Supporting Information

Chouikha and Hinnebusch 10.1073/pnas.1413209111

SI Materials and Methods

Bacterial Mutagenesis. Deletion of the *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* urease cluster (URE, ~6 kb) or *ureD* gene (~1 kb) (Fig. 2*B*) was performed by allelic exchange using pCVD442 (1) containing a Km resistance cassette flanked with long-homology sequences of the target DNA (2). Briefly, the Km cassette and its flanking FLP recognition target (FRT) sites were amplified from the pKD4 plasmid (3) with primer pair pKD4-KmF/pKD4-KmR (Table S2). Long-flanking regions (~500 bp) upstream and downstream of the target sequence were amplified using the primer pairs Fup URE/Rup URE-Km and Fdown URE-Km/Rdown URE for the URE cluster deletion and Fup *ureD* /Rup *ureD*-Km and Fdown URE-Km /Rdown URE for the *ureD* deletion (Table S2 and Fig. 2*B*).

The conjugative knock-out plasmids, pCVD-Δ*ureD*:: Km and pCVD-ΔURE:: Km (Table S4), were introduced into *Escherichia coli* S17-1 λ-pir and transferred to *Y. pseudotuberculosis* and *Y. enterocolitica* via conjugation. Positive transconjugants, in which the deletion of the URE cluster and the *ureD* gene had occurred, were checked by urease activity assays and by PCR using primers *ureF* F/R and/or *ureD* F/R. The correct insertion of the Km cassette was verified with primers pairs encompassing one extremity of the Km cassette (k1 or kt-c) and the DNA region adjacent to the target gene (Fup *ureD*, Fup URE, and Rdown URE).

Site-Directed Mutagenesis. *Yersinia pestis* KIM6+ urease revertants were generated by overlap extension PCR as described (4, 5). *Y. pestis* KIM6+ genomic DNA was used as a template for PCR amplification. The primers used to delete a single guanidine residue from a G-rich region in the *Y. pestis* KIM6+ *ureD* gene (Fig. 2*B*) are listed in Table S2, with the degenerate primers (*ureD*ΔG KIM-F/R) containing the mutation of interest sharing complementary sequence. In the first round of PCR, two ~500-bp fragments of *ureD* were amplified using the primer pairs Fup *ureD*ΔG/*ureD*ΔG KIM-R and *ureD*ΔG KIM-F/ Rdown *ureD*ΔG KIM. The generated PCR products were used as template DNA for the second PCR using the primers Fup *ureD*ΔG/Rdown *ureD*ΔG KIM. The resulting amplified ~1-kb fragment was gel-purified and cloned into the SmaI site of pCVD442. The resulting recombinant plasmid (pCVD-*ureD*ΔG_{KIM6+}) was checked by endonuclease restriction and used to introduce the mutation into *Y. pestis* KIM6+ by conjugation as described in the previous section. Revertants were selected according to their ability to hydrolyze urea on modified urea segregation agar (MUSA) plates (6) and confirmed by sequencing using primers *ureD* seqF/R (Table S2).

Transcomplementation of Urease-Negative Strains. Before complementation of *Y. pseudotuberculosis* IP32953 ΔURE (IPΔURE), the Km cassette was cured by homologous recombination at the FRT sites mediated by the FLP recombinase carried on the plasmid pCP20 (7) as described (3). The Km cassette excision was verified by streaking isolated colonies simultaneously on LB agar plates with or without Km and by PCR using primers FupURE/RdownURE. Complementation plasmid pWKS-URE_{pstb} was constructed by ligating a PCR product encompassing the entire *Y. pseudotuberculosis* urease cluster plus ~500 bp of upstream and downstream sequence into the *Eco*RV site of pWKS130 (Table S4). The plasmid pWKS-URE_{pstb} was transformed in *E. coli* TOP10 (Life Technologies) and transformants were screened by PCR for the presence of all urease genes (Fig. 2*B*; see Table S2 for primers used) and selected based on their ability to hydrolyze urea on MUSA plates. Plasmid pWKS-URE_{pstb} isolated from positive clones was transformed into *Y. pseudotuberculosis* IPΔURE_{KmS} and *Y. pestis* KIM6+.

Complementation of the nonfunctional *ureD* gene was performed by introducing the plasmid pCR*ureG-D* containing the promoter and bicistronic *ureG* and *ureD* genes of IP32953 cloned in the high-copy plasmid pCR XL-TOPO TA cloning into *Y. pestis*.

Mathematical Modeling. Assuming that the population of susceptible hosts is not limiting, the flea density per host (m) required to sustain a plague transmission cycle (*Y. pestis* successfully transmitted from a single infected animal to at least one new host; $R_0 = 1$) can be estimated from a modification of Macdonald's equation (8), $m = r/abp^n$, with $1/r$ being the duration of infectivity of the primary host and a, b, p, and n being vector competence factors. The effect of urease-related toxicity on the vector-specific parameters corresponding to biofilm-dependent transmission by blocked *X. cheopis* fleas is indicated in Table S3. For wild-type (urease-negative) *Y. pestis* the threshold flea density is estimated to be 3, but this threshold increases to 6 for a urease-positive clone.

1. Donnenberg MS, Kaper JB (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* 59(12):4310–4317.
2. Derbise A, Lesic B, Dacheux D, Ghigo JM, Carniel E (2003) A rapid and simple method for inactivating chromosomal genes in *Yersinia*. *FEMS Immunol Med Microbiol* 38(2): 113–116.
3. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
4. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR (1989) Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77(1):51–59.
5. Reikofski J, Tao BY (1992) Polymerase chain reaction (PCR) techniques for site-directed mutagenesis. *Biotechnol Adv* 10(4):535–547.
6. Hu LT, Nicholson EB, Jones BD, Lynch MJ, Mobley HL (1990) *Morganella morganii* urease: Purification, characterization, and isolation of gene sequences. *J Bacteriol* 172(6):3073–3080.
7. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158(1):9–14.
8. Lorange EA, Race BL, Sebbane F, Hinnebusch BJ (2005) Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. *J Infect Dis* 191(11):1907–1912.

Table S1. 2-DE/mass spectrometry identification of *Y. pseudotuberculosis* membrane-associated proteins that are absent from, differentially expressed, or have a different pI in the *Y. pestis* 2-DE profile (Fig. 2)

Spot no.	Accession no.	Gene name	Gene ID	Coverage*	AA†	Molecular weight, kDa	Calculated pI	Score‡	Description	% ID to *Y. pestis*§
1	Q66CY8	*ompC*	YPTB1261	36.12	371	41.0	5.16	816.98	Outer membrane protein C, porin	99
2	Q66CY8	*ompC*	YPTB1261	31.27	371	41.0	5.16	1,046.76	Outer membrane protein C, porin	99
3	Q66CY8	*ompC*	YPTB1261	16.71	371	41.0	5.16	1,450.25	Outer membrane protein C, porin	99
4	Q66CY8	*ompC*	YPTB1261	28.03	371	41.0	5.16	1,511.31	Outer membrane protein C, porin	99
5	Q66FD5	*groEL*	YPTB0405	62.59	548	57.4	5.01	11,323.09	60-kDa chaperonin	100
6	Q663Q8	*atpD*	YPTB3967	54.35	460	50.1	5.06	5,068.73	ATP synthase subunit beta	100
7	Q663Q8	*atpD*	YPTB3967	28.91	460	50.1	5.06	1,127.69	ATP synthase subunit beta	100
8	Q66DA1	*sucB*	YPTB1148	24.32	407	44.3	5.69	1,368.55	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate	100
9	Q66FD5	*groEL*	YPTB0405	9.36	545	57.1	5.01	279.77	60-kDa chaperonin	100
10	**P52313**	***ureC***	**YPTB2942**	**11.71**	**572**	**61.0**	**5.81**	**341.35**	**Urease subunit alpha**	**100**
11	Q66EH8	*lpdA*	YPTB0715	24.68	474	50.5	6.11	1,291.95	Dihydrolipoyl dehydrogenase	100
12	**P52316**	***ureD***	**YPTB2938**	**13.71**	**321**	**35.7**	**6.96**	**968.76**	**Urease accessory protein UreD**	**99**¶
13	Q664U6	*rpoA*	YPTB3673	33.43	329	36.5	5.06	697.36	RNA polymerase subunit alpha	100
14	Q66CY8	*ompC*	YPTB1261	27.49	371	41.0	5.16	1,054.05	Outer membrane protein C, porin	99
15	**P69993**	***ureG***	**YPTB2939**	**42.27**	**220**	**24.0**	**4.98**	**955.35**	**Urease accessory protein UreG**	**100**
16	Q66BY2	*manX*	YPTB1634	23.18	302	32.9	5.82	768.09	PTS system, mannose-specific	100
17	P38399	*ompA*	YPTB1453	30.03	353	37.9	7.27	1,511.91	Outer membrane protein A	100
18	P38399	*ompA*	YPTB1453	17.56	353	37.9	7.27	1,250.79	Outer membrane protein A	100
19	Q56957	*ail*	YPTB2867	22.35	179	19.8	8.50	2,058.25	Attachment invasion locus protein	99
20	Q664R9	*bfr*	YPTB3700	55.41	157	18.2	4.87	632.50	Bacterioferritin	99
21	Q66BV7	*ftnA*	YPTB1659	21.30	169	19.2	5.16	295.67	Ferritin	99
22	Q66FA2	*rpsF*	YPTB0438	30.91	110	12.6	5.53	955.97	30S ribosomal protein S6	100
23	Q669E1	*dps*	YPTB2546	51.50	167	18.9	6.29	2,930.60	DNA protection during starvation protein	100
24	Q66F99	*rplI*	YPTB0441	40.67	150	15.9	6.80	2,366.43	50S ribosomal protein L9	100
25	Q669E1	*dps*	YPTB2546	32.34	167	18.9	6.29	553.01	DNA protection during starvation protein	100
26	Q56957	*ail*	YPTB2867	39.11	179	19.8	8.50	3,264.24	Attachment invasion locus protein	99
27	**P69991**	***ureB***	**YPTB2943**	**36.11**	**144**	**15.8**	**9.44**	**854.41**	**Urease subunit beta**	**100**
28	Q66A25	*lpp*	YPTB2307	60.26	78	8.3	8.84	402.25	Major outer membrane lipoprotein	100
29	Q669E1	*dps*	YPTB2546	22.16	167	18.9	6.29	281.52	DNA protection during starvation protein	100
31	**P52313**	***ureC***	**YPTB2942**	**47.03**	**572**	**61.0**	**5.81**	**1,3911.12**	**Urease subunit alpha**	**100**
32	**P52313**	***ureC***	**YPTB2942**	**36.71**	**572**	**61.0**	**5.81**	**6,969.14**	**Urease subunit alpha**	**100**
33	**P52313**	***ureC***	**YPTB2942**	**36.54**	**572**	**61.0**	**5.81**	**2,857.35**	**Urease subunit alpha**	**100**

Identified urease structural and accessory subunit proteins are in bold.

*Percentage of amino acid sequence identified by matching tryptic peptides.

†Number of amino acids in the intact protein.

‡Number of matches calculated by comparing query masses with database masses.

§Percentage identity to homolog in *Y. pestis* KIM10+.

¶The 99% identity of UreD (spot 12) compares the predicted 277-aa truncated protein encoded by the *Y. pestis ureD* pseudogene with the 321-aa UreD of *Y. pseudotuberculosis*.

Table S2. Primers used in this study

Primer	Sequence (5′–3′)*	Gene target/primer use
Fup URE (1)	CGTAATGGATTTTATTATC	URE deletion in *Y. pseudotuberculosis*
Rup URE- Km (2)	gaagcagctccagcctacacCTGCATAAGCCCTCCTGTATCATG-TTAAGAAAGTGAAAGTGAGAAAAAGC	
Fup *ureD* (3)	GACGACCGAGGATGCCAAAC	*ureD* deletion
Rup *ureD*- Km (4)	gaagcagctccagcctacacCTGTCATGCCTGTTCTCCTTCCGGC-TGTACGTGGGTAAACAGGAAATCTC	
Fdown URE- Km (5)	ggaccatggctaattcccatGGCGCTGATTTTGCTGTTTTGATGCG-CCATTTTAAGGGGAGAAAAGGGTG	URE and *ureD*
Rdown URE (6)	CCGGTCAATGGGTTATTCTG	Deletion in *Y. pseudotuberculosis*
ureD F	ATTTCATTGTGGAGGAAGGCG	*ureD* PCR
ureD R	GCGTCAAGGCTTTCTACTTTTGG	
ureD endR	CGGCGTCTTTACGATCTGCC	*ureG-ureD* locus PCR (used with primer *ureF* F)
ureD seqF	GACGCAGTGGGTGTTATGC	*ureD* sequencing
ureD seqR	CCTTAAAATGGCGCATCAAAACAG	
ureA F	CTACACGCTGTCTGATGTGGCG	*ureA-G* locus PCR
ureA R	CGTGTACCGTGACCAGACGGC	
ureB F	CGTGACCAAAGTTAAAGTCCG	
ureB R	GCGCTCACTGTTGGGTACCAC	
ureC F	CGATAGCCTGAATGAATGTGG	
ureC R	GAGAACATGGAGATAACGCCC	
ureE F	CTGGATCTGGGCATTTCTCTCG	
ureE R	GTGAATGTGCAGACCAGAGCC	
ureF F	GCATTATCCGTGCTGATTGGG	
ureF R	GGTCATTGCCACACCGTATTG	
ureG F	GCTGCGGTAGAAGAGATGGAAG	
ureG R	CGTGGGTAAACAGGAAATCTC	
pKD4-KmF	GCGATTGTGTAGGCTGGAGCTGCTTC	PCR of pKD4 *km* cassette flanked with FRT
pKD4 KmR2	GCTGACATGGGAATTAGCCATGGTCC	
k1	CAGTCATAGCCGAATAGCCT	pKD4 primers to verify correct insertion of *km* (1)
kt-c	GGATTCATCGACTGTGGCCG	
Yent Fup URE	GTAGCTATGGCATTAGCTATAG	URE deletion in *Y. enterocolitica* 8081
Yent Rup URE Km	gaagcagctccagcctacacGCTGCATAAGCCCTCCTGTATCTGGTTAA-GAAAGTGAAAGTGAGAAAAACAACC	
Yent Fdown URE Km	ggaccatggctaattcccatGGCGTTAAGGTGTTGCTCAAAGGTATTGG-CGTTGCAGCAAAACATCGCTGC	URE and *ureD* deletion
Yent Rdown URE	CAATGACCGACACGATGCTACC	In *Y. enterocolitica*
Yent Fup *ureD*	GCCTCTGATCATTACCAATGAC	*ureD* deletion in *Y. enterocolitica*
Yent Rup *ureD* Km	gaagcagctccagcctacacGCGATGTCATGCATGTTCTCCTTGTGGCT-GCACATGGGTAAACAAGAAGTCGCGC	
Yent *ureD* F	GCCGTGTGTCACCATGATCTCG	PCR of internal sequences of *ureD* and *ureF* in *Y. enterocolitica*
Yent *ureD* R	CGCATCAAATGATTGCATGACC	
Yent *ureF* F	GCCGACGTTAAAAGGCTTCGTG	
Yent *ureF* R	GGCGAACGTGCGCTTTCACATGC	
*ureD*ΔG KIM-F	CCGGCTCATTTTGACATCAAAGGGGGGATTGCCAGCGGCGCGACG	Single guanosine deletion in *Y. pestis ureD*
*ureD*ΔG KIM-R	CGTCGCGCCGCTGGCAATCCCCCCTTTGATGTCAAAATGAGCCGG	
Rdown *ureD*ΔG	GCCGGGTTACCTTCACCATAAGC	
Fup *ureD*ΔG	GGAAGCGGGCGCTTGTGCTCACG	

Numbers in parentheses refer to primer indicated in Fig. 2*B*.
*Sequences complementary to the kanamycin resistance cassette are in lowercase letters.

1. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.

Table S3. Effect of *Yersinia* urease toxicity on flea vectorial capacity

Variable	Definition	Value for fleas infected with	
		Urease −	Urease +
a	Daily biting rate after becoming infective (blocked)	1.0	1.0
b	Probability of a flea becoming infective after an infectious blood meal and transmitting (per bite)	0.19	0.15
n	Days (mean) between infectious blood meal and infectivity (extrinsic incubation period)	14	14
p^n	Probability of a flea to survive the extrinsic incubation period	0.9	0.53
1/r	Life expectancy (days) of host after reaching the threshold septicemia level	2	2

Biofilm-dependent transmission can occur during any of several nearly continuous feeding attempts from the time partial blockage develops until the flea dies from starvation, a period of 3–4 d. Therefore, with regard to transmission and the equation above, the value of *a* is effectively 1.0. The probability of infectivity (with the potential to transmit) after an infectious blood meal is the blockage rate for biofilm-dependent transmission; the probability of a blocked flea transmitting (transmission rate) during a single blood meal attempt is 0.5. The product of the two probabilities is *b*. Values are from this study and ref. 1.

1. Lorange EA, Race BL, Sebbane F, Hinnebusch BJ (2005) Poor vector competence of fleas and the evolution of hypervirulence in *Yersinia pestis*. *J Infect Dis* 191(11):1907–1912.

Table S4. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype/description	Urease phenotype	Source
Y. pseudotuberculosis			
IP32953	Wild type	+	(1)
IPΔ*ureD*	IP32953 deleted of the gene *ureD*, Km^R	–	This study
IPΔURE	IP32953 deleted of the entire urease cluster (*ureA-ureD*), Km^R	–	This study
$IP\Delta URE_{KmS}$	IPΔURE cured of the Km cassette		This study
$IP\Delta URE(pWKS\text{-}URE_{pstb})$	$IP\Delta URE_{KmS}$ complemented with the whole urease cluster, Km^R	+	This study
Y. pestis			
KIM6+	Wild type (pCD–)	–	(2)
$KIM6+(pCRureGD_{pstb})$	KIM6+ expressing the *ureG* and *ureD* genes of *Y. pseudotuberculosis* IP32953 on the pCRXL TOPO TA high-copy plasmid	+	This study
KIM6+(pWKS130)	KIM6+ containing the empty plasmid pWKS130		This study
$KIM6+(pWKS\text{-}URE_{pstb})$	KIM6+ containing the urease cluster of *Y. pseudotuberculosis* IP32953 on a low-copy plasmid	+	This study
KIM6+*ureD*ΔG	KIM6+ deleted of one guanidine residue	+	This study
Y. enterocolitica			
Ye8081	Biotype 1B, wild type	+	(3)
Ye8081Δ*ureD*	*Y. ent* 8081deleted of the gene *ureD*, Km^R	–	This study
Ye8081ΔURE	*Y. ent* 8081 deleted of the entire urease cluster (*ureA-ureD*), Km^R	–	This study
E. coli			
TOP10	F^-*mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL endA1 nup*G	–	Invitrogen
S17-1 λ*pir*	RP4 2-Tc::Mu-Km::Tn*7 pro thi recA* $HsdR^-M^+$ λ*pir*	–	(4)
TOP10(pWKS130)	*E. coli* TOP10 containing the empty plasmid pWKS130	–	This study
$TOP10(pWKS\text{-}URE_{pstb})$	*E. coli* TOP10 containing the pWKS130 plasmid carrying the urease cluster of *Y. pseudotuberculosis* IP32953	+	This study
Plasmids			
pKD4	Ap^R, Km^R	N/A	(5)
pCP20	Flp recombinase gene, Ap^R, Cm^R	N/A	(6)
pCVD442	Suicide vector, Ap^R	N/A	(7)
pWKS130	Low copy plasmid, Km^R	N/A	(8)
$pWKS\text{-}URE_{pstb}$	pWKS130 plasmid carrying the *Y. pseudotuberculosis* IP32953 urease cluster	N/A	This study
pCRXL TOPO TA	High-copy plasmid, Ap^R	N/A	Invitrogen
pCR*ureG-D*	pCRXL containing the promoter and bicistronic *ureG* and *ureD* genes of IP32953	N/A	This study
pCVD-Δ*ureD*::Km	Suicide vector for deletion of *ureD* in *Y. pstb* IP32953	N/A	This study
pCVD-ΔURE:: Km	Suicide vector for deletion of URE in *Y. pstb* IP32953	N/A	This study
$pCVD\text{-}\Delta ureD_{Yent}$:: Km	Suicide vector for deletion of *ureD* in *Y. ent* 8081	N/A	This study
$pCVD\text{-}\Delta URE_{Yent}$:: Km	Suicide vector for deletion of URE in *Y. ent* 8081	N/A	This study
$pCVD\text{-}ureD\Delta G_{KIM6+}$	Suicide vector for single nucleotide deletion in *Y. pestis* KIM6+ *ureD*	N/A	This study

N/A, not applicable.

1. Chain PS, et al. (2004) Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. *Proc Natl Acad Sci USA* 101(38):13826–13831.
2. Deng W, et al. (2002) Genome sequence of *Yersinia pestis* KIM. *J Bacteriol* 184(16):4601–4611.
3. Thomson NR, et al. (2006) The complete genome sequence and comparative genome analysis of the high pathogenicity *Yersinia enterocolitica* strain 8081. *PLoS Genet* 2(12):e206.
4. Simon R, Priefer U, Pühler A (1983) A broad range mobilization system for in vivo genetic engineering: Transposon mutagenesis in Gram-negative bacteria. *Biotechnology* 1:784–791.
5. Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* 97(12):6640–6645.
6. Cherepanov PP, Wackernagel W (1995) Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* 158(1):9–14.
7. Donnenberg MS, Kaper JB (1991) Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. *Infect Immun* 59(12): 4310–4317.
8. Wang RF, Kushner SR (1991) Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. *Gene* 100:195–199.