonic plague, occurs by seeding of the lungs with the organism following spread from bubonic or septicemic disease. Secondary pneumonic plague occurs by spread of respiratory droplets through close contact with infected humans or animals. Secondary pneumonic infection results in a fulminate pneumonia followed by spread to the deeper organs. Without antibiotic treatment, death can occur within 24 hours. In contrast, *Y. pseudotuberculosis* does not generally cause illness in healthy humans, but can cause a mesenteric lymphadenitis in immunocompromised individuals following ingestion of contaminated food, while *Y. enterocolitica* causes gastroenteritis in healthy humans but not lethal disease.[3,17]

The difference between *Y. pestis* and *Y. pseudotuberculosis* is particularly striking considering that the genomic similarity of these species is very high and that their 16S rRNA sequences are identical.[18] Attributes contributing to the difference in disease potential between these two species were summarized previously by Brubaker.[19] Table 1 summarizes shared and unique properties of *Y. pestis* and *Y. pseudotuberculosis* that relate to virulence and physiologic differentiation.[20-37,64]

ROLE OF PLASMIDS IN *Y. pestis* VIRULENCE

The vast majority of virulence factors described in Yersiniae, including *Y. pestis*, are plasmid borne. Most documented *Y. pestis* virulence factors are encoded by a 70 kb plasmid that is common to all three *Yersinia* pathogen species and is, therefore, not species specific.[20,38-40] The 70 kb plasmid is called low calcium response plasmid (pLCR/pYV), since it confers the requirement for calcium in order to grow at 37ºC.[33] The specific nomenclature of pLCR varies in different *Yersinia* species. pLCR was designated pCD1 (denoting calcium dependence) in *Y. pestis*, pYVe (denoting *Yersinia* virulence) in *Y. enterocolitica*, and pYV or pIB1 in *Y. pseudotuberculosis*.[41] In all animal models examined, pLCR

Table 1. Characteristics of *Y. pestis* compared with *Y. pseudotuberculosis*.

Characteristic	Yps[a]	Yptb[b]	Function	Reference
pYV/pLCR	+	+	YOP production; absolutely necessary for virulence	20
pPst	+	-	Plasminogen activator production	21
pFra	+	-	Murine toxin and capsule synthesis	22
HPI	+	+/-[c]	Yersiniabactin siderophore production	23, 24
pgm loci	+	+[d]	Pigmentation on Congo red agar; promotes biofilm formation in flea vector for *Y. pestis*	25, 26
pH 6 antigen	+	+	Putative adhesin, antiphagocytic	27, 64
YadA	-	+	Adhesin	28
LPS O antigen	-	+	Cell structure	16
Invasin (Inv)	-	+	M cell translocation	29, 30
Ail	+/-[e]	+	Host cell attachment and serum resistance	31
IS1541 and IS100	++[f]	+	Insertion sequence elements	6, 29, 32
Low calcium response	++[f]	+	Regulation of YOPs and reduced growth at 37ºC in the absence of added Ca^{+2}	33
Motility at 26˚C	-	+	Chemotaxis	34
Rhamnose fermentation	-[g]	+	Sugar metabolism	34
Melibiose fermentation	-[g]	+	Sugar metabolism	34
Urease	-[g]	+	Nitrogen assimilation	35

[a] Yps, *Y. pestis*.
[b] Yptb, *Y. pseudotuberculosis*.
[c] Non pathogenic strains are negative.
[d] The 68-kb *pgm* locus is present in *Y. pseudotuberculosis* but is usually silent.[36,37]
[e] In some strains of *Y. pestis*, *ail* is interrupted by a copy of IS*285* but in others this locus is intact.[1]
[f] The ++ and + refers to the higher number of these insertion sequence elements or relative intensity of the LCR seen in *Y. pestis* vs. *Y. pseudotuberculosis*, respectively.
[g] Metabolic capability known to undergo reversion.

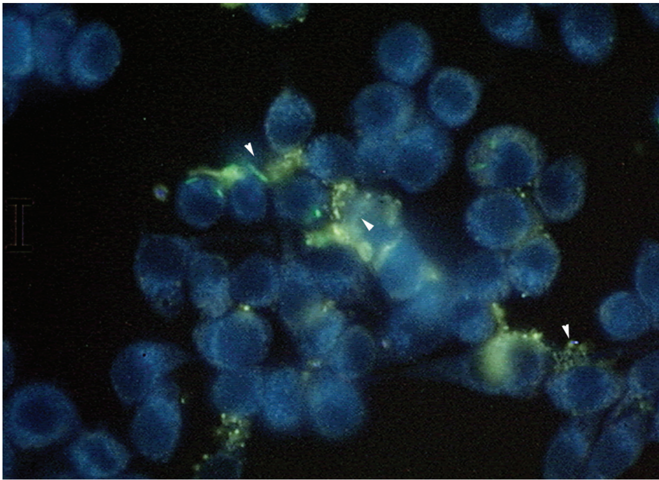

Figure 1. Fluorescence microscopy of *Y. pestis* interacting with mouse macrophage cells (RAW 264.7). Arrows: *Y. pestis* bacteria harboring GFP plasmid to produce green fluorescence.

has been shown to be absolutely critical for virulence.[33] Loss of this molecule results in complete avirulence of any of the pathogenic *Yersinia*, including *Y. pestis*.[33] The major virulence factors encoded by pLCR are the type III secretion system and the associated effector proteins, generally designated as *Yersinia* outer proteins (Yops).[17] Established pLCR-encoded effector proteins and virulence properties have been reviewed in greater detail elsewhere,[41] but will be summarized briefly here. YopE is a GTPase-activating protein and YopH is a protein tyrosine phosphatase, both of which are antiphagocytic. YopO/YpkA is a serine threonine kinase, YopM can transit to the host cell nucleus and has no known function, YopJ/P inhibits the proinflammatory cytokine, tumor necrosis factor-α, production and induces macrophage apoptosis. YopT is a cytotoxin that causes actin filament disruption. All the effector Yops listed here that have defined functions have been shown to disrupt intracellular signaling or result in cytoskeletal changes that interfere with phagocytosis.

The low calcium response (LCR) core region also encodes six genes that are involved in the control and translocation of the effector Yops to the target cell: *yop*N, *yop*B, *yop*D, *tye*A, *lcr*G and *lcr*V.[41] Recently, an *in vivo* experiment demonstrated that *Y. pestis* targets specific immune cells, dendritic cells, macrophages and neutrophils for intracellular delivery of Yop proteins, but does not target B and T lymphocytes.[42] Figure 1 demonstrates GFP-labeled *Y. pestis* bacteria interacting with mouse macrophage cells. It has also been suggested the Yops are used to disable these specific cell populations in order to annihilate the host immune response during plague infection. The DNA sequence of the chief LCR genes (known as the LCR gene core) is 98% identical between the *Y. pestis* plasmid, pCD1, and two sequenced *Y. enterocolitica* plasmids, pYVe8081 and pYVe227.[42-45] In contrast, the overall genomic homology between *Y. pestis* and *Y. enterocolitica* is approximately 23%.[46] Most identified virulence-associated genes encoded on pLCR are >92% identical in both DNA and deduced amino acid sequences between all the pLCR molecules sequenced to date across the Yersiniae.[41] However, the *Y. pestis* allele of *yop*M contains two extra copies of a repeat sequence (insertion sequence (IS) element) when compared with the DNA sequence on both sequenced *Y. enterocolitica* plasmids. This may be evidence of the importance of these elements in the emergence of pathogens from less virulent organisms. The impact that these DNA sequence differences have on the pathogenesis of *Y. pestis* versus *Y. enterocolitica* is yet to be determined.

The unique ability of *Y. pestis* to promote acute disease may be caused in part by accessory virulence factors mediated by two species-specific plasmids, pMT1/pFra and pPCP1/pPst, which are not shared by the enteropathogenic Yersiniae. There are two well-known virulence factors encoded by the approximately 100 kb plasmid, pMT1/pFra: murine toxin and fraction 1 (F1) capsular antigen.[22,47,48] The murine toxin is required for *Y. pestis* survival in the flea, and the F1 capsule is a highly immunogenic protein often assumed to be essential for full virulence of *Y. pestis*.[48] It is also thought that pFra may carry more potential virulence factors.[49] Du et al[50] reported F1 antigen has anti-phagocytic activity; however, strains of *Y. pestis* lacking F1 antigen expression (either containing pFra or not) have been reported to be virulent.[51-54] Although F1-negative strains examined differ biochemically and genetically, their demonstrated virulence suggests that F1 antigen and the entire pFra plasmid probably are not required for full virulence in tested laboratory animal hosts.[17]

Another plasmid specific to *Y. pestis* is pPCP1, a 9.6 kb plasmid which encodes a particulate plasminogen activator named Pla (Pst).[21,55] In host tissues, Pla facilitates dissemination of *Y. pestis*. Presence of Pla is essential for full virulence via transmission by flea bite and other peripheral routes of infection in epidemic strains of *Y. pestis*.[56] In contrast, in the mouse model some endemic strains of *Y. pestis*, as represented by strain Pestoides F, do not require pPCP1 to be fully infectious by the subcutaneous route.[57] Expression of the plague plasminogen activator in *Y. pseudotuberculosis* and *Escherichia coli* did not significantly influence virulence in mice,[58] and a Pla$^-$ mutant of *Y. pestis* retained virulence in guinea pigs. Lack of pPst did not lead to an increase in LD50 with either subcutaneous or respiratory challenge, but when challenged by aerosol with cultures grown at 37ºC to mimic the person-to-person pneumonic plague cycle, the average LD50 values decreased two-fold for pPst-containing *Y. pestis* and 10-fold for pPst-negative *Y. pestis* variants, respectively. These data indicate that historical outbreaks of human pneumonic plague in the Caucasus region, where there are natural plague foci in common voles, may have been caused by pPst-negative *Y. pestis* strains.[59] Taken together, the data pertaining to Pla and virulence suggest this protein may be more important for virulence by the subcutaneous route of infection compared to the respiratory route.

METHODS FOR IDENTIFYING NOVEL *Y. pestis* VIRULENCE FACTORS

The identification of a small number of *Y. pestis* unique genes encoded on plasmids that have been shown to be involved in virulence[41,60] suggests that other chromosomally encoded factors may be responsible for the enhanced pathogenicity of this organism. Recently, studies to identify chromosomally encoded proteins of *Y. pestis* that may serve as chromosomally encoded virulence factors (CEVF)[61] have been undertaken. Methods successfully used for finding *Yersinia* CEVFs are summarized elsewhere[61] and include functional complementation screening, targeted disruption studies and large-scale mutant screening. Such recombinant DNA experiments using *Yersinia* DNA led to the identification of three chromosomally encoded virulence genes: *yst*, *psa* and *inv*. Targeted disruption of presumed virulence genes in *Yersinia* has been undertaken based on studies in other bacteria.[61] Functional complementation screening consist of large-scale mutant screens for potential virulence determinants. *In vivo* expression technology (IVET) and signature-tagged transposon mutagenesis (STM) both proved useful techniques for identifying CEVFs.[62,63] However, most characterized CEVFs were identified in enteropathogenic Yersiniae; Revell and Miller[61] listed 24 in their review. Only *psa* and the high pathogenicity island (HPI) were characterized as virulence factors of *Y. pestis* with functions of anti-phagocytosis[64] and iron acquisition.[65] Darwin[66] recently summarized the techniques of IVET and STM applied to screening virulence genes in the *Yersinia* spp. IVET has been used only to study the virulence of *Y. enterocolitica*,[62,67,68] while STM has been employed to identify *Y. pestis* virulence associated genes.[69] Below we outline some methods that have been used by different groups or could potentially be used to screen for *Y. pestis* virulence factors. Genomic approaches will be emphasized.

Signature-tagged Transposon Mutagenesis (STM)

STM was developed for the isolation of bacterial virulence genes and is an insertional mutagenesis system that uses transposons carrying unique DNA sequence tags.[70] Tags from a mixed population of bacterial mutants represented in inocula and then in bacteria recovered from infected hosts were compared using DNA amplification, radiolabeling and hybridization analysis. When applied to a murine model, mutants with attenuated virulence were revealed by detecting tags absent in bacteria recovered from infected mice but present in the inocula.[63,70] This approach allows one to screen pools of mutants together to identify those specific mutants unable to survive *in vivo*[63] and has been used successfully for many different bacterial species, including the enteric *Yersinia* and *Y. pestis*.[69,71-73] Sixteen putative attenuated mutants of *Y. pestis* were identified using STM, including mutants with interruptions in genes such as the virulence regulator, *lcr*F; virulence-associated genes involved in global bacterial physiology (e.g., *pur*H, *pur*K, *dna*E and *gre*A); and genes encoding hypothetical polypeptides.[69] Flashner et al[69] also found that one of the avirulent mutant strains was disrupted in the *pcm* locus which was previously shown to be involved in bacterial response to environmental stress.

Table 2. Genetic loci of *Y. pestis* (strains CO92 and KIM) reported to be unique or highly divergent in comparison with *Y. pseudotuberculosis*.

Hinchliffe et al.[10]	Zhou et al.[11, 12]	Chain et al.[9]
YPO0387-0397	YPO0387-0396	YPO0387-397
YPO1087-1088	YPO1087-1098	YPO1668
YPO1094-1098	YPO2087-2095	YPO1670-1671
YPO1668-1672	YPO2096-2135	YPO2084
YPO2084-2130	YPO2271-2280*	YPO2087-2088
YPO2261		YPO2090-2097
YPO2271-2281*		YPO2100
YPO2503		YPO2114
YPO2380		YPO2261
YPO3910		YPO2484-2486
YPO4031-4032		YPO3609-3610

*Genes found in strain CO92 but not in strain KIM

Comparative Genomic Studies

Since diverse *Yersinia* genome sequences have now been completed, it is possible to use comparative genome analysis to select genes for further investigation of contributions to pathogenesis, particularly of genes unique to *Y. pestis*. Directed mutagenesis followed with animal testing is an expedient approach to the identification of undiscovered *Y. pestis* virulence factors. Generally, comparative genomic studies include DNA microarray hybridizations and *in silico* computational genomic analysis. The application of both methods to *Y. pestis* studies will be discussed in the following sections.

DNA microarray analysis

DNA microarrays have provided a powerful tool in genomics research. They are widely used to determine genome-wide mRNA expression levels in a single experiment[74] and are also used to probe for genetic differences and changes between strains at the intra- or interspecies level.[75,76] DNA microarray analysis represents an ideal methodology to compare closely related genomes. The technique was applied to compare the genomic contents of different strains, serotypes and biovars of *Y. pestis*, and its ancestor *Y. pseudotuberculosis*, before the DNA sequence of the latter was released.[10-12] Hinchliffe et al[10] reported that 11 regions in the genome for the strain CO92 were absent or highly divergent in all 10 *Y. pseudotuberculosis* strains tested by DNA microarray hybridization. In the same year, Zhou et al[11,12] also reported similar regions that were specific to *Y. pestis* (table 2).

These findings provide critical information, which can be used for directed mutagenesis of *Y. pestis*-specific genes, and thus, are useful for identifying *Y. pestis*-specific virulence factors or vaccine candidates. Indeed, a recent protein microarray study showed that the *Y. pestis*-specific genes YPO0388, YO2090 and YPO2093 encoded proteins that induced significant antibody response in the immunized rabbit.[77] These results yield clues about the role of these genes in pathogenicity and immunity, and beg for further investigation into these possibilities and for the potential use of these immunogens as vaccine candidates.

The DNA microarray is also an ideal tool for genome-wide analysis of gene regulation at the transcriptional level, as mentioned above. *Y. pestis*, the etiologic agent of plague, must acclimatize itself during its life cycle to shifts between the temperature of the flea (26ºC) and that of the warm-blooded host (37ºC). It is known that in the adaptation to these temperature shifts, significant phenotype variations occur such as in virulence, nutrient requirements, enzyme activity, capsule formation, and pigmentation production.[14] Differential gene expression at these two temperatures is thought to allow the bacterium to colonize its host efficiently, leading to its pathogenesis. Temperature-induced changes occurred in the transcription of genes encoding proven or predicted virulence factors, regulators, metabolism-associated proteins, prophages and hypothetical proteins.[14]

A few groups have used whole-genome DNA microarray analysis to investigate transcriptional regulation upon the upshift of growth temperature from 26ºC to 37ºC in a chemically defined medium with or without 2.5 mM CaCl.[13,14] Genes which were upregulated quickly after the shift to 37ºC may be valid *Y. pestis* virulence factor candidates, since early changes in transcriptional expression due to temperature shift probably reflect adaptation to the host rather than changes solely integral to the onset of bacteriostasis. Transcriptional profiles reported by Motin et al[13] revealed thermal induction of 51, 4 and 13 genes or open reading frames on pCD1, pPCP and pMT, respectively. In addition to type III secretion-associated proteins (e.g., Yops) that are known to be induced upon the upshift to 37ºC, 15 novel thermoregulated genes that may contribute to Yersiniae virulence were found on the three plasmids. The majority of the thermoregulated genes on pCD1 were downregulated by Ca^{2+} and matching previous reports that Yop genes and their regulators are all upregulated at 37ºC.[13,20] In contrast, Ca^{2+} concentration had little effect on chromosomal or plasmid-borne (i.e., pCP1, pMT1) genes. Motin et al[13] demonstrated that of the genes tested, 235 were thermally upregulated and 274 were thermally downregulated. Chromosomal genes involved in carbon and energy metabolism, nitrogen and amino acid metabolism, lipid metabolism, nucleotide metabolism and macromolecular synthesis were found to be strongly or modestly downregulated when the temperature was shifted to 37ºC. Additionally, a significant number of genes encoding putative exported, membrane or unknown proteins were upregulated at 37ºC.[13] Overall, the results of Motin et al[13] indicated that, in nature, plague bacilli favor fermentative patterns of metabolism during slow growth within the flea, but exhibit pronounced oxidative catabolism during rapid proliferation in the host.

Differential transcription during temperature shift also provided a useful list of putative virulence-associated genes,[13] particularly those genes that are upregulated at 37ºC. Han et al[14] reported similar findings in that of the available 2,646 genes represented on their microarray, 401 genes (15%) were differentially regulated by the same temperature shift. Within these differentially regulated genes, 155 genes (39%) were upregulated at 37ºC, while 246 (61%) were upregulated at 26ºC. Of these differentially regulated genes, 340 were chromosomally located and fell into 20 functional categories according to the *Y. pestis* strain CO92 genome annotation.

These studies provided a genome-wide profile of gene transcription induced by temperature shift that could shed light on the pathogenesis of this deadly pathogen and its interaction with host systems. However, actual measurement of *Y. pestis* gene expression within the host is a more direct way to examine genetic involvement in the process of pathogenesis. Lathem et al[78] used a gene-specific microarray to examine the expression of bacterial genes within the lungs of mice in an intranasal challenge model of primary pneumonic plague. Of the 4,037 *Y. pestis* open reading frames represented on their microarray, 405 were differentially regulated in bacteria recovered from lungs 48 hours after infection compared with bacteria grown in a rich broth medium at 37ºC for 12 hours.[78] Of these differentially regulated genes, 349 were chromosomally located, 48 on pCD1, and 4 each on pMT1 and pPCP1. The 235 *Y. pestis* genes that were upregulated included 27 genes involved in the biosynthesis of a number of amino acids, 33 genes associated with the Yop-ysc type III secretion system, and genes encoded in the virulence–associated pigmentation (*pgm*) region for yersiniabactin iron acquisition and hemin uptake. Interestingly, genes for two other virulence factors, the pH 6.0 antigen (*psa* operon) and the plasminogen activator Pla, were downregulated under these conditions. Genes encoding enzymes that deactivate reactive oxygen species were also downregulated, similar to what was observed by Motin et al[13] and again indicated these genes may not be involved in virulence. This study yielded both new and reinforcing information about the involvement (or lack thereof) of identified virulence factors in primary pneumonic infection, and also the potential involvement of previously unlinked systems. These genes can now be considered for more focused pathogenesis studies.

Genome comparisons-based computational analysis

Before the *Y. pseudotuberculosis* sequence was published, some novel virulence-associated genes were identified by comparing the genome sequences of six human pathogens known to cause persistent or chronic infections in humans (*Y. pestis, Neisseria gonorrhoeae, Helicobacter pylori, Borrelia burgdorferi, Streptococcus pneumoniae* and

Table 3. Genes found in *Y. pestis* strains CO92 and KIM but not in avirulent strain 91001 nor in *Y. pseudotuberculosis* strain IP32953[6-9]

Gene in CO92	Gene in KIM	Predicted product
YPO2096		Hypothetical phage protein
YPO2097		Putative phage protein
YPO2098	y2216	Putative phage lysozyme
YPO2099	y2215	Putative prophage endo-peptidase
YPO2100	y2214	Phage regulatory protein
YPO2101	y2213	Hypothetical phage protein
YPO2102	y2212	Hypothetical phage protein
YPO2103	y2211	Putative phage terminase (pseudogene)
YPO2104	y2210	Transposase for the IS285 insertion element
YPO2106	y2209	Hypothetical phage protein
YPO2108	y2207	Hypothetical phage protein
YPO2109	y2206	Hypothetical phage protein
YPO2110	y2205	Putative phage protein
YPO2111	y2204	Hypothetical phage protein
YPO2112	y2203	Conserved hypothetical phage protein
YPO2113	y2202	Hypothetical phage protein
YPO2114	y2201	Hypothetical phage protein
YPO2115	y2200	Hypothetical phage protein
YPO2116	y2199	Putative phage protein
YPO2117	y2198	Putative phage tail protein
YPO2118		Putative phage minor tail protein
YPO2119	y2197	Putative phage tail protein
YPO2120	y2196	Putative phage protein
YPO2122	y2195	Putative phage protein
YPO2123	y2194	Putative phage minor tail protein
YPO2124	y2193	Putative phage protein
YPO2125	y2192	Putative phage regulatory protein
YPO2126	y2191	Putative phage regulatory protein
YPO2127	y2190	Putative phage-related membrane protein
YPO2128		Putative phage-related lipoprotein
YPO2129	y2189	Putative phage tail assembly protein
YPO2130		Putative phage protein
YPO2131	y2188	Putative phage host-specificity protein
YPO2132	y2187	Hypothetical phage protein
YPO2133	y2186	Hypothetical phage protein
YPO2134	y2185	Putative phage tail fiber assembly protein
YPO2135		Hypothetical phage protein

Treponema pallidum).[79] Using a bioinformatics approach, 17 conserved genes (*vag* genes) with unknown function were identified among a selection of these six human pathogens' genes. Five of those *vag* genes were directly associated with virulence in animal experiments. For example, *Y. pestis vag*A insertion and nonpolar deletion mutants were not affected in growth, yet were strongly attenuated for virulence compared to a wild-type strain.[79]

Whole-genome DNA sequences were completed of two fully virulent *Y. pestis* strains, CO92 and KIM;[6,7] one avirulent *Y. pestis* strain, 91001;[8] and one *Y. pseudotuberculosis* strain, IP32953.[9] This provided an opportunity to make comparisons of these genomes in order to gain a better understanding of genetic contributions to the differences in the virulence and pathogenicity between these two species. Chain et al[9] completed whole-genome comparisons of *Y. pestis* (strains KIM and CO92) and *Y. pseudotuberculosis*. Their computational analysis identified 112 genes specific to *Y. pestis* (represented by strains KIM and CO92) distributed in 21 clusters throughout the genome and ranging in size from 300 bp to 41.7 kb (table 2). Approximately three categories of genes were identified in these 21 regions: 39 (35% of the total) were hypothetical or conserved hypothetical genes, 59 (53%) were phage or transposon-related genes and 14 genes (12%) were attributed another putative function. Among those genes with an ascribed function were membrane proteins, lipoproteins, a putative esterase, a DNA-binding protein and a methyltransferase. A 9 kb filamentous prophage region in strain CO92 previously believed to be specific to the Orientalis biovar of *Y. pestis* was also found in some members of the Antiqua biovar, but was absent from all *Y. pseudotuberculosis* strains tested. Of the 112 genes uniquely associated with the two *Y. pestis* genomes, only 32 genes located in six clusters were present in all 19 *Y. pestis* strains and absent from all *Y. pseudotuberculosis* strains examined. These 32 *Y. pestis*-unique genes are obvious targets for mutagenesis in pathogenesis studies.

A whole genome sequence of *Y. pestis* strain 9100l[8] provides an even wider window to more fully understand the genetic pathogenesis of *Y. pestis*. Strain 91001 is reported to be representative of an atypical *Y. pestis* biovar that causes endemic plague in local rodents, such as Brandt's voles, but is avirulent in humans and therefore diverged from strains capable of causing epidemic disease.[80] The genome of 91001 consists of one chromosome and four plasmids (pPCP1, pCD1, pMT1 and pCRY). The pPCP1 plasmid of strain 91001 is almost identical to the counterparts from reference strains (CO92 and KIM). Compared with the molecules encoded by epidemic strains CO92 and KIM, plasmids pCD1 and pMT1 have slightly different architecture. The pCRY molecule is a novel cryptic plasmid identified during this study. It is a 21,742 bp plasmid that harbors a cryptic type IV secretory system and proved to be unnecessary for pathogenicity. Many genetic variations were found in comparing the chromosomes of these three *Y. pestis* strains. Rearrangements mediated by insertion elements appear to have dramatically altered the structure of the strain 91001 chromosome in comparison with strains CO92 and KIM. A very interesting finding was the absence of a large genomic region in the 91001 chromosome (table 3) that may contribute to its host specificity and unique nonpathogenicity in humans. This 33 kb region specific to strains CO92 and KIM seems to encode prophage proteins with no obvious virulence motifs. Most of the other genes found to be specific to *Y. pestis* versus *Y. pseudotuberculosis* were putative phage genes or phage-related genes.[10,11] It is thought that prophages are a major driving factor in the evolution of bacterial genomes by mediating lateral gene transfer.[81] It is well known that virulence genes are encoded on bacteriophage.[82-85] Therefore, it is reasonable to assume that this 33 kb prophage-like fragment might provide additional underscribed virulence mechanisms that enhance pathogenicity in strains CO92, KIM and other fully virulent *Y. pestis*. Directed mutagenesis of this 33 kb region may be an effective approach to determine *Y. pestis* genes that are involved in virulence in humans and in the ability to cause epidemic disease.

Genome structure of Y. pestis *and pathogenesis*

The genome of *Y. pestis* is altered, relative to that of *Y. pseudotuberculosis,* on a number of levels. *Y. pestis* genome sequences contain many pseudogenes.[9,86] In comparison with *Y. pseudotuberculosis*, Chain et al[9] found 149 pseudogenes in *Y. pestis*. They also determined that 317 genes were entirely absent from the *Y. pestis* genome. Their comparative analyses indicated that as many as 13% of *Y. pseudotuberculosis* genes are absent or otherwise no longer function in *Y. pestis.*[2] The *Y. pestis* genome appears to have been altered through extensive insertion sequence-mediated genome rearrangements, including deletions. Chain et al[9] concluded that reductive evolution through massive gene loss in *Y. pestis*, which resulted in elimination and modification of preexisting gene expression pathways, appears to be a more important force than acquisition of genes in the evolutionary divergence of *Y. pestis* from *Y. pseudotuberculosis*. The host and vector of plague foci can differ markedly; these environments are driving forces that contribute to shaping the bacterial genome. Adaptive microevolutionary analysis of different natural plague foci in China, based on pseudogene profiles, was undertaken by Tong et al.[86] This group suggested that adaptation to different niches by *Y. pestis* was mediated by different mechanisms of genome change such as gene acquisition or loss, and point mutations. The accumulation of these changes allows *Y. pestis* a diversity of genotypes with each potentially exhibiting different epidemiological, etiological and virulence features.[86] These results suggest a sobering example of how a highly virulent epidemic clone can suddenly emerge from a less virulent one by genetic changes in a closely related progenitor.

Selection of Target Genes for Directed Mutagenesis

The availability of complete *Yersinia* genome sequences has provided ample opportunity for genomic comparisons to identify genes involved in enhanced human virulence by *Y. pestis*. A systematic mutational analysis of genes in their

normal location can provide significant insight into their function. A number of general allelic replacement methods can be used to inactivate bacterial chromosome genes, but these all require a relatively complicated process, such as creating the gene disruption on a suitable plasmid before recombining it onto the chromosome. Recently a very efficient one-step recombination system was developed for chromosome engineering in *E. coli*.[87-89] The phage lambda red recombinase system was exploited for this purpose.[87] By using conditions favoring efficient electroporation of appropriately designed polymerase chain reaction products into lambda red backgrounds, any gene of choice can be recombined directly into the chromosome while requiring only a single marker encoded within the polymerase chain reaction product. The only information needed to do this is the sequence of the gene and its immediate flanking region. This efficient genomic engineering process has also been successfully adapted to *Yersinia* mutagenesis[90] and used as a tool for directed mutagenesis for *Y. pestis* virulence factor identification.

MODELS FOR *Y. pestis* PATHOGENESIS STUDIES

Mice, rats and guinea pigs have been used as animal models of plague since the discovery of the plague bacillus, primarily to confirm the identity of *Y. pestis* isolates from suspected plague cases.[91,92] Rats and guinea pigs infected with *Y. pestis* show the enlargement of regional lymph nodes, septicemia and rapid death that resembles human bubonic plague.[93] In contrast, mice do not develop typical buboes, although their lymph nodes are infected and sometimes enlarged. However, the considerable variation in susceptibility to plague among individual rats[94] has made mice the most commonly used model for molecular pathogenesis and vaccine studies since the 1950s.[95] The monkey has also been used as an animal model for plague,[96] though not extensively due to the model's high cost. Recently Sebbane et al[97] developed and characterized a rat model of bubonic plague in the inbred Brown Norway strain of *Rattus norvegicus* that closely resembled descriptions of human bubonic plague. This animal model should be useful in pathogenesis studies to examine the role of *Y. pestis* virulence factors at each stage of the disease.[97]

Although mammalian animal models are widely accepted for assessment of *Y. pestis* pathogenesis, an alternative approach using non-mammalian, genetically tractable host organisms is attractive.[98] Animal models for studying the pathogenesis of bacteria as mentioned above are genetically unwieldy and do not lend themselves easily to experimental analysis.[98] Also, there are cost and ethical restraints on the use of laboratory mammals. It has been demonstrated that the nematode *Caenorhabditis elegans* can be infected by human *Yersinia* pathogens as well as other bacteria.[99] Moreover, the mechanisms of invasion and host responses may parallel that in mammalian cells. Recently a *C. elegans* model of *Yersinia* infection was used to investigate pathogenicity and the evolutionary divergence of the genus.[100] In this study, three strains of *Y. pestis*, including a strain lacking pMT1, caused blockage and death of *C. elegans*. One strain lacking the haemin storage (*hms*) locus had no effect on the host. Similarly, 15 strains of *Y. enterocolitica* caused no deleterious effect on the host. In contrast, *Y. pseudotuberculosis* exhibited different levels of pathogenicity in this model.

CONCLUSION

Plague research now stands at the precipice of a new era with the biological implications of recently generated genomic sequences still largely unexplored. The genomic approaches outlined here will significantly benefit the search for novel *Y. pestis* virulence determinants and will further our understanding of global regulatory systems that are integral to this pathogen's life cycle, and its survival in disparate hosts. Comparative genetic studies of *Y. pestis* and *Y. pseudotuberculosis*, including DNA microarray analysis and computational sequence analysis, can provide basic information on the genetic differences between these two highly related species. These genetic differences will then be targeted for more intensive study to determine their contribution to the contrast in disease potential between these species. Undiscovered genes involved in virulence are likely to be identified as newly developed technologies are employed, including molecular tools such as more efficient and powerful mutagenesis systems and also more suitable infection models, including refined mammalian and novel nonmammalian systems. Discoveries of new *Y. pestis* virulence factors can, in turn, be exploited for medical applications including diagnostic, vaccine and drug development. Application of these techniques to study the life cycle of *Y. pestis* in the arthropod host may yield a more detailed understanding of molecular events within that host and may reveal potential applications to control endemic foci. Finally, a deeper understanding of the plague disease process will provide new information about bacterial pathogenesis, including a detailed molecular model of the rapid emergence of a lethal pathogen from a moderate pathogen, a potential to understand the molecular basis of host range and epidemic eruptions, as well as the possible elucidation of shared mechanisms of bacterial pathogenesis that are yet to be described.

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