

Bioscience

Innovation for Health and Security

Backgrounds and Near-neighbor Studies Underscore Challenges of Designing Assays to Support Biosurveillance

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Joint U.S. Environmental Protection Agency,
Department of Homeland Security
Conference on Real-World Applications and
Solutions for Microbial Risk Assessment



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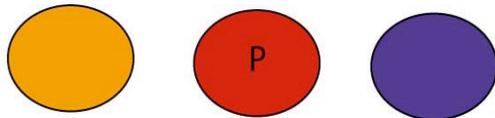
Backgrounds and Near-neighbor Studies

This talk is a synthesis of two lines of study at conducted at Los Alamos National Laboratory

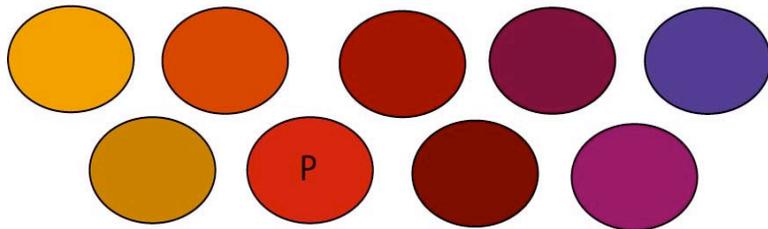
- Backgrounds surveys for pathogen and neighbor groups in the environment performed by Cheryl Kuske. Funded by DOE-CBNP and DHS.
- Re-evaluation of pathogen DNA signatures and assays considering new genomic sequence data and diverse neighbor panels performed by Jason Gans and Norman Doggett. Funded by DHS.

and a consideration of relevant works in the literature.

Closely related species pose the greatest potential for false positive detection.



Pathogen and related cultured species

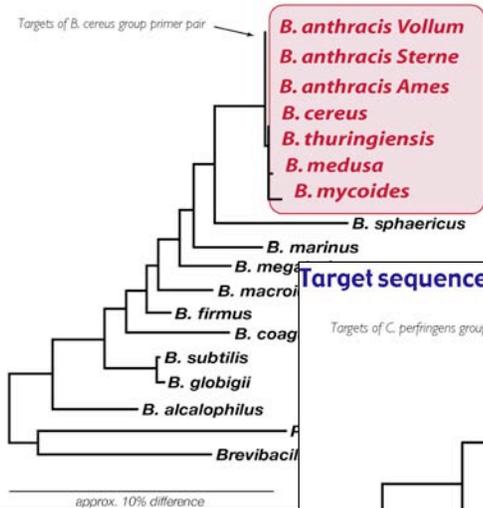


Pathogen and related environmental species

- Bacterial genomes are fluid, and genetic exchange across closely related species is not unusual.
- Near neighbors share DNA sequences and therefore protein, cell surface and physiological traits with target pathogens.
- Close relatives may or may not be pathogenic (depends on clinical evidence) and may share virulence factors.
- Many near neighbor species in the environment may be difficult to culture and absent in culture collections.

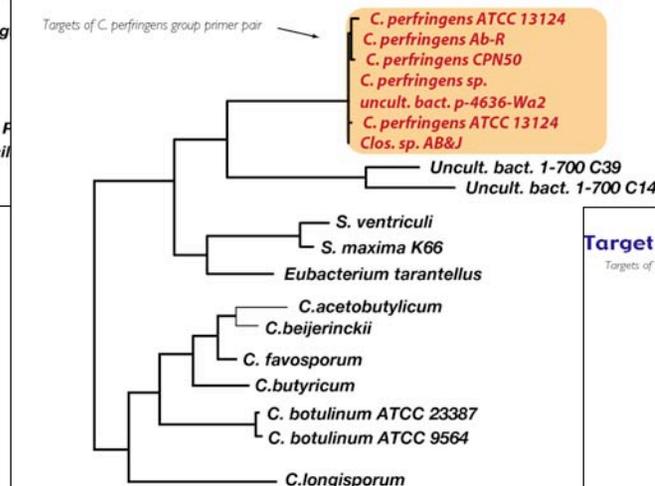
Developed suite of Pathogen Phylogenetic Group Specific Primers

Target organisms for Bacillus cereus group 16S rDNA primers



Bacillus anthracis
Yersinia pestis
Clostridium perfringens
Francisella tularensis
Brucella melitensis

Target sequences of the Clostridium perfringens group primers

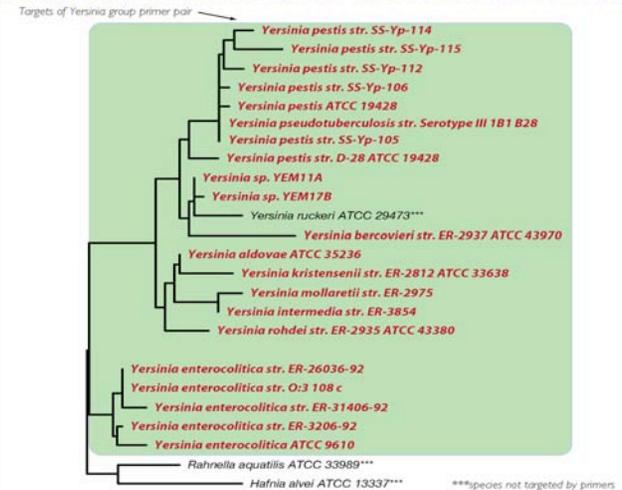


Burkholderia pseudomallei
Clostridium botulinum

Cheryl Kuske

Cloning and sequencing of PCR products identifies species or strains.

Target species of Yersinia genus- specific 16S rDNA primers



Environmental Backgrounds Surveyed

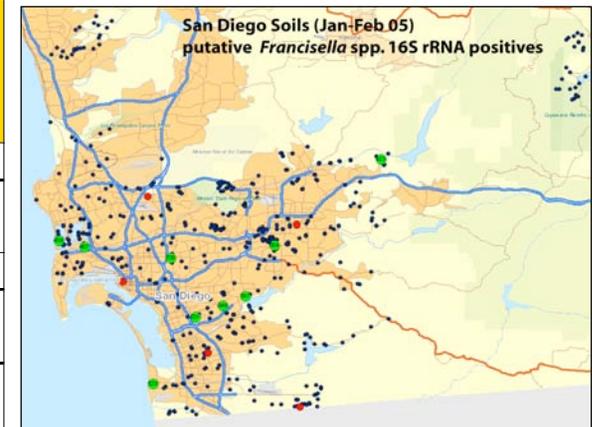
1. 11,000 EPA PM2.5 filters from 15 US cities - all pathogens
2. 100 national soil samples - all pathogens
3. Denver & Chicago 50 soils each - follow up on EPA findings
4. DC 150 soils/2 seasons - all pathogens
5. Houston 350 soils, 50 soils, 270 soils - Francisella & Brucella
6. San Diego 400 soils plus 5 months/5 locations Aerosol filters - all pathogens
7. Martha's Vineyard 3 months/10 locations Aerosol filters - Francisella



Example of BioWatch City Survey Results - San Diego

- Relatives of *B. anthracis* found in 90% of soils and 95% of aerosols
- Novel *Francisella* spp. detected in ten soils,
- *Brucella* relatives detected in 12-15% of samples, 2 soils and 1 aerosol positive with BASIS *Brucella* primer set.
- *Clostridium perfringens* also widespread in soils and aerosols
- *Yersinia* spp. present in soils and aerosols, but no *Y. pestis* detected
- A subset of samples was analyzed by LRN assays (SD, Kevin Poth)

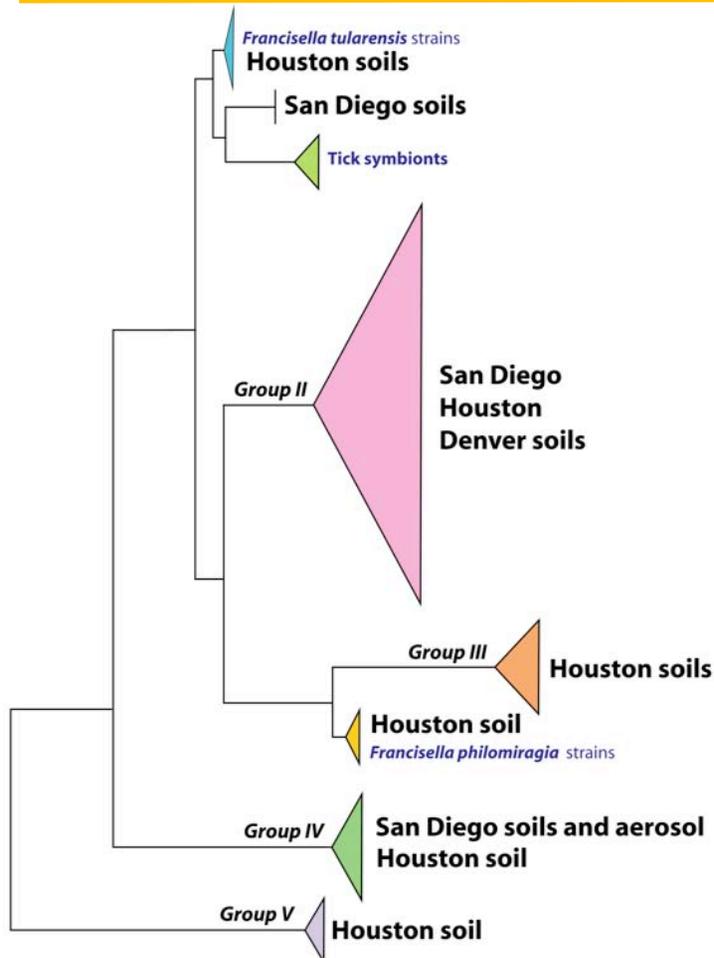
Phylogenetic Group Primer Set	% of soils positive (of 424)	% of aerosols positive (of 86)	Virulence Genes & Specific Primers	San Diego LRN Assays
<i>B. cereus</i> group	90%	95%	all <i>pag</i> negative	two aerosol 1° +
<i>Brucella</i> and rels	15%	12%	three BASIS positives	no +
<i>Francisella</i> spp.	2%	1%	in progress	one aer. 1° +
<i>Yersinia</i> spp.	4%	16%	all other <i>Yersinia</i> spp.	no +
<i>C. perfringens</i>	57%	85%	toxin gene in progress	no +



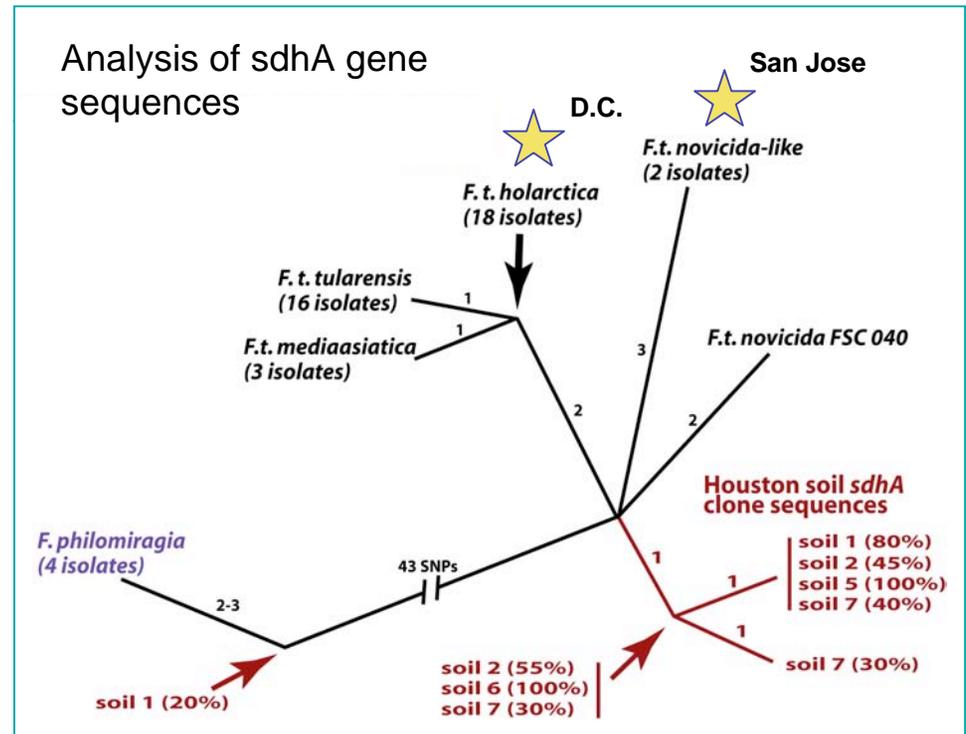
Cheryl Kuske

Slide 6

Identification of *Francisella* species in soils and aerosols of 5 BioWatch cities



Schematic phylogenetic tree of environmental *Francisella* spp. 16S rRNA sequences



Our analysis of additional samples indicates that novel *Francisella* spp. are diverse and widespread in soils.

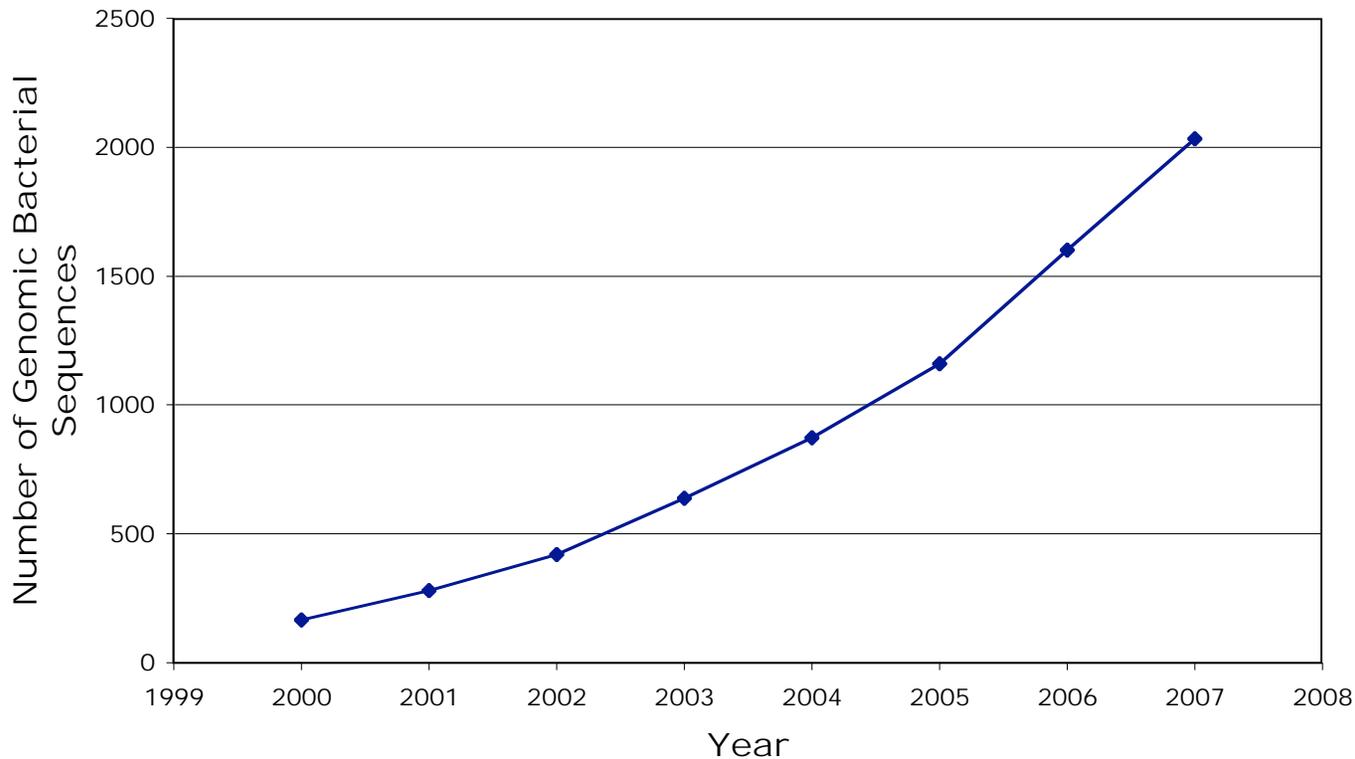
Parallel testing of DC, San Diego, and Martha's Vineyard samples by CDC/BioWatch

General Trends from Environmental Backgrounds Surveys

- *Bacillus* spp. very closely related to *B. anthracis* are present in all cities surveyed (15 cities EPA samples + 5 cities soil samples + 2 cities BioWatch samples). Present in most samples, both aerosol and soil. Some of the close relatives may be pathogens and/or share plasmid traits with *B. anthracis*.
- We have discovered at least 5 new (potential) species of *Francisella*, and new biovars of *F. tularensis* in urban environments, for which we have no data on pathogenicity. One of the new groups was found in San Diego, Houston & Denver in limited surveys.
- While we find many *Yersinia* species in soils, we rarely find them in aerosols, and we have not found *Y. pestis* in the soil or aerosols tested.
- *Brucella*-related organisms are widespread and abundant in soils in the cities we have surveyed. *Ochrobactrum* is prevalent in San Diego aerosols.
- *Clostridium perfringens* is also very prevalent in urban soils and aerosols.
- In San Diego, the relative frequency of positives for each pathogen group was similar for soils (collected at one time point) compared with 6 months of weekly aerosol samples, suggesting that a single soil sample survey may predict what is seen more sporadically in aerosols.

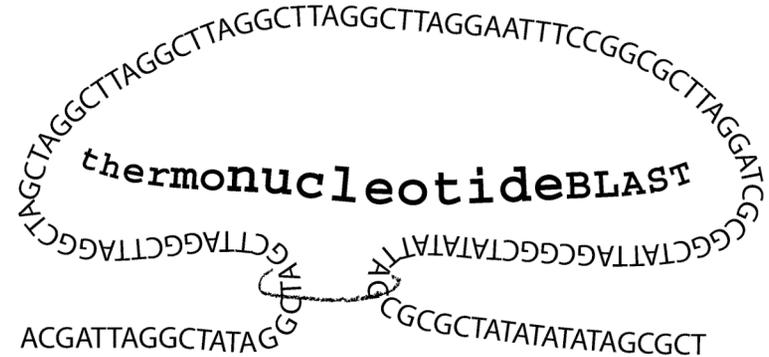
Why consider re-screening of signatures?

Genbank Growth (2000 - 2007)



- Significantly more sequences available now than when assays were originally designed

In silico screening with ThermonucleotideBLAST



- Hybridization-based alignments
 - SantaLucia parameters (salt and strand parameters to match Primer3)
- Allows for insertions/deletions, loops and bulges
- Complete (all possible primer combinations considered)
- Dynamic programming generates alignments minimizing ΔG
- Huge database search capability
 - All of Genbank searched with target assays in 30 min
- Multi-processor (using MPI or OpenMP)

5' AGCTAATGAGCGATTAGAC 3' 3' TCGATTACTCGCTAATCTG 5' Dimer alignment size = 19 Dimer T_m = <u>53.6082 °C</u>	5' AGCTAATGAGCG-ATTAGAC 3' 3' TCGATTACTCGCaTAATCTG 5' Dimer alignment size = 20 Dimer T_m = <u>43.0653 °C</u>	5' AGCTA-ATGAGCG-ATTAGAC 3' 3' TCGATtTACTCGCaTAATCTG 5' Dimer alignment size = 21 Dimer T_m = <u>33.1816 °C</u>
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In the example above, T_m drops $\sim 10^\circ\text{C}$ per gap

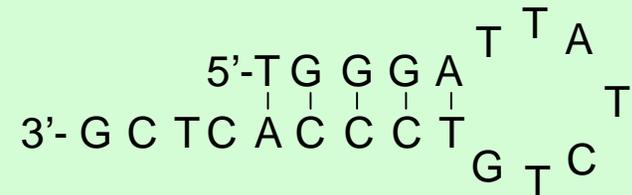
Bacillus anthracis

ba28

- Predicted to detect *B. cereus* G9241, *B. cereus* ATCC 14579 (66.8 °C; perfect match) and *B. thuringiensis israelensis* (58.8 °C)

ba29 & ba30

- Potential hairpin formation
 - Probe ba29 hairpin @ 52.7 °C
 - Forward ba30 hairpin @ 50.5 °C
 - Reverse ba30 hairpin @ 51.8 °C



Yersinia pestis

yp10

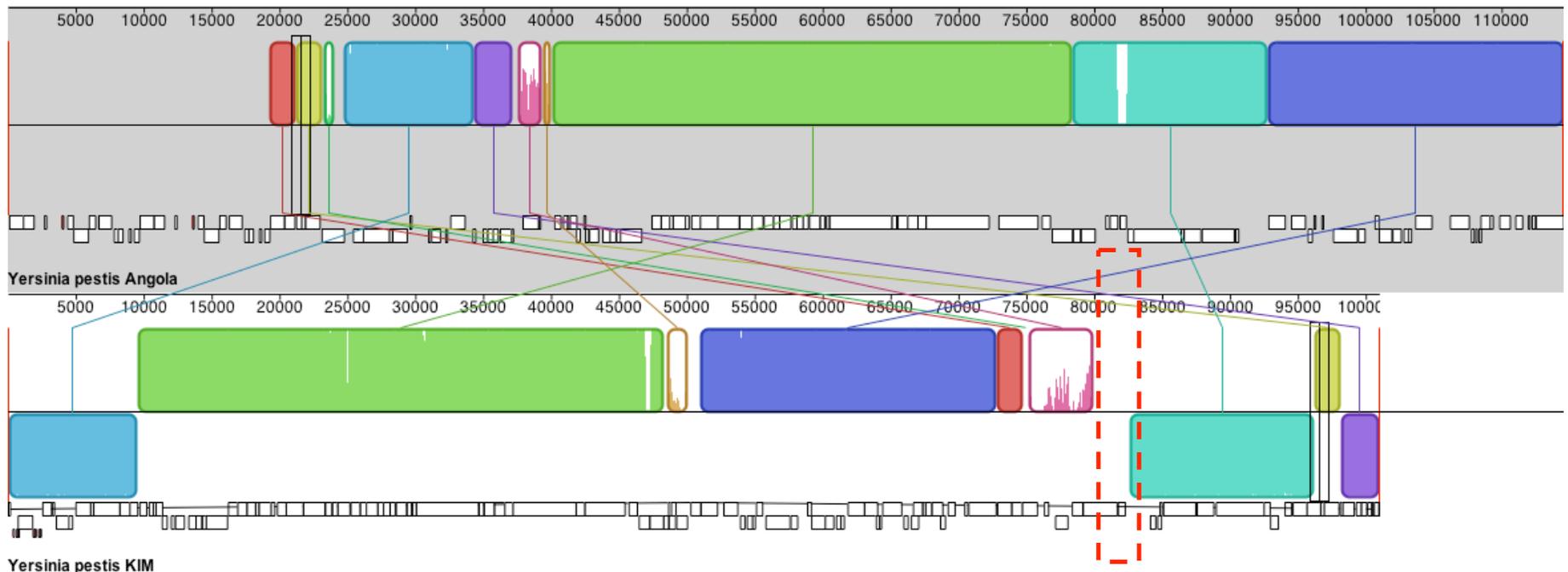
- Fails to detect *Y. pestis* Angola. This strain is detected by yp12 which also targets the pMT1 plasmid.

yp15

- Predicted to detect *Salmonella enterica* Typhi plasmid pHCM2 (57.4 °C); 2 base 3' clamp
- Fails to detect the pMT1 plasmid in *Y. pestis* Antiqua

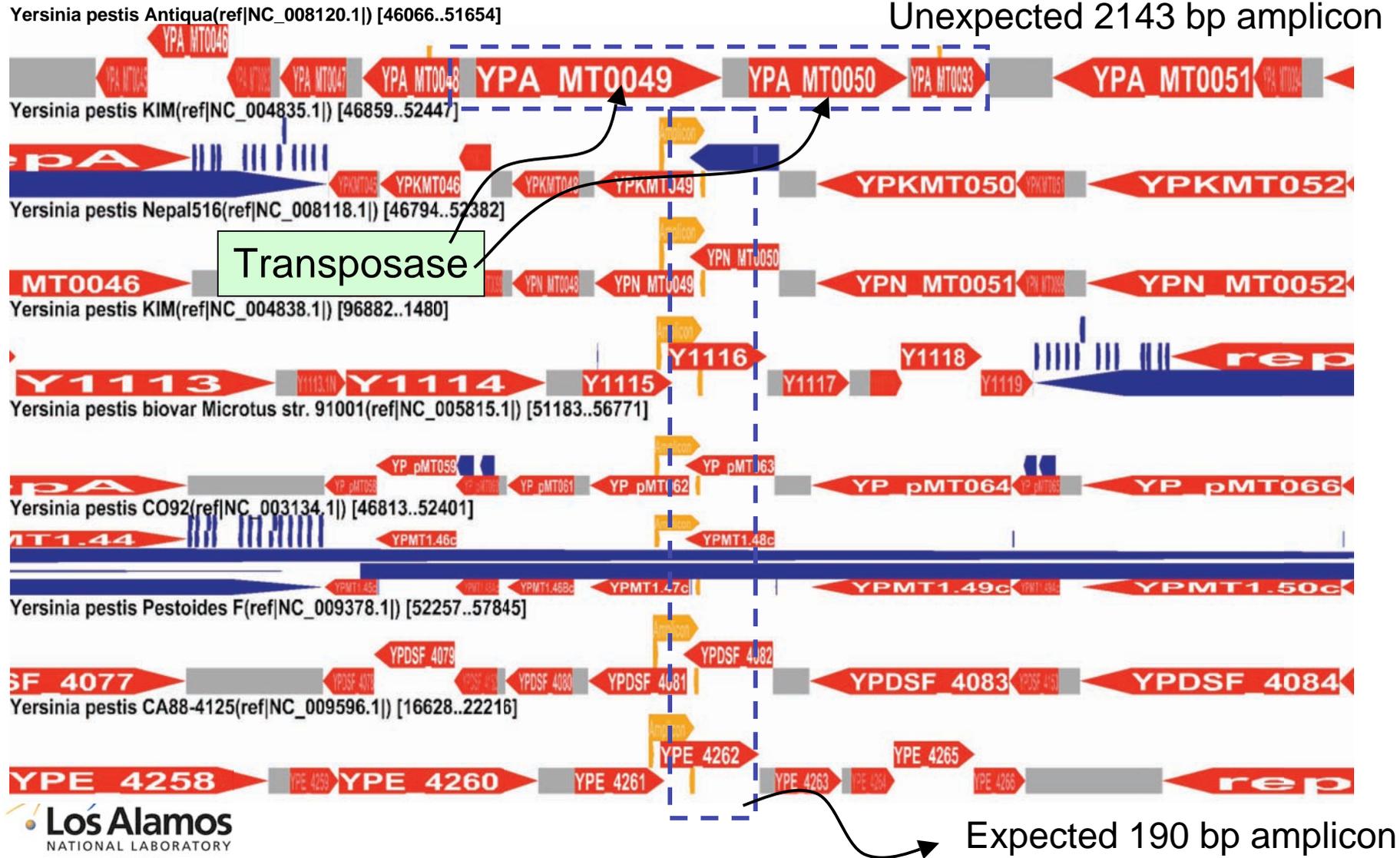
Deletion within pMT1 plasmid of *Y. pestis* Angola accounts for false negative of yp10 signature

Alignment of pMT1 plasmid in *Y. pestis* KIM and pMT-pPCP plasmid in *Y. pestis* Angola



caf1 gene (F1 capsule antigen) targeted by yp10 not present in *Y. pestis* Angola

Insertion in pMT1 plasmid of *Y. pestis* Antiqua accounts for failure of yp15 signature.



Francisella tularensis

ft16 and ft19 (overlapping amplicons)

- Matches multiple uncultured proteobacterium clones in Genbank (from Barns *et al.* 2005)
- Reduced affinity to ITIC *Ft* isolate

ft18

- Predicted to detect *F. philomiragia* (57.2 °C, 3 base 3' clamp)
- Partial match to 30% of available *Ft* genomes (primer T_m ~10 °C less)
- Targets high copy number transposase

Bacillus anthracis: TaqMan real-time assays

B. anthracis target results

Genus/species	Strain	ba25	ba25	ba26	ba26	ba27	ba27	ba28	ba28	ba29	ba29	ba30	ba30	ba31	ba31
B. anthracis	K4596	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K7441	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K6835	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K2802	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K7222	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K0123	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K4516	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K2762	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K7948	+	+	+	+	+	+	+	+	+	+	+	+	+	+
B. anthracis	K1340	+	+	+	+	-	-	+	+	+	+	+	+	+	+

All but one assay, ba27 were positive as expected in all target strains.

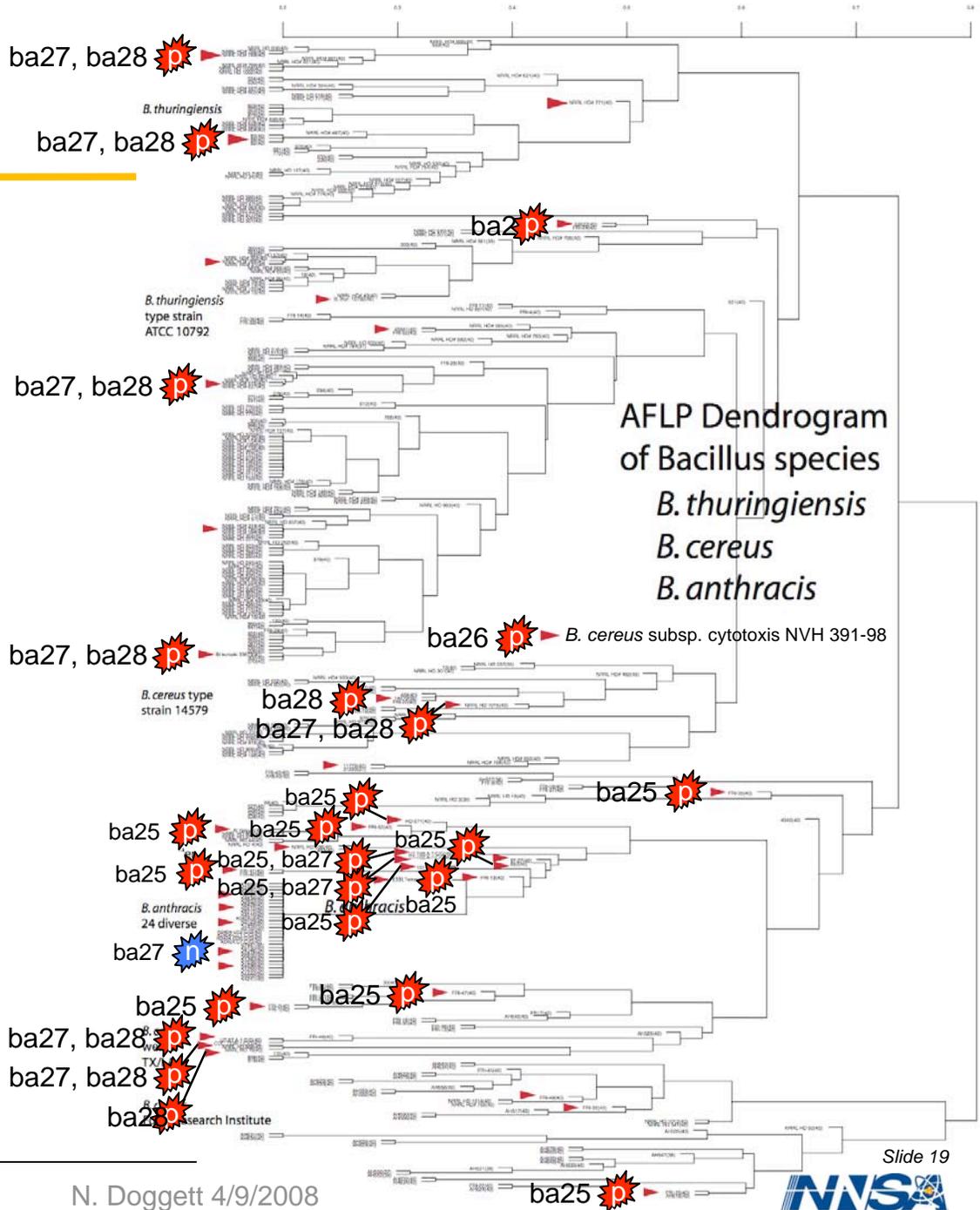
ba27 is the only *Ba* TaqMan assay targeting pX01 plasmid. We had previously found that MLVA primers targeting pXO1 in *Ba* strain K1340 failed, providing further evidence for plasmid loss in this strain. The original MLVA data for K1340 reported by the Keim lab showed that this strain did contain pXO1, thus it appears that plasmid loss has occurred during laboratory culturing.

Bacillus summary

39 strains tested to be representative of Bacillus groups.

p false positives
n false negatives

57% of *B. anthracis* TaqMan assays fail largely due to false positives.



Bacillus anthracis pXO1 Plasmid Sequence Conservation among Closely Related Bacterial Species

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Received 13 June 2001/Accepted 3 October 2001

The complete sequencing and annotation of the 181.7-kb *Bacillus anthracis* virulence plasmid pXO1 predicted 143 genes but could only assign putative functions to 45. Hybridization assays, PCR amplification, and DNA sequencing were used to determine whether pXO1 open reading frame (ORF) sequences were present in other bacilli and more distantly related bacterial genera. Eighteen *Bacillus* species isolates and four other bacterial species were tested for the presence of 106 pXO1 ORFs. Three ORFs were conserved in most of the bacteria tested. Many of the pXO1 ORFs were detected in closely related *Bacillus* species, and some were detected only in *B. anthracis* isolates. Three isolates, *Bacillus cereus* D-17, *B. cereus* 43881, and *Bacillus thuringiensis* 33679, contained sequences that were similar to more than one-half of the pXO1 ORF sequences examined. The majority of the DNA fragments that were amplified by PCR from these organisms had DNA sequences between 80 and 98% similar to that of pXO1. Pulsed-field gel electrophoresis revealed large potential plasmids present in both *B. cereus* 43881 (341 kb) and *B. thuringiensis* ATCC 33679 (327 kb) that hybridized with a DNA probe composed of six pXO1 ORFs.

DNA sequence conservation between the *Bacillus anthracis* pXO2 plasmid and genomic sequence from closely related bacteria

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Abstract

Background: Complete sequencing and annotation of the 96.2 kb *Bacillus anthracis* plasmid, pXO2, predicted 85 open reading frames (ORFs). *Bacillus cereus* and *Bacillus thuringiensis* isolates that ranged in genomic similarity to *B. anthracis*, as determined by amplified fragment length polymorphism (AFLP) analysis, were examined by PCR for the presence of sequences similar to 47 pXO2 ORFs.

Results: The two most distantly related isolates examined, *B. thuringiensis* 33679 and *B. thuringiensis* AWO6, produced the greatest number of ORF sequences similar to pXO2; 10 detected in 33679 and 16 in AWO6. No more than two of the pXO2 ORFs were detected in any one of the remaining isolates. Dot-blot DNA hybridizations between pXO2 ORF fragments and total genomic DNA from AWO6 were consistent with the PCR assay results for this isolate and also revealed nine additional ORFs shared between these two bacteria. Sequences similar to the *B. anthracis* *cap* genes or their regulator, *acpA*, were not detected among any of the examined isolates.

Conclusions: The presence of pXO2 sequences in the other *Bacillus* isolates did not correlate with genomic relatedness established by AFLP analysis. The presence of pXO2 ORF sequences in other *Bacillus* species suggests the possibility that certain pXO2 plasmid gene functions may also be present in other closely related bacteria.

Cereulide synthetase gene cluster from emetic *Bacillus cereus*: Structure and location on a mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXO1

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Conclusion: The *ces* gene cluster that is located on a pXO1-like virulence plasmid represents, beside the insecticidal and the anthrax toxins, a third type of *B. cereus* group toxins encoded on megaplasmids.

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0095-1137/06/\$08.00+0 doi:10.1128/JCM.00154-06

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Vol. 44, No. 7

Bacillus anthracis Virulent Plasmid pX02 Genes Found in Large Plasmids of Two Other *Bacillus* Species

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Center for Biological Defense, College of Public Health, University of South Florida, Tampa, Florida¹; Florida Department of Health, Bureau of Laboratories, Tampa, Florida²; and Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma³

We report the presence of 10 genes (*acpA*, *capA*, *capB*, *capC*, *capR*, *capD*, IS1627, ORF 48, ORF 61, and *repA*) and the sequence for the capsule promoter normally found on pX02 in *Bacillus circulans* and a *Bacillus* species closely related to *Bacillus luciferensis*.

Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax

Alex R. Hoffmaster^{*†}, Jacques Ravel^{†‡}, David A. Rasko^{†‡}, Gail D. Chapman[§], Michael D. Chute[§], Chung K. Marston^{*}, Barun K. De^{*}, Claudio T. Sacchi^{*}, Collette Fitzgerald^{*}, Leonard W. Mayer^{*}, Martin C. J. Maiden[¶], Fergus G. Priest^{||}, Margaret Barker^{||}, Lingxia Jiang[‡], Regina Z. Cer[‡], Jennifer Rilstone[‡], Scott N. Peterson[‡], Robbin S. Weyant^{*}, Darrell R. Galloway[§], Timothy D. Read^{†§}, Tanja Popovic^{*†}, and Claire M. Fraser^{†***}

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Communicated by John B. Robbins, National Institutes of Health, Bethesda, MD, April 5, 2004 (received for review March 24, 2004)

Bacillus anthracis is the etiologic agent of anthrax, an acute fatal disease among mammals. It was thought to differ from *Bacillus cereus*, an opportunistic pathogen and cause of food poisoning, by the presence of plasmids pXO1 and pXO2, which encode the lethal toxin complex and the poly- γ -D-glutamic acid capsule, respectively. This work describes a non-*B. anthracis* isolate that possesses the anthrax toxin genes and is capable of causing a severe inhalation anthrax-like illness*. Although initial phenotypic and 16S rRNA analysis identified this isolate as *B. cereus*, the rapid generation and analysis of a high-coverage draft genome sequence revealed the presence of a circular plasmid, named pBCXO1, with 99.6% similarity with the *B. anthracis* toxin-encoding plasmid, pXO1. Although homologues of the pXO2 encoded capsule genes were not found, a polysaccharide capsule cluster is encoded on a second, previously unidentified plasmid, pBC218. A/J mice challenged with *B. cereus* G9241 confirmed the virulence of this strain. These findings represent an example of how genomics could rapidly assist public health experts responding not only to clearly identified select agents but also to novel agents with similar pathogenic potentials. In this study, we combined a public health approach with genome analysis to provide insight into the correlation of phenotypic characteristics and their genetic basis.

**B. cereus* G9241 was the causative agent of a severe pneumonia in a Louisiana welder in 1994.

Anthrax?, but Not *Bacillus anthracis*

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0095-1137/06/\$08.00+0 doi:10.1128/JCM.00561-06

Vol. 44, No. 9

Characterization of *Bacillus cereus* Isolates Associated with Fatal Pneumonias: Strains Are Closely Related to *Bacillus anthracis* and Harbor *B. anthracis* Virulence Genes†

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Bacillus cereus is ubiquitous in nature, and while most isolates appear to be harmless, some are associated with food-borne illnesses, periodontal diseases, and other more serious infections. In one such infection, *B. cereus* G9241 was identified as the causative agent of a severe pneumonia in a Louisiana welder in 1994. This isolate was found to harbor most of the *B. anthracis* virulence plasmid pXO1 (13). Here we report the characterization of two clinical and one environmental *B. cereus* isolate collected during an investigation of two fatal pneumonia cases in Texas metal workers. Molecular subtyping revealed that the two cases were not caused by the same strain. However, one of the three isolates was indistinguishable from *B. cereus* G9241. PCR analysis demonstrated that both clinical isolates contained *B. anthracis* pXO1 toxin genes. One clinical isolate and the environmental isolate collected from that victim's worksite contained the *cap A, B, and C* genes required for capsule biosynthesis in *B. anthracis*. Both clinical isolates expressed a capsule; however, neither was composed of poly-D-glutamic acid. Although most *B. cereus* isolates are not opportunistic pathogens and only a limited number cause food-borne illnesses, these results demonstrate that some *B. cereus* strains can cause severe and even fatal infections in patients who appear to be otherwise healthy.

Yersinia pestis: TaqMan real-time assays

Targets plasmid pMT1. Java 9 is known to be a pMT1 deficient strain.

Genus/species	Strain	yp8	yp8	yp9	yp9	yp10	yp10	yp11	yp11	yp12	yp12	yp13	yp13	yp14	yp14	yp15	yp15
Y. pestis	CO92	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Java 9	+	+	+	+	-	-	+	+	-	-	+	+	-	-	-	-
Y. pestis	La Paz	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	195P	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Harbin 35	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Kim-10-Variant	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Pest F	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Pest A	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Y. pestis	Angola	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
Y. pestis	Antiqua	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
Y. pestis	Pest J	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Ev76-51	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	III	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis	Pest G	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Y. pestis (vaccine strain)	ATCC A1122	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+	+
Y. kristensenii	ATCC 29911	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. mollaretii	ATCC 43969	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. boreovieri	ATCC 42970	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. enterocolitica 0001	16155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC B11YP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC 29833	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC 907	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC 13979	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC 27802	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y. pseudotuberculosis	ATCC 29910	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Angola is missing caf1 gene (F1 capsule antigen)

Targets pPCP1 plasmid which is now known to be lacking in Pestoides F.

Vaccine strain lacks pCD1 plasmid

While Antiqua strain contains pMT1 plasmid, as confirmed with yp10, yp12 and yp14, amplicon size for yp15 is 2143 bp instead of 190 bp due to plasmid rearrangements.

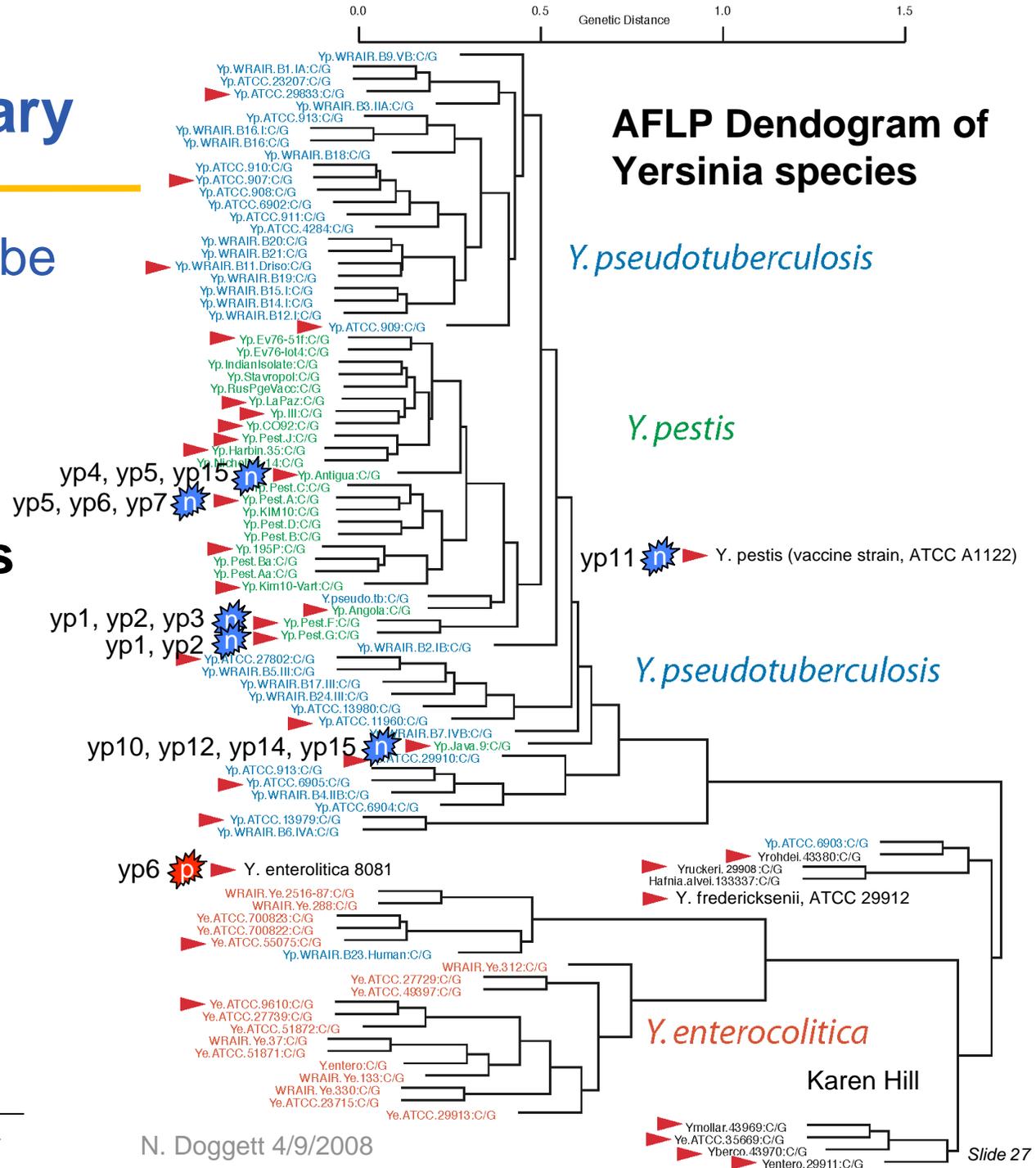
Yersinia summary

33 strains tested to be representative of *Yersinia* diversity.

p false positives
n false negatives

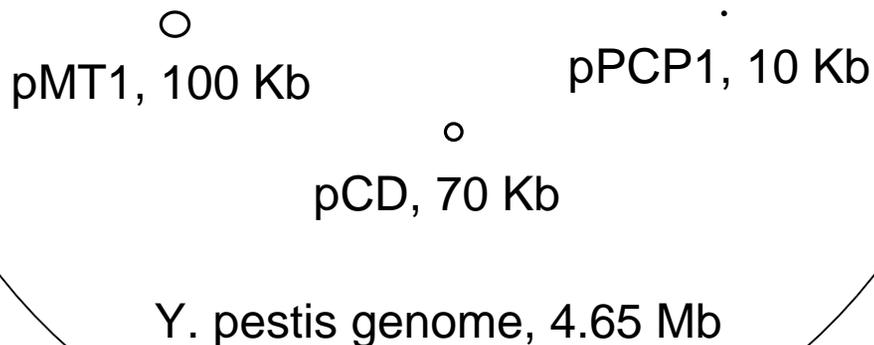
All *Y. pestis* PCR assays and 75% of TaqMan assays failed, largely due to false negatives.

AFLP Dendrogram of *Yersinia* species



Y. pestis Java 9 is a pMT1 deficient strain

Most classical isolates of *Y. pestis* (e.g. Antiqua, Nepal516), contain three 'virulence' plasmids: pMT1 (100 Kb), pCD (70 Kb), and pPCP (10 Kb).



The *Y. pestis* Java 9 strain was negative in all TaqMan assays which targeted plasmid pMT1, but positive in the chromosomal assays. A literature review reveals that Java 9 is a pMT1-deficient strain, which has nonetheless been shown to be virulent, killing African green monkeys. pMT1 encodes the F1 antigen among other virulence factors. Several studies over the years have reported F1 antigen-negative *Y. pestis* strains isolated from different animal species and in one case, such a strain was isolated from a fatal human infection.

Y. pestis Pestoides F is a pPCP1 deficient strain



Details

Organism

Genus species strain: *Yersinia pestis* Pestoides F
JGI project id: 2773191
Culture collection:
Year of funding: 2002 DOE
Public website date: 4/23/07

Assembly

Phase: Finished
Assembly Date: 4/13/07 GenBank accepted date of final
Size (Mb): Chromosome - 4.517347 CP000668
Plasmid pCD - 0.071509 CP000669
Plasmid pMT - 0.137012 CP000670
Total - 4.725868
Major Contigs: 3 (≥ 10 reads and ≥ 2 Kbp)
Coverage:
GC content: 47.7%
GenBank CP000668 - CP000670

The *Y. pestis* Pestoides F strain was negative in real-time PCR assays (yp1, yp2, yp3, yp8) which targeted plasmid pPCP1, but positive in the chromosomal assays.

Recent finished sequence from the JGI confirms that Pestoides F lacks the pPCP1 plasmid. Pestoides F does contain the pCD plasmid (71.5 Kb) and the pMT1 plasmid (13.7 Kb).

Y. pestis Pestoides G appears to be a pPCP1 deficient strain

Known pPCP1 plasmid deficient strains.

Y. pestis subsp. *pestis*
231pPst-
M-231 #5
358pPst-
parent KM219
parent A-250
KIM1002
KIM1008
CO92 pPst-
CO92 Pla-12
Alexander pPst-
Y. pestis subsp. *caucasica*
w.t. 6499 (pPst-)
6499 #2
w.t. Pestoides F

[From Anisimov et al., Clin. Micro. Rev. 17: 434–464 (2004).]

- *Y. pestis* Pestoides G-8786 from Georgia was shown to be negative for the Pla gene by PCR suggesting that this strain is deficient in plasmid pPCP1. [H. Tomaso et al. FEMS Immuno. Med. Micro. 38:117-126 (2003).]
- A total of 1.2% to 2% of the *Y. pestis* variants from Ural-Emba foci had no autonomous pPCP1 (pFra or pPst) plasmid. [Anisimov et al., Clin. Micro. Rev. 17: 434–464 (2004).]
- The numbers of pPst- *Yersinia pestis* subsp. *altaica* isolates in some years reached 10% of all isolates. [Aparin et al., Zh. Mikrobiol. Epidemiol. Immunobiol. 11:16–20. (1987); Balakhonov, S. V. Mol. Gen. Mikrobiol. Virusol. 4:39–42 (1989).]
- Several strains of *Yersinia pestis* have been shown to be naturally devoid of plasmid pPCP1 but are nonetheless virulent. The only known virulence factor encoded by pPCP1 is the coagulase/fibrinolysin also known as plasminogen activator. This protein is thought to promote the systemic spread of *Y. pestis* from peripheral sites. The bacteriocin, pesticin, and the pesticin immunity protein add selective pressure for plasmid maintenance. [Aparin, et al., Zh. Mikrobiol. Epidemiol. Immunobiol. 11:16–20 (1987); Filippov, et al. FEMS Microbiol. Lett. 67:45–48 (1990); Ivanova, et al., Mol. Gen. Mikrobiol. Virusol. 3:16–88 (1990); Filippov, et al., Zh. Mikrobiol. Epidemiol. Immunobiol. 3:10–13 (1992); Samoilova, et al. J. Med. Microbiol. 45:440–444 (1996); Samoilova, et al., J. Med. Microbiol. 45:440–444 (1996); Welkos, et al., Microb. Pathog. 23:211–223 (1997).]

Problems of *Y. pestis* plasmid targets

The value of plasmid signatures as a diagnostic tool for the identification of *Y. pestis* is questioned. Plasmids can be lost during cultivation in the laboratory and plasmid-deficient *Y. pestis* strains exist in nature. **pPCP1**-deficient *Y. pestis* strains have been isolated from voles in natural plague foci of the Caucasus and Daghestan mountains. In rare cases, *Y. pestis* strains do not express the F1 antigen, suggesting loss of the **pMT1** plasmid in these strains. Java 9 is a pMT1-deficient strain isolated from a rat in Java. **pCD1** is shared by all pathogenic members of *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis* and can be lost spontaneously. *Y. pestis* strains deficient of one or more plasmids can cause mild, chronic or even fatal infections. Thus a combination *Y. pestis*-specific real-time PCR assays including chromosomal targets is needed for confirmative detection.

- The *Y. pestis*-specific **9.5-kb pPCP1** plasmid codes for a plasminogen activator/coagulase (Pla). This protease may be important for invasiveness of *Y. pestis* transmitted by fleas because pPCP- strains are avirulent in mice infected subcutaneously, but fully virulent by aerosol challenge.
- The *Y. pestis*-specific **100-110-kb pMT1 plasmid** codes for the highly immunogenic fraction 1 capsule antigen (F1), a glycoprotein that is expressed at temperatures above 33°C. F1-deficient highly virulent *Y. pestis* strains have been reported. The *Y. pestis* murine toxin gene codes for Ymt protein which is highly toxic for mice when injected intraperitoneally. Ymt is a phospholipase that is important for the survival of *Y. pestis* in the flea vector and thus crucial for arthropod-borne transmission.
- The **64-kb pCD1 plasmid** contains a number of genes coding for several virulence factors including a type III secretion system. These *Yersinia* outer proteins (Yops) are required for full virulence in the mouse. Yops modulate host defense mechanisms such as phagocytosis and stress-activated signaling pathways. Plasmid pCD1 is shared by all pathogenic members of the species *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*.

and Chromosomal targets are not immune...

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Yersinia pestis Evolution on a Small Timescale: Comparison of Whole Genome Sequences from North America

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Background. *Yersinia pestis*, the etiologic agent of plague, was responsible for several devastating epidemics throughout history and is currently of global importance to current public health and biodefense efforts. *Y. pestis* is widespread in the Western United States. Because *Y. pestis* was first introduced to this region just over 100 years ago, there has been little time for genetic diversity to accumulate. Recent studies based upon single nucleotide polymorphisms have begun to quantify the genetic diversity of *Y. pestis* in North America. **Methodology/Principal Findings.** To examine the evolution of *Y. pestis* in North America, a gapped genome sequence of CA88-4125 was generated. **Sequence comparison with another North American *Y. pestis* strain, CO92, identified seven regions of difference (six inversions, one rearrangement), differing IS element copy numbers, and several SNPs. Conclusions/Significance.** The relatively large number of inverted/rearranged segments suggests that North American *Y. pestis* strains may be undergoing inversion fixation at high rates over a short time span, contributing to higher-than-expected diversity in this region. These findings will hopefully encourage the scientific community to sequence additional *Y. pestis* strains from North America and abroad, leading to a greater understanding of the evolutionary history of this pathogen.

Francisella tularensis: TaqMan real-time assays

Genus/species	Strain	ft16	ft16	ft17	ft17	ft18	ft18	ft19	ft19	ft20	ft20	ft21	ft21	ft22	ft22	ft23	ft23	ft24	ft24
<i>Ft subsp. tularensis</i>	WY96-3418	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	SCHU S4 (FSC237)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	94-2338	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	97-1365	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	97-1518	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	98-1815	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	99-3570	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. tularensis</i>	FRAN0008	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. holarctica</i>	LVS (FSC155)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. novicida</i>	U112 (ATCC 15482)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Ft subsp. novicida</i>	Fx1 (FSC165)	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+
<i>Francisella philomiragia</i>	25015	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Francisella philomiragia</i>	25017	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Francisella piscicida</i>	GM2212 [DSM	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Francisella</i> sp. DAS																			
<i>Beggiatoa alba</i>	B18LD																		
<i>Cycloclasticus pugetii</i>	PS-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Leucothrix mucor</i>	1 [NCMB 2222]																		
<i>Methylophaga alcaliphila</i>	M39 [DSM 14953]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Methylophaga marina</i>	222 [NCMB 2244]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Thiomicrospira frisia</i>	JB-A2 [DSM 12351]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Thiothrix eikelboomii</i>	AP3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>Thiothrix fructosivorans</i>	Q	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

False negative for *F. tularensis* subsp. *novicida* Fx1, a clinically significant strain, isolated from the blood of a compromised patient with pneumonia in Galveston, TX.

Note that ft16 and ft19 are predicted to yield false positive amplification with uncultured environmental near neighbors, which could not be tested experimentally due to a lack of these source DNAs.

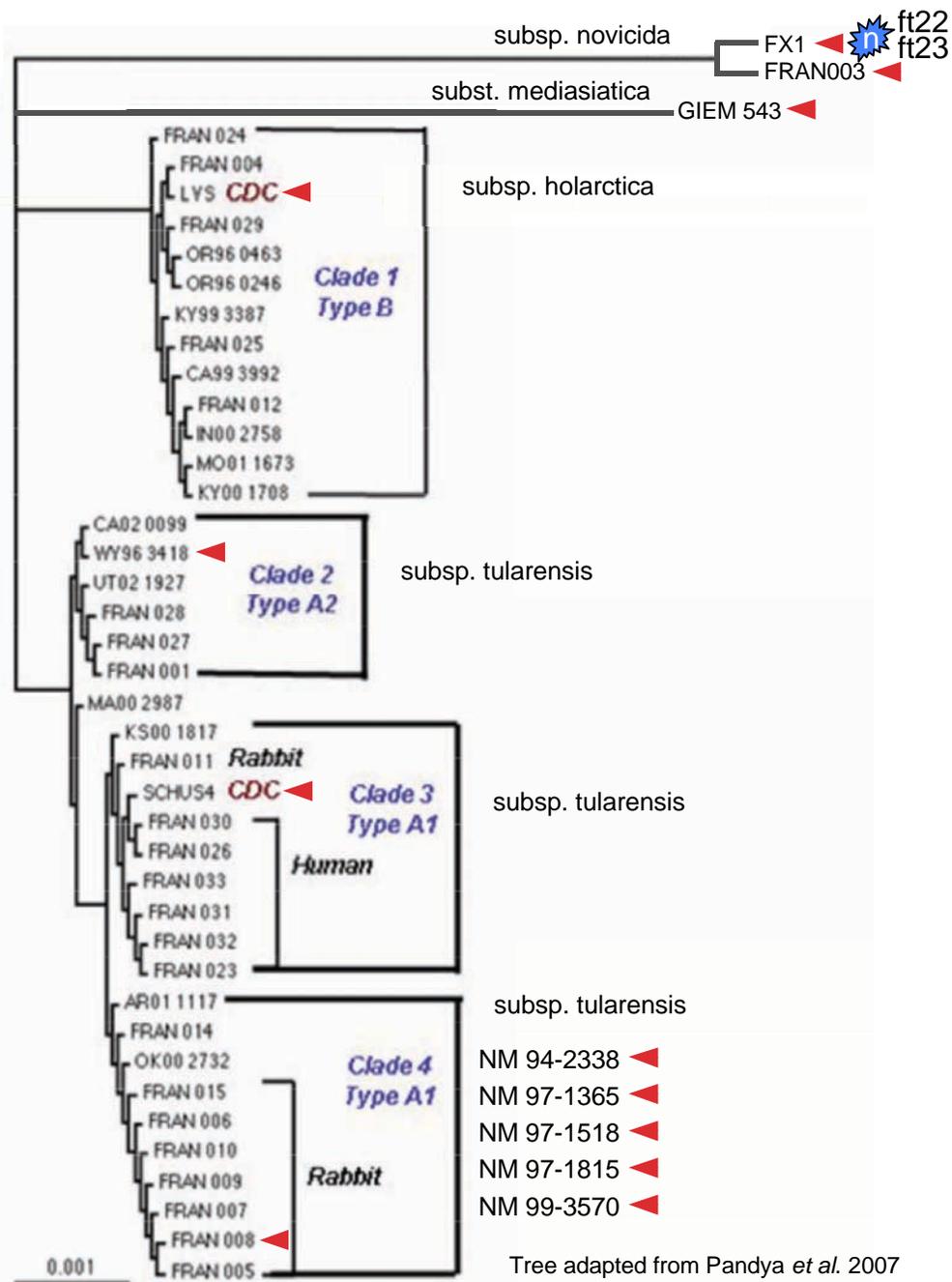
Francisella tularensis

Representatives of each *F. tularensis* subsp. & clade and *F. philomiragia*, *F. piscicida* were tested.

p false positives

n false negatives

22% of TaqMan assays failed experimentally due to false negatives. An additional 22% predicted to fail due to false positives.



Characterization of Two Unusual Clinically Significant *Francisella* Strains

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We have isolated two phenotypically distinct nonfastidious *Francisella* strains (Fx1 and Fx2) from the blood of compromised patients with pneumonia and compared them with eight other *Francisella* strains, including *Francisella tularensis* biovar *tularensis*, *F. tularensis* biovar *novicida*, and *F. philomiragia*. Our isolates grew well on sheep blood agar, chocolate agar, modified Thayer-Martin agar, and Trypticase soy agar. Fx1 and Fx2 were determined to be within the *Francisella* genus by cellular fatty acid analysis and by the utilization of glucose, production of H₂S and catalase, and lack of motility, oxidase, nitrate reductase, and gelatinase. They were additionally shown to belong to the species *F. tularensis* by sequencing of two variable regions comprising approximately 500 nucleotides of the 16S rRNA gene. Also, RNA probe hybridization confirmed their belonging to the species *F. tularensis*. However, the new strains, which are not identical, are distinguished from other *F. tularensis* strains by growth characteristics, repetitive extragenic palindromic PCR fragment pattern, and some biochemical tests. Key biochemical differences included the findings that Fx1 was positive for β-galactosidase and arabinose hydrolysis and that both strains were citrulline ureidase positive and glycerol negative. Commercial *F. tularensis* antiserum agglutinated stock *F. tularensis* strains but not Fx1, Fx2, *F. tularensis* biovar *novicida*, or *F. philomiragia*; serum from either patient failed to agglutinate or only weakly agglutinated commercial antigen but showed agglutination when tested against each patient's respective isolate. Fx1 and Fx2 produced β-lactamase. Because of their good growth, negative serology, and biochemical profile, the organisms could be misidentified in the clinical laboratory if standard strategies or commercial identification systems are used.

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Detection of Diverse New *Francisella*-Like Bacteria in Environmental Samples†

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Received 7 January 2005/Accepted 4 April 2005

Following detection of putative *Francisella* species in aerosol samples from Houston, Texas, we surveyed soil and water samples from the area for the agent of tularemia, *Francisella tularensis*, and related species. The initial survey used 16S rRNA gene primers to detect *Francisella* species and related organisms by PCR amplification of DNA extracts from environmental samples. **This analysis indicated that sequences related to *Francisella* were present in one water and seven soil samples.** This is the first report of the detection of *Francisella*-related species in soil samples by DNA-based methods. **Cloning and sequencing of PCR products indicated the presence of a wide variety of *Francisella*-related species. Sequences from two soil samples were 99.9% similar to previously reported sequences from *F. tularensis* isolates and may represent new subspecies.** Additional analyses with primer sets developed for detection and differentiation of *F. tularensis* subspecies support the finding of very close relatives to known *F. tularensis* strains in some samples. **While the pathogenicity of these organisms is unknown, they have the potential to be detected in *F. tularensis*-specific assays. Similarly, a potential new subspecies of *Francisella philomiragia* was identified.** The majority of sequences obtained, while more similar to those of *Francisella* than to any other genus, were phylogenetically distinct from known species and formed several new clades potentially representing new species or genera. The results of this study revise our understanding of the diversity and distribution of *Francisella* and have implications for tularemia epidemiology and our ability to detect bioterrorist activities.

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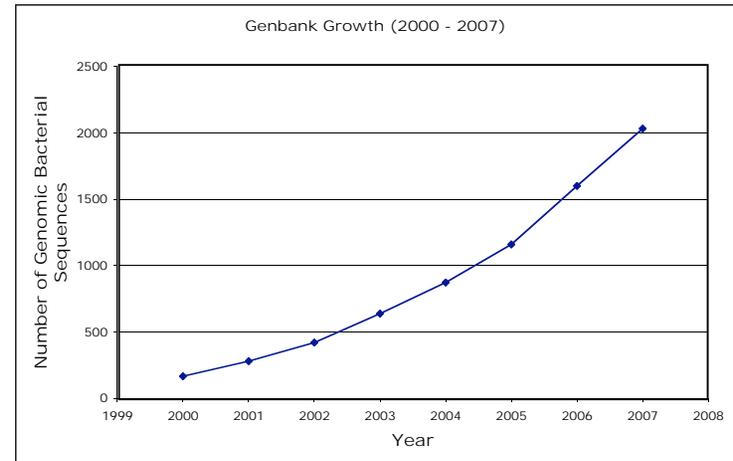
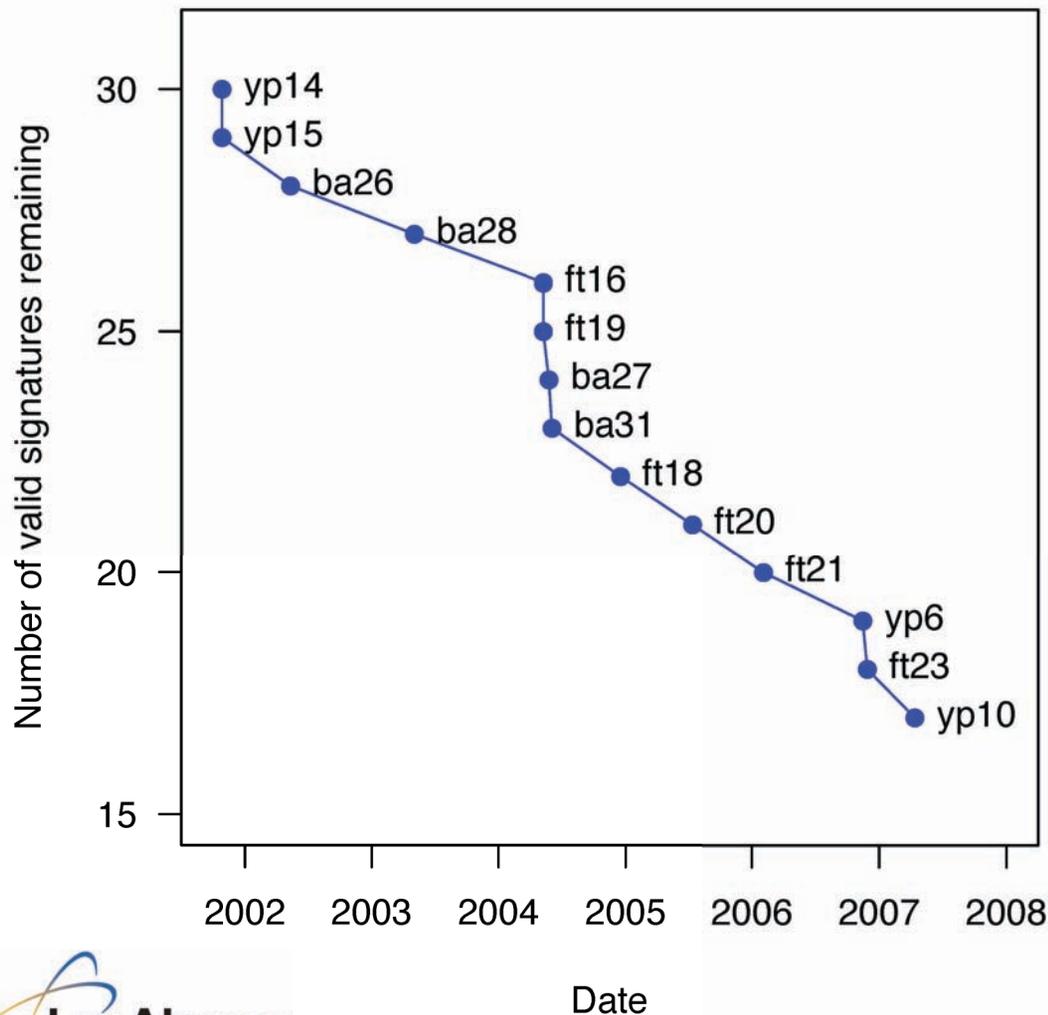
Comparison of *in silico* and experimental screening

DHS style warning indicates level of concern from *in silico* screening.

- All Yp and Ba *in silico* failures were found in the wet chemistry screening.
- Wet chemistry screening identified 1 additional assay failure for Ba and 4 additional assay failures for Yp.
- False positive *in silico* failures for Ft due to environmental samples could not be tested experimentally.

Organism	Name	Low	Guarded	Elevated	Severe	Expt.
Y. pestis	yp1				X	X
	yp2				X	X
	yp3				X	X
	yp4	X				X
	yp5	X				X
	yp6				X	X
	yp7	X				X
	yp8				X	X
	yp9	X				
	yp10				X	X
	yp11	X				
	yp12	X				X
	yp13	X				X
	yp14					X
	yp15					X
F. tularensis	ft16			X		
	ft17	X				
	ft18				X	
	ft19			X		
	ft20		X			
	ft21		X			
	ft22			X		X
	ft23		X			X
	ft24	X				
B. anthracis	ba25		X			X
	ba26				X	X
	ba27				X	X
	ba28				X	X
	ba29		X			
	ba30		X			
	ba31			X		

TaqMan Signature erosion over time



Signature erosion rate is approximately linear in time during which there has been an exponential growth in bacterial genomes sequenced. This may reflect that the sequencing of neighbor species is lagging behind the total growth of bacterial sequences.

A linear extrapolation of the rate of signature erosion suggests that all of the TaqMan signatures for Ba, Yp and Ft will erode by 2014.

Conclusions

- Regular signature re-evaluation screening is needed as new pathogen and neighbor sequences become available. This is ideally suited for an automated informatic process.
- Experimental screening of diverse strain panels that are selected to be representative of the known phylogenetic diversity of pathogens and their close neighbors is critical.
- Additional genome sequencing of pathogen and in particular near neighbors is needed to support the identification of robust signature development biosurveillance.
- We have only scratched the surface of environmental microbial diversity related to pathogens as is currently represented in cultured collections.

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