Supplemental Information

Retracing the Evolutionary Path that Led to Flea-borne Transmission of *Yersinia pestis*

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Table S1. Strains and Plasmids Used in This Study, Related to Figures 2, 3, and 4

Strain or plasmid	Genotype and/or description	Reference or source
Y. pestis KIM6+ strains:		
KIM6+ wt	wild type (pCD1-negative)	(Deng et al., 2002)
Δ*hmsS*	*hmsS* (y2356*)* deleted; unable to produce extracellular matrix required for Pgm and biofilm phenotypes	(Sun et al., 2008)
rcsA-pstb	*rcsA* (y1741*)* replaced with *Y. pseudotb rcsA* allele (YPTB2486)	(Sun et al., 2008)
PDE2-*pstb*	PDE2 gene (*rtn*; y2909) replaced with *Y. pseudotb rtn* allele (YPTB1308)	this study
PDE3-*pe*’	C to T point mutation in promoter region (*Y. pestis* KIM allele replaced by *Y. pestis* Pestoides A/91001 allele; cf. Fig. S3A)	this study
PDE3-*pstb*	PDE3 gene (y3389) replaced with *Y. pseudotb* PDE3 allele (YPTB3308)	this study
PDE3-pe^R	C to T promoter region point mutation and TGG to TAG stop codon mutation restored to C and TGG (as in *Y. pseudotuberculosis* IP32953; cf. Fig. S3A)	this study
rcsA-pstb PDE2-*pstb* PDE3-*pe*’	*rcsA* and PDE2 (*rtn*) genes replaced with *Y. pseudotb* alleles; PDE3 gene (y3389) replaced with PDE3’	this study
Y. pseudotuberculosis IP32953 strains:		
IP32953 wt	wild type	(Chain et al., 2004)
wt+ymt	wt transformed with pCH16 (*ymt* on plasmid)	this study
wt::ymt	YPTB1884-1891 replaced by *ymt* (*ymt* insertion in chromosome)	this study
+*ymt rcsA-pe* ΔPDE2 PDE3-*pe*	*rcsA* and PDE3 genes replaced by *Y. pestis* alleles, PDE2 (*rtn*) replaced by Cm cassette, (pCH16)	this study
::ymt rcsA-pe ΔPDE2 PDE3-*pe*	YPTB1884-91*::ymt, rcsA* and PDE3 genes replaced by *Y. pestis* alleles, PDE2 (*rtn*) replaced by Cm cassette	this study
Plasmids:		
pCH16	*Y. pestis ymt* gene (Y1069) cloned in pACYC177	(Hinnebusch et al., 2002)
p-PDE2-*pe*	*Y. pestis* PDE2 gene (*rtn*) cloned in pLG339	this study
p-PDE2-*pstb*	*Y. pseudotb* PDE2 gene (*rtn*) cloned in pLG339	this study
p-PDE3-*pe*	*Y. pestis* PDE3 gene cloned in pUC18	this study
p-PDE3-*pstb*	*Y. pseudotb* PDE3 gene cloned in pUC18	this study
p-PDE2-$pstb_{E509A}$	*Y. pseudotb* PDE2 (*rtn*) with mutated EAL domain (E509A) cloned in pLG339	this study
p-PDE3-$pstb_{E638A}$	*Y. pseudotb* PDE3 with mutated EAL domain (E638A) cloned in pUC18	this study
p-PDE3-$pstb_{D512A,E513A}$	*Y. pseudotb* PDE3 with mutated GGDEF domain (D512A, E513A) cloned in pUC18	this study
pAcGFP1	contains GFP gene	Clontech
pLG339	low-copy-number cloning vector	(Cunningham et al., 1993)
Notes:		

a) All *Y. pseudotuberculosis* strains other than wt lacked the pYV virulence plasmid. As with the *Y. pestis* virulence plasmid pCD1(Hinnebusch et al., 1996), absence of this plasmid did not change the flea infection phenotype. YPTB1884-1899 deletion was also confirmed not to affect flea infection phenotype.
b) Gene annotations in *Y. pseudotuberculosis* IP32953 and Y. *pestis* KIM, respectively: *hmsS,* YPTB1952, y2356; *rcsA*, YPTB2486, y1741; PDE2 (*rtn*), YPTB1308, y2909; PDE3, YPTB3308, y3389 (see Fig. 4 for details of the different PDE3 alleles).
c) Strains transformed with pAcGFP1 were used for flea dissections (Figure 2).
d) Cm, chloramphenicol; GFP, green fluorescent protein.

Table S2. Primer and Probe Sets Used in this Study, Related to Figures 2, 3, and 4 and Table S1

Target gene	Gene ID[a]	Use	Sequence[b] (5' to 3')
PDE2 (rtn)	y2909; YPTB1308	cloning into pCVD442/allelic exchange	TGA**CGCATGC**GATCTCGGCAAGAAAGTCCA; CTA**AGAGCTC**AATTGCTGCAAGAGTCGTGA
		cloning into pLG339	CTC**GTCGAC**CCCTATCACCTTCCACTGCG; GATC**GGATCC**TATTGTTGGCAATCTCCTGCGAA
		PDE2 deletion/ Cm insertion via λ Red recombinase	ACTT**CCCGGG**TATCCCACTGAGCACCCAAT; TGAAC**CCGGG**ACAAAATATTGCGGGAGA (for PDE2 gene) GTGTAGGCTGGAGCTGCTTCG; CATATGAATATCCTCCTTAG (for Cm cassette)
		EAL to AAL site-specific mutation	TAAACCTGATATTGTCTGATTATGTG; AGTGTGCGCAGCCCTTATCCGTTGGCAACAC
PDE3	y3389; YPTB3308	cloning into pCVD442, pLG339, and pUC18	TGA**CGCATG**CTTCTCGGGGTCAGTCATA; CTA**AGAGCT**GGTAAACGCCGATCTCCTGTC
		GGDEF to GGAAF site-specific mutation	TCCGCCTTGGCGGGAGACTA; AGTATGCGCAGCGTTTGTTTTGATTATTTCCAATCTCTC
		EAL to AAL site-specific mutation	TGCCCCTATCAGTTGCATGTCG; AGTATGCGCAGCGTTGGTACGCTGGAATCAC
		PDE3-*pe*' mutation via allelic exchange	CTA**AGAGCT**CGGACACGCGGATCCTCTC; TGAC**GCATGC**TTCTCGGGGTCAGTCATA
		PDE-3-*pe*^{R} mutation via allelic exchange	TGAC**GCATGC**TAAAGAATGGCGGTGTTGCT; CTAA**GAGCTC**GGCCCCATATTTTGGCTAAT
		RT-qPCR primers	AACGGGTCTGCCGAATCC; CTGAGGCGGTACCACGAAA
		RT-qPCR probe[c]	FAM - TGCCAAGCCGATCATTCAGCAGG - TAMRA
crr	y1485; YPTB2717	RT-qPCR primers	GCCCTCTGGCAATAAAATGG; AGCATGGTTGGTCTCGAAAATT
		RT-qPCR probe[c]	FAM-CTCCTGTTGACGGCATCGGT-TAMRA
ymt	Y1069	cloning	TGAC**AAGCTT**GATATTCGCCCAAGAAGG ; CG**GGATCC**AAAACTGCGACGAAACAGA
		cloning upstream insertion site (YPTB1884)	TGAC**GCATGC**AACGTGTGGTGGTGGTAGT; CG**GGATCC**AAATGGTTGCCAGAAATGGT
		cloning downstream insertion site (YPTB1891)	CG**GGATCC**TTGTTGGCTGCATTAGCTTG; CTAA**GAGCTC**AACAGGCCGCATAAATCAAC

[a]gene annotation number for *Y. pestis* KIM and *Y. pseudotuberculosis* IP32953
[b]6-nt sequences in bold type are restriction enzyme sites added to facilitate cloning
[c]RT-qPCR probes contain the reporter 5'-6-carboxyfluorescein (5'-FAM) and the quencher 3'-6-carboxy-tetramethyl-rhodamine (3'-TAMRA)

Figure S1. Restoration of *Yersinia pestis* PDE2 and PDE3 Phosphodiesterase Pseudogenes Results in Decreased Levels of Intracellular c-di-GMP (This figure is related to Figure 1). HPLC profiles of a 0.1 nM solution of c-di-GMP (A) or samples prepared from cell lysates of *Y. pestis* KIM6+ strains with the indicated genotypes (B-N). A c-di-GMP peak (indicated by the arrow) was not detected in wt *Y. pestis* (D) but could be detected in a strain deleted of *hmsP* (F), the only functional phosphodiesterase (PDE) gene in *Y. pestis* (Bobrov et al., 2011; Ren et al., 2013; Sun et al., 2011). Transformation of the Δ*hmsP* strain with a plasmid containing the functional PDE2 or PDE3 gene of *Y. pseudotuberculosis* (K, N) eliminated the c-di-GMP peak, and this was dependent on an intact EAL catalytic domain (L). Transformation of the Δ*hmsP* strain with plasmids containing the PDE2 or PDE3 pseudogenes of *Y. pestis* had no effect (J, M). *Y. pestis* encodes two functional diguanylate cyclase (DGC) genes: *hmsT* (y3756) and *hmsD* (y3730) (Bobrov et al., 2011; Ren et al., 2013; Sun et al., 2011). As expected, no c-di-GMP was detected when these two genes were deleted, even in the Δ*hmsP* background (B, C). *Y. pestis hmsP*, *hmsT*, and *hmsD* mutant strains and sample preparation and analysis methods are described in (Sun et al., 2011).

Figure S2. Restoration of Three *Y. pestis* Pseudogenes Attenuates in vitro Biofilm and Pigmentation Phenotypes (This figure is related to Figures 2, 3 and 4). The colony pigmentation phenotype on Congo red agar (+, red; –, white) and the relative amount of adherent biofilm following growth in tissue culture plate wells of *Y. pestis* KIM6+ strains are indicated. See Table S1 for strain descriptions.

Figure S3. Loss of PDE3 Activity in *Y. pestis* by Two Sequential Mutations (This figure is related to Figures 2, 3, and 5 and to Table 1).

(A) PDE3 alleles in *Y. pseudotuberculosis* and *Y. pestis* strains. Short vertical bars indicate single nucleotide polymorphisms (SNPs) relative to *Y. pseudotuberculosis* IP32953. A transition mutation (C to T) in the promoter region 171 bp upstream of the gene is present in all *Y. pestis* and some *Y. pseudotuberculosis* strains. The PDE3 gene is intact in two Branch 0 Pestoides *Y. pestis* strains (the PDE3-pe' allele), but is a pseudogene in all other *Y. pestis* due to a nonsense (stop codon) mutation; i.e. the PDE3 of 0.ANT2a and of all Branch 1-2 and unclassified biovar Orientalis strains examined are identical to KIM (the PDE3-*pe* allele). The PDE3-pe^R allele is an artificial construct that differs from PDE3-pe' only by reversion of the C to T transition. Alleles of *Y. pseudotuberculosis* ST42 (IP32953) and ST14 (YPIII); and *Y. pestis* 0.PE4b (Pestoides A), 0.PE4c (Microtus or 91001), and 2.MED1c (KIM) are depicted.

(B) Relative expression of different PDE3 alleles in *Y. pestis*. PDE3 transcript levels in *Y. pestis* KIM6+ wt and KIM6+ strains in which the native PDE3-*pe* allele had been replaced with the PDE3-*pstb*, PDE3-*pe*', or PDE3-pe^R allele were measured by RT-qPCR. Expression levels represent the mean and SEM of the averaged ΔΔCt values of three independent experiments performed in triplicate. Transcript levels of the *Y. pestis* PDE3-*pe*' and PDE3-*pe* alleles were significantly lower than the PDE-*pstb* allele and PDE3-pe^R alleles. *, $P < 0.0001$ by 1-way ANOVA with Tukey's multiple comparison post-test. Consistent with these expression data, *Y. pestis* containing the gene with just the promoter mutation (the PDE3-*pe*' allele) produced significant biofilm and pigmented colonies, whereas *Y. pestis* containing the PDE3-*pstb* allele did not (Figure S2). *Y. pestis*

KIM6+ containing the PDE3-*pe*' allele instead of the wild-type PDE3-*pe* allele was also able to produce proventricular blockage in fleas, unlike the *Y. pestis* PDE3-*pstb* strain (Figure 3A).

Supplemental Experimental Procedures

Pigmentation Phenotype and Microtiter Plate Biofilm Assays

Strains were streaked onto Congo red agar plates (Surgalla and Beesley, 1969) and colonies were observed visually for red pigmentation (Pgm) phenotype (adsorption of the Congo red dye) after growth for two days at room temperature. For quantitative in vitro biofilm comparisons, bacteria were grown in LB broth supplemented with 4 mM $CaCl_2$ and 4 mM $MgCl_2$ for 24 h at room temperature and diluted to A_{600} 0.02 in the same medium. 100 µl aliquots were added to wells of 96-well polystyrene dishes, which were incubated with shaking at 250 rpm for 24 h at room temperature. Media and planktonic cells were removed, the wells were washed four times with water, and the adherent biofilm was stained with 200 µl of 0.01% crystal violet for 15 min. The wells were washed four times with water, bound dye was solubilized with 200 µl of 80% ethanol-20% acetone, and the A_{600} was measured. Background absorbance for uninoculated control wells was subtracted. The mean and SD was calculated from three independent experiments with at least three replicates.

Quantitative Expression Analysis of Different PDE3 Alleles in *Y. pestis*

Y. pestis KIM6+ wt and KIM6+ strains in which the native PDE3-*pe* allele had been replaced with the PDE3-*pstb*, PDE3-*pe*', or PDE3-pe^R alleles were cultured in LB at

21°C to mid-log phase. Total RNA was isolated from harvested bacteria (RNeasy MiniKit; Qiagen) and contaminating DNA removed (DNA-free kit; Ambion) as described (Rebeil et al., 2013). Transcript levels were measured by reverse transcription-quantitative PCR (RT-qPCR) using an ABI Prism 7900 and Taqman Universal PCR Master Mix (Applied Biosystems). For each primer-probe set assay, a standard curve was prepared using known concentrations of *Y. pestis* KIM6+ total RNA. Relative levels of expression were determined by normalizing the expression of the PDE3 gene to that of the reference gene *crr* using the comparative ΔΔCt method (Schmittgen and Livak, 2008).

Supplemental References

Bobrov, A.G., Kirillina, O., Ryjenkov, D.A., Waters, C.M., Price, P.A., Fetherston, J.D., Mack, D., Goldman, W.E., Gomelsky, M., and Perry, R.D. (2011). Systematic analysis of cyclic di-GMP signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis*. Mol. Microbiol. *79*, 533-551.

Chain, P.S.G., Carniel, E., Larimer, F.W., Lamerdin, J., Stoutland, P.O., Regala, W.M., Georgescu, A.M., Vergez, L.M., Land, M.L., Motin, V.L., *et al.* (2004). Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc. Natl. Acad. Sci. USA *101*, 13826-13831.

Cunningham, T.P., Montelaro, R.C., and Rushlow, K.E. (1993). Lentivirus envelope sequences and proviral genomes are stabilized in *Escherichia coli* when cloned in low-copy-number plasmid vectors. Gene *124*, 93-98.

Deng, W., Burland, V., Plunkett, G., Boutin, A., Mayhew, G.F., Liss, P., Perna, N.T., Rose, D.J., Mau, B., Zhou, S., *et al.* (2002). Genome sequence of *Yersinia pestis* KIM. J. Bacteriol. *184*, 4601-4611.

Hinnebusch, B.J., Perry, R.D., and Schwan, T.G. (1996). Role of the *Yersinia pestis* hemin storage (*hms*) locus in the transmission of plague by fleas. Science *273*, 367-370.

Hinnebusch, B.J., Rudolph, A.E., Cherepanov, P., Dixon, J.E., Schwan, T.G., and Forsberg, Å. (2002). Role of Yersinia murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. Science *296*, 733-735.

Rebeil, R., Jarrett, C.O., Driver, J.D., Ernst, R.K., Oyston, P.C., and Hinnebusch, B.J. (2013). Induction of the *Yersinia pestis* PhoP-PhoQ regulatory system in the flea and its role in producing a transmissible infection. J. Bacteriol. *195*, 1920-1930.

Ren, G.X., Yan, H.Q., Zhu, H., Guo, X.P., and Sun, Y.C. (2013). HmsC, a periplasmic protein, controls biofilm formation via repression of HmsD, a diguanylate cyclase in *Yersinia pestis*. Environ. Microbiol. *Nov 5. doi: 10.1111/1462-2920.12323*.

Schmittgen, T.D., and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative Ct method. Nature Protocols *3*, 1101-1108.

Sun, Y.-C., Hinnebusch, B.J., and Darby, C. (2008). Experimental evidence for negative selection in the evolution of a *Yersinia pestis* pseudogene. Proc. Natl. Acad. Sci. USA *105*, 8097-8101.

Sun, Y.-C., Koumoutsi, A., Jarrett, C., Lawrence, K., Gherardini, F.C., Darby, C., and Hinnebusch, B.J. (2011). Differential control of *Yersinia pestis* biofilm formation *in vitro* and in the flea vector by two c-di-GMP diguanylate cyclases. PLoS One *6*, e19267.

Surgalla, M.J., and Beesley, E.D. (1969). Congo red agar plating medium for detecting pigmentation in *Pasteurella pestis*. Appl. Microbiol. *18*, 834-837.