Title: Increased Plasmid Copy-number is Essential for *Yersinia* T3SS

Function and Virulence

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Abstract: Pathogenic bacteria have evolved numerous virulence mechanisms that are essential for establishing infections. The enterobacteria, *Yersinia*, uses a Type III Secretion System (T3SS), encoded by a 70-kb, low-copy, IncFII-class virulence plasmid. Here, we report a novel virulence strategy in *Y. pseudotuberculosis* in which this pathogen up-regulates the plasmid copy-number during infection. We show that increased dose of plasmid-encoded genes is indispensable for virulence and substantially elevates the expression and function of the T3SS. Remarkably, we found direct, tight coupling between plasmid replication and T3SS function.

This regulatory pathway provides a framework for further exploration of the environmental sensing mechanisms of pathogenic bacteria.

One Sentence Summary: Regulation of plasmid copy-number is an indispensable virulence strategy in pathogenic *Yersinia*.

Main Text: Three human pathogenic *Yersinia* strains: *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, share a common 70-kb virulence plasmid (IncFII) that encodes a set of virulence proteins, called Yersinia Outer Protein (Yops) (1, 2). These bacterial toxins are secreted by a plasmid-encoded organelle, the *ycs/yop* Type III Secretion System (T3SS) (3-5). The T3SS is composed of ~20 proteins that span the inner and outer bacterial membranes (6, 7). Upon contact with eukaryotic cells, *Yersinia* deploys the T3SS to translocate Yops into the cytoplasm of target cells via a translocon formed in the cell membrane (8, 9). This process is strictly regulated, and Yop expression and secretion increases after the bacterium establishes intimate contact with the eukaryotic target cell (10). This cell contact-dependent regulation can be mimicked *in vitro* at 37°C by depleting Ca^{2+} from the growth medium (11).

Incubation of *Yersinia* at 37°C in Ca^{2+} -deficient medium leads to T3SS induction and growth restriction after approximately two generations (4). Growth arrest may be due to the metabolic burden caused by excess expression of plasmid-encoded T3SS proteins (12). Thus, the function of the T3SS is paradoxical, because conditions that promote Yop-secretion result in bacterial growth restriction. This feature is incompatible with infection; consequently, we reasoned that *Yersinia* must have evolved a mechanism to circumvent this problem. We observed that increased levels of virulence plasmid DNA were recovered from wild-type *Y. pseudotuberculosis* cells grown under T3SS-inducing conditions compared with bacteria grown under T3SS repressive conditions. Therefore, we hypothesized that *Yersinia* may undergo rapid changes in

gene expression by increasing and decreasing virulence plasmid copy-numbers. Rapid gene-dose changes could adjust the T3SS output to trade off virulence costs and the pathogen's metabolic capacity to optimize growth.

To explore a potential connection between virulence and plasmid copy-numbers, we first determined plasmid (pIBX) copy-numbers in *Y. pseudotuberculosis* YpIII (YpIII/pIBX) (13) cultures under different conditions with a PCR-free whole-genome sequencing approach (14). The depth of coverage (the number of times a nucleotide is read during the sequencing process) reflects the concentrations of chromosomal DNA and plasmid DNA molecules in the sample. We found that the pIBX copy-number increased from ~1 to ~3 per chromosomal equivalent when conditions were repressive (26°C) or inductive (37°C, Ca²⁺-free), respectively, to T3SS activity (Fig 1A). At 37°C in the presence of Ca²⁺ (T3SS repressed conditions), the copy-number only increased modestly (1.6 per chromosomal equivalent). Similar differences in plasmid copy-numbers were found with quantitative real-time PCR (qPCR) (Fig S1A) and Southern Blot analysis (Fig S1B).

We used qPCR to determine the time course of pIBX copy-number variations. We found that the copy-number increased and plateaued 1 hour after the temperature shift to T3SS-inducing conditions (Fig 1B). Bacteria grown at 37°C under T3SS-repressing conditions plateaued at ~2 chromosome equivalents (Fig 1B). These results confirmed that the plasmid copy-number increased in *Y. pseudotuberculosis* under T3SS-inducing conditions.

To analyze the control of plasmid copy-number in *Yersinia*, we investigated copy-number changes in T3SS regulatory mutants. Earlier studies have shown that both Ca²⁺ and temperature regulates the T3SS in *Yersinia* (15, 16). LcrF activates transcription of the T3SS-regulon in

response to elevated temperature (37°C) and low Ca²⁺. *lcrF* mutants lack Yop expression and growth is not restricted in Ca²⁺-depleted medium at 37°C (16). In contrast, *yopD* mutants have Ca²⁺-insensitive Yop-expression and restricted growth at 37°C, irrespective of Ca²⁺ concentrations (17). Thus, YopD is involved in a negative-feedback regulatory pathway controlling T3SS expression (18). We found that plasmid copy-number control was not affected in a $\Delta lcrF$ mutant (YpIII/pIB73) (Fig 1C). In contrast, in a $\Delta yopD$ mutant (YpIII/pIB621), the plasmid copy-number was elevated at 37°C under T3SS-repressive conditions (+Ca²⁺, Fig 1C). Thus, YopD is directly or indirectly inhibiting a default temperature regulated plasmid copy-number increase in *Yersinia*.

The plasmid replication initiation protein RepA initiates IncFII plasmid replication. The level of RepA is controlled at the translational level by the antisense RNA CopA (19). To investigate if the IncFII replicon *per se* is responsible for increased virulence plasmid copy-number, we expressed the *Yersinia copA* (20) under control of the *yopE* promoter. This should increase CopA levels, and consequently, reduce the rate of initiation of virulence plasmid replication under T3SS-inducing conditions. This construct was integrated *in cis* into the virulence plasmids of wild-type *Yersinia* and a $\Delta yopD$ mutant. CopA overexpression resulted in reduced plasmid copy-numbers under T3SS-inducing conditions in both strains (Fig 1D). This shows that the IncFII replicon, *per se*, is essential for the T3SS regulated copy-number increase. Furthermore, the reduced plasmid copy-number in the CopA-overexpressing strains suppressed T3SS-related growth defects and reduced Yop-expression under T3SS-induced conditions (Fig S2, Fig 1D insert). This indicates that *Yersinia* has evolved a T3SS-dependent mechanism to down-regulate the copy-number of its virulence plasmid, likely to counteract the metabolic burden associated with induction of the T3SS.

To investigate whether increased plasmid copy-number is involved in virulence, we designed a mutant strain that could not change virulence plasmid copy-number during infection. This was achieved by inserting pIBX, without the replication function (IncFII⁻), into the chromosome of *Y. pseudotuberculosis* (Fig 2A). The final mutant, YpIII:(cIBX)_{n=1}, contained one copy of the replication-deficient pIBX inserted into the chromosomal gene *YPK_3687* (Fig 2A). *YPK_3687* was previously shown to be redundant for *Y. pseudotuberculosis* virulence (21). Insertion of a single copy of the replication-deficient pIBX plasmid, (cIBX)_{n=1}, into *YPK_3687* was verified by DNA-sequencing and qPCR (Fig 2B and S3). As expected, the mutant strain was unable to change the gene-dose of plasmid-encoded genes in response to T3SS activity (Fig S3). Secretion analysis showed that YpIII:(cIBX)_{n=1} retained temperature- and Ca²⁺-dependent regulation of Yop-expression and secretion, but Yop levels were severely reduced by comparison with wild-type (Fig 2C). Yop-translocation experiments showed that YpIII:(cIBX)_{n=1} could translocate the reporter protein, YopH₁₋₉₉-Bla (22), into HeLa-cells, in a dose dependent manner, but less than the parental YpIII/pIBX wild-type strain (Fig 3A). Importantly, the YpIII:(cIBX)_{n=1} mutant was severely attenuated in an oral BALB/c mouse infection model. YpIII:(cIBX)_{n=1} initially colonized Peyer's-patches (PP), cecum, and mesenteric lymph nodes (MLN, Fig 3B and S4) but all infected mice survived and recovered their initial body weight (Fig 3B insert), showing that the YpIII:(cIBX)_{n=1} strain is avirulent.

To confirm if the reduced virulence of YpIII:(cIBX)_{n=1} was a result of a reduced gene-dose of virulence-plasmid encoded genes, we amplified the integrated virulence plasmid in the mutant. This was achieved by selecting for a gene-dose dependent increase in chloramphenicol resistance conferred by the *cat* gene encoded by the integrated construct (Fig 2A) (23, 24). Amplification of the *cat* gene was possible through homologous recombination of the duplicated (831 bp)

YPK_3687 sequences flanking the integrated IncFII⁺ pIBX plasmid (Fig 2A). When YpIII:(cIBX)_{n=1} was plated on to agar plates with different chloramphenicol concentrations, we recovered clones resistant to 150 µg/ml chloramphenicol at a frequency of 1×10^{-6} and found duplication of the integrated virulence plasmid. After initial duplication, further amplification was achieved after selection on plates with 300 µg/ml chloramphenicol at a frequency of 1×10^{-4} . As expected, in the absence of selective pressure (23), the amplified virulence plasmid-containing clones were unstable and reverted to the original single-copy genotype at a frequency of 2×10^{-1} . These revertants showed the same phenotype as the original YpIII:(cIBX)_{n=1} variant.

A clone with three copies of the plasmid (YpIII:(cIBX)_{n=3}) was selected for further analysis of T3SS function and virulence. We verified the high- and low-copy genotypes of YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3}, respectively, by whole-genome sequencing and qPCR (Fig 2B and S3).

YpIII:(cIBX)_{n=3} showed Yop-expression, secretion and translocation profiles similar to those of the wild-type strain (Fig 2C and 3A). While the YpIII:(cIBX)_{n=1} strain showed a similar growth rate as the plasmid cured YpIII (T3SS⁻) strain, the YpIII:(cIBX)_{n=3} strain showed reduced growth at 37°C under T3SS-induced conditions similar to that of the wild-type strain (Fig S5).

Importantly, YpIII:(cIBX)_{n=3} was virulent in an oral BALB/c mouse infection model (Fig 3B). All infected mice developed a systemic infection and were consequently euthanized. Bacterial loads 2 days after infection were 100-fold higher in homogenized PPs from animals infected with virulent YpIII:(cIBX)_{n=3} compared with those infected with avirulent YpIII:(cIBX)_{n=1} (Fig S4). The high- and low-copy genotypes of bacterial strains were retained after passage through the mouse. YpIII:(cIBX)_{n=3} colonies recovered from PPs, MLNs, and cecum 2 days after infection showed variable numbers of virulence plasmid equivalents (Fig 3C). Remarkably, several isolated colonies possessed greater plasmid equivalents than the original three, indicating that the

integrated plasmid was under selective pressure for further amplification. Collectively, the observed gene-dose dependent virulence of YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3} indicates that plasmid copy-number amplification is essential for *Yersinia* virulence.

To determine whether the plasmid copy-number increases during infection, we isolated total DNA from PPs of mice after infection with wild-type YpIII/pIBX. The copy-number of pIBX was determined by whole genome DNA-sequencing and qPCR in the complex DNA sample. Both independent methods showed a six-fold increase in virulence plasmid copy-number in the PPs compared with the infecting inoculum (Fig 4A). Increased virulence plasmid copy-number during infection was verified by qPCR of lysates prepared from animal tissues recovered at different times after oral infections with wild-type YpIII/pIBX (Fig 4B). We observed substantial increases in virulence plasmid copy-numbers in all infected samples analyzed.

It has been shown that reduced ratios of *copA* antisense RNA compared to *repA* mRNA leads to elevated RepA levels and thus to an increase in plasmid replication initiation (19). Analysis of *copA* and *repA* transcript levels in *Yersinia* during infection showed that the ratio of *copA* and *repA* RNA decreased significantly during PP colonization compared to bacteria grown *in vitro* (Fig 4C), corroborating the findings presented above. Our results show that the T3SS related copy-number regulation affects the stability or transcription of both RNA species (Table S2, Fig. S6). Importantly, considering the actual gene dose of plasmid-encoded genes under the different conditions, we found that the major change was a marked decrease in *copA* levels while the *repA* mRNA levels per plasmid copy remained basically unchanged (Table S2, Fig. S6). This favors a model where the T3SS related copy-number regulation operates via a change in the *copA* antisense RNA levels.

In summary, we show that increased gene-dose of plasmid-encoded genes is essential for *Yersinia* virulence. One copy of the virulence plasmid that encodes the T3SS-regulon is insufficient to establish a systemic infection in mice; thus, *Yersinia* has evolved a mechanism to rapidly increase the copy-number during an infection. Our results also show that, despite the increased metabolic burden caused by three T3SS-regulon copies, during infection, there was selective pressure to increase the T3SS gene-dose. Moreover, the secreted protein, YopD, is involved in regulation of the copy-number. This feature indicates that the system is tightly controlled, linking external sensors to T3SS activity and the plasmid copy-number.

In *Yersinia*, high T3SS activity is deleterious for growth *in vitro*. Yet, T3SS expression is essential for successful infection and proliferation in hosts. We found an inverse correlation between virulence plasmid gene-dose and growth rate under T3SS induced conditions. Therefore, we propose that *Yersinia* maintains the entire T3SS virulence system in a plasmid, which enables rapid adjustments in T3SS expression in response to prevailing environmental conditions. This basic regulatory tactic is likely to apply to any plasmid-encoded genes by analogous mechanisms. Our findings provide a framework for further functional investigations of the regulatory pathways that allows pathogens to respond to environmental cues and may be important in bacterial antibiotic resistance.

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Fig. 1.

Fig. 1. *Y. pseudotuberculosis* differentially regulates virulence plasmid copy-number *in vitro*. **(A)** Virulence plasmid copy-numbers in DNA isolated from *Y. pseudotuberculosis* YpIII/pIBX grown 3 hours under different conditions determined by whole-genome sequencing; 26°C (white bar), 37°C under T3SS-repressive (gray bar) and T3SS-inductive (black bar) conditions. The copy-number was calculated as the ratio of the average depth of plasmid DNA coverage:chromosomal DNA coverage ($N=2$). **(B)** Time-course of plasmid copy-number increase determined by qPCR. At time zero, *Y. pseudotuberculosis* cultures were shifted from 26°C to 37°C under T3SS-repressive (gray bars) and T3SS-inductive (black bars) conditions. Plasmid copy-number is defined as the number of plasmid equivalents per chromosome ($N=6$). **(C)** Plasmid copy-number changes in YpIII/pIB73 ($\Delta lcrF$) and YpIII/pIB621 ($\Delta yopD$) determined by qPCR after 3 h growth under different conditions (same as A) ($N=6$). **(D)** qPCR results showing plasmid copy-numbers in YpIII/pIBX (wt) and a YpIII/pIB621 ($\Delta yopD$) overexpressing the antisense *copA*-RNA fused to the *yopE* promoter *in cis* (*pyopE-copA*) after 3 h growth at 37°C under T3SS-repressive (gray bars) and T3SS-inductive (black bars) conditions. Copy numbers in YpIII/pIBX (wt) is presented as control ($N=4$). *Insert*: immunoblot of whole-cell lysates from the indicated strains probed with

anti-Yop antibodies. Data presented: mean $\pm$ S.E.M. (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, ns, not significant by Mann-Whitney U -test).

Fig. 2.

Fig 2. Integration of the pIBX (IncFII⁻) amplicon into the chromosome. **(A)** Homologous recombination of pNQΔ*oriRI-sacB* (Cm^r) into pIBX (Km^r) results in a replicating pIBX:pNQΔ*oriRI-sacB* intermediate (Cm^r, Km^r, *sacB*⁺). SacB counter-selection in the presence of Km and Cm, selects for *sacB*⁻ clones where the pIBX:pNQ plasmid (Cm^r, Km^r) has been integrated into the chromosome. The final Cm^r, Km^r, *sacB*⁻ strain results from the deletion of 3426 bp encoding the native pIBX IncFII replicon and *sacB* (gray). Dashed lines indicate homologous recombination events. The integrated construct, flanked by duplicated *YPK_3687* sequences (blue arrows), can be amplified by selecting for gene-dose dependent increase in Cm^r conferred by the *cat* gene (orange box). Black arrows and corresponding numbers: the experimentally derived frequencies of indicated genetic events. **(B)** Number of reads of pIBX and chromosome/kb determined by whole genome DNA sequencing. Red: pIBX sequences; blue: chromosomal sequences. Gap in the plasmid sequence alignments shows the 3426-bp deletion of the IncFII replicon. Arrowhead: increased *YPK_3687* coverage after gene duplication/multimerization. The figure is a composite where the pIBX reads have been inserted into the correct location in the chromosome. **(C)** Western-blot analysis of Yop-expression and secretion by T3SS-induced *Y. pseudotuberculosis* cultures (-Ca²⁺). Whole-cell samples (upper panel) and proteins secreted into the culture medium (lower panel) were collected at the indicated times after a shift from 26°C to 37°C.

Fig. 3.

Fig. 3. Increased gene-dose of plasmid-encoded genes is essential for *Yersinia* virulence. **(A)** Translocation of YopH₁₋₉₉-Bla into HeLa-cells after 90 min. Histogram represent the mean $\pm$ S.E.M. of a representative experiment. Translocation deficient $\Delta yopBD$ mutant (YpIII/pIB219) expressing YopH₁₋₉₉-Bla is included as a negative control. Response ratio: blue/green ratio divided by background ratio. Lower panel shows representative micrographs of cells infected at a multiplicity of infection (MOI) of 50. **(B)** *In vivo* images showing anesthetized mice infected with YpIII:(cIBX)_{n=1} (left) or YpIII:(cIBX)_{n=3} (right). Pseudo-colors indicate levels of light emitted by bioluminescent bacteria. *Insert*: changes in average body-weights of infected mice over time (open circles: YpIII:(cIBX)_{n=1}; solid circles: YpIII:(cIBX)_{n=3}). **(C)** Virulence plasmid gene-dose measured in bacterial colonies isolated from Peyer's patches (PP), mesenteric lymph node (MLN), and cecum from infected mice, 2 days after infection. Each point represents the virulence plasmid copy-number per chromosome of a single colony determined by qPCR (open circles: YpIII:(cIBX)_{n=1}; solid circles: YpIII:(cIBX)_{n=3}). Data represent mean $\pm$ S.E.M. (***) $P \leq 0.001$, based on the Mann-Whitney *U*-test).

Fig. 4.

Fig. 4. *Yersinia* virulence plasmid copy-number increases during infection in mice. **(A)** Virulence plasmid copy-number of wild-type YpIII/pIBX in total DNA extracted from infected Peyer's patches (PP) 48 h after infection, determined by whole genome sequencing (TruSeq) (Table S1) and by qPCR. Plasmid copy-number of infecting inoculum determined with qPCR is shown as control ($N=4$). **(B)** Plasmid copy-numbers of wild-type YpIII/pIBX in lysates from homogenates from indicated organs determined by qPCR. Control: plasmid copy-number of infecting inoculum ($N\geq 3$). **(C)** The ratio of *copA:repA* RNA decreases in *Yersinia* during infection. The *copA:repA* ratio was calculated as Reads Per Kilobase of transcript per Million mapped reads (RPKM) of *copA* and *repA* (Table S2) from bacteria grown at 25°C (Inoculum), 37°C and infected PP (*in vivo*) ($N=3$). Data represent mean $\pm$ S.E.M. (Differences between control and the respective conditions were analyzed by unpaired *t* test, ns: not significant, $*P\leq 0.05$, $**P\leq 0.01$, $***P\leq 0.001$).

Supplementary Materials:

Materials and Methods

Figures S1-S7

Tables S1-S4

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Supplementary Materials for

Increased Plasmid Copy-number is Essential for *Yersinia* T3SS Function and Virulence

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Materials and Methods

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Materials and Methods

Growth conditions and growth curves

Bacterial strains and plasmids used in this study are listed in Table S3. *Yersinia pseudotuberculosis* and *Escherichia coli* strains were routinely grown in Luria-Bertani (LB) broth or agar at 26°C for *Y. pseudotuberculosis* strains and 37°C for *E. coli* strains supplemented with appropriate antibiotics (Kanamycin (Km) 50 µg/ml, Chloramphenicol (Cm) 25 µg/ml and Carbenicillin (Cb) 50 µg/ml), unless otherwise stated. For T3SS induction experiments, overnight *Y. pseudotuberculosis* cultures were inoculated in fresh LB (1:20) supplemented with 20 mM MgCl₂ and 5 mM CaCl₂ (T3SS repressed conditions, +Ca²⁺) or 5 mM EGTA (T3SS induced conditions, -Ca²⁺) followed by 1 hour incubation at 26°C before shifting the cultures to 37°C.

Growth curves were obtained from 2 independent bacterial cultures assayed in duplicates. Bacteria were grown overnight at 26°C in Brain Heart Infusion (BHI) and were thereafter inoculated in fresh BHI (1:400) supplemented with 20 mM MgCl₂ and 20 mM sodium oxalate (-Ca²⁺, T3SS induced conditions). The cultures were grown 1 hour at 26°C before two 100 µl aliquots of each culture were distributed in 96 well clear bottom assay plates (Costar). The plate was assayed at 37°C using a TECAN Infinite F200 micro-plate reader (TECAN) by measuring OD₅₉₅ every 10 min with 20 seconds orbital shaking before each measurement.

DNA techniques

Total DNA (both genomic and plasmid DNA) from bacterial cells and infected tissues was purified using GeneJet Genomic DNA Purification Kit (Thermo Scientific). Bacterial DNA was enriched in total DNA samples from infected tissues using a NEBnext Microbiome DNA Enrichment Kit (New England Biolabs). *Y. pseudotuberculosis* virulence plasmids were extracted using alkaline lysis methods (25). Pure virulence plasmid preparations were isolated using standard CsCl₂ gradient centrifugation methods. For qPCR analysis, total DNA lysates of bacterial cells and infected mouse tissues were prepared using a *mericon* DNA Bacteria Kit (Qiagen). The concentration of dsDNA was determined using a Qubit 3.0 fluorometer according to manufacturer's recommendations (Thermo Scientific).

Mutant construction

Two DNA fragments flanked by *SphI* sites (pUCpyopE-*copA* and pUCΔ*oriR1-sacB*) were purchased from GenScript USA (Fig. S7). The 860 bp fragment in pUCpyopE-*copA* included 560 bp encoding *yerA* (*sycE*) and the *yopE* promoter sequence fused to a 288 bp fragment starting with the reported *copA*-antisense RNA start site (21) and downstream sequence encoding putative termination sequences (Fig. S7A). The 4497 kb fragment in pUCΔ*oriR1-sacB* included 831 bp of the *YPK_3687* gene, a 798 bp fragment starting 1107 bp downstream of the pIBX *repA* stop codon, a 2001 bp fragment encoding the *Bacillus subtilis* *sacB* gene, and a 837 bp fragment starting 1453 bp upstream of the pIBX *repA* start codon (Fig. S7B). The fragments were excised with *SphI* and ligated into pNQ705 (26), giving pNQpyopE-*copA* and pNQΔ*oriR1-sacB* respectively.

The pNQpyopE-*copA* suicide plasmid was introduced into wild-type *Y. pseudotuberculosis* (YpIII/pIBX) and Δ*yopD* *Y. pseudotuberculosis* (YpIII/pIB621) by conjugation using standard techniques. Cm^r and Km^r YpIII/pIBX-pyopE-*copA* and YpIII/pIB621-pyopE-*copA* clones

resulting from integration of pNQpyopE-copA into the *yerA* (*sycE*) locus in the respective virulence plasmids, were isolated on *Yersinia* selective (CIN) agar plates. The mutants were re-streaked on selective LA-plates and verified by PCR and sequencing.

pNQ Δ oriR1-sacB was integrated into the pIBX virulence plasmid using the same procedure presented in the previous section. SacB counter-selection of the resulting *sacB*⁺, Cm^r and Km^r YpIII/pIBX:pNQ Δ oriR1-sacB strain on agar plates containing 10 % sucrose, 50 μ g/ml Km, and 25 μ g/ml Cm, recovered SacB⁻, Cm^r and Km^r *Yersinia* clones where pIBX (lacking 3426 bp encoding the IncFII replicon) fused to pNQ705, had been integrated into the *YPK_3687* gene in the YpIII chromosome (Fig 2A). The final clone (YpIII:(cIBX)_{n=1}) was verified by PCR and sequencing. Proper integration of pIBX (IncFII⁻) into the chromosome in YpIII:(cIBX)_{n=1} was further verified by loss of pIBX after purification of the plasmid using alkaline lysis.

Amplification of the pIBX (IncFII⁻) amplicon in the chromosome by chloramphenicol selection

Overnight YpIII:(cIBX)_{n=1} cultures were diluted 1:20 in fresh LB and grown at 26°C for 3 hours. Serial, 10-fold dilutions of the culture were plated on LA plates with 50 μ g/ml Km and increasing concentrations of Cm (25, 50, 100, 150, 200, 250 and 300 μ g/ml). The plates were incubated at 26°C for 48 hours followed by counting of recovered colonies. The relative number of virulence plasmid equivalents compared to the chromosome in recovered colonies was determined by qPCR. The pIBX (IncFII⁻) duplication frequency was calculated from the number of colonies appearing on LA plates with 150 μ g/ml Cm after 48 hours incubation, compared to total bacteria plated. The procedure was repeated on duplicated clones after the first round of selection giving the frequency for further amplification. The genotype of YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3} was verified by whole-genome sequencing and qPCR.

The gene-dose of the integrated pIBX (IncFII⁻) amplicon correlated with the recorded bioluminescence of individual colonies due to increased *lux*-operon copies encoded by the integrated construct. 48 hours after plating *Y. pseudotuberculosis* strains with multiple copies of the chromosomally integrated plasmid on non-selective LA plates, the reversion rate was determined by counting the number of bioluminescent colonies exhibiting low, YpIII:(cIBX)_{n=1} levels of bioluminescence after exposure with a Chemidoc Darkbox (Bio-Rad). The low, single copy genotype of the colonies was verified using qPCR.

Yop expression and secretion experiments

Overnight *Y. pseudotuberculosis* cultures were diluted 1:20 in fresh LB with 20 mM MgCl₂ and 5 mM EGTA (T3SS induced conditions, -Ca²⁺). The cultures were incubated 1 hour at 26°C before shifting the cultures to 37°C. Aliquots were extracted at the indicated times. Whole-cell samples were prepared from the bacterial culture, and samples containing secreted proteins were prepared from bacteria-free supernatants recovered after centrifugation. The samples were separated by 12 % SDS-PAGE, transferred to Immobilon PVDF membranes (Millipore) and probed with polyclonal rabbit antibodies raised against Yop proteins. The final blots were developed by enhanced chemiluminescence (ECL, GE-healthcare).

β -lactamase translocation experiments

HeLa-cells (2 x 10⁴) were seeded in DMEM lacking Phenol Red (Gibco) supplemented with 10 % fetal calf serum (FCS) in μ CLEAR 96-well black chimney well plates (Greiner) one day before experiments. Overnight cultures of YopH₁₋₉₉-Bla expressing *Y. pseudotuberculosis* strains (YpIII/pIBX, YpIII:(cIBX)_{n=1}, YpIII:(cIBX)_{n=3}, and YpIII/pIB219) carrying pET101yopH99-bla were diluted 1:100 in fresh DMEM lacking Phenol Red with 10 % FCS and grown at 26°C for 2

hours before infection. The amount of bacteria present in the cultures was determined by OD₆₀₀ measurements and the bacteria were diluted and added to the cultured HeLa-cells at the indicated multiplicity of infection (MOI). The infections were ended by adding Gentamicin to a final concentration of 25 µg/ml before labeling the cells with CCF2-AM loading solution (LiveBLAzer FRET-B/G Loading Kit, Thermo Scientific) supplemented with 2.5 mg/ml Probenecid (Molecular Probes) for 2 hours at room temperature. Translocation of YopH₁₋₉₉-Bla was determined by measuring the cleavage of the FRET substrate CCF2-AM by translocated YopH₁₋₉₉-β-lactamase fusion protein at wavelengths 409/520 nm (green) and 409/450 nm (blue) employing a Tecan infinite M200 (TECAN). The response ratio was calculated by dividing the recorded blue to green ratio in the infected wells by the average negative ratio recorded from CCF2-AM labeled uninfected HeLa-cells. Images of the cells were also acquired by Live cell imaging microscopy (Nikon Eclipse Ti-E) using a Nikon DS-2Fi color camera, a 20 × objective, and a filter with 405/20 nm excitation, 425 nm dichroic mirror, 435 nm (long-pass) emission, designed specifically for visualization of the CCF2-AM reaction (Chroma, USA). The MOIs of the infections were verified by viable counts of diluted infecting cultures after each experiment.

Quantitative Real-Time PCR (qPCR)

Y. pseudotuberculosis cells (~10⁸ cells) were harvested by centrifugation 6,000 x g for 5 min at 4°C. The bacterial pellet was resuspended in 150 µl of Fast Lysis Buffer from a *mericon* DNA Bacteria Kit (Qiagen). For isolation of bacterial DNA from infected mouse tissues, the organs were homogenized in 1 ml pre-cooled PBS using a Dispomix drive homogenizer (Medic Tools, Switzerland). Tissue homogenates were cleared by centrifugation 2,000 x g for 1 min at 4°C. Total DNA lysates were prepared using *mericon* DNA Bacteria Kit (Qiagen) for Gram-negative bacteria according to the manufacture's instructions.

Quantitative Real-Time PCR was performed using an iCycler iQ Real-Time PCR detection System (Bio-Rad) with iQ SYBR Green Supermix (Bio-Rad). After amplification, a melting curve analysis was performed to confirm the specificity of the reaction. Samples were analyzed with three different specific genomic DNA primer pairs and two specific plasmid primer pairs (Table S4). Plasmid copy number was defined as the ratio of plasmid DNA per chromosome, using the formula $2^{-\Delta CT}$, where ΔCT is the difference in threshold cycles between the plasmid gene and the chromosomal gene.

Southern blot analysis

A sample with 200 ng *Bam*HI digested total DNA isolated from *Y. pseudotuberculosis* bacterial strains and increasing amounts of *Bam*HI digested CsCl₂ purified pIBX were separated on a 1 % agarose gel. The DNA was transferred to a Zeta-probe Blotting Membrane (Bio-Rad) using standard capillary DNA transfer. The DNA was fixed to the membrane by UV irradiation (Spectrolinker, Spectronics Corporation), and the membrane was hybridized with Rapid-Hyb Buffer (GE Healthcare) with indicated P³² labeled DNA probes (Random Primed DNA Labeling Kit, Roche) according to manufacturer's recommendations. The probes were PCR amplified using primers presented in Table S4. The washed membranes were exposed to a Storage Phosphor Screen (Molecular Dynamics) and visualized using a Typhoon FLA 9500 Biomolecular Imager (GE Healthcare). The signals were quantified using the Quantity One software package (Bio-Rad).

Whole genome sequencing and data analysis

Purified total DNA (1 µg) was fragmented using an Adaptive Focused Acoustics S220 (Covaris). 350 bp, single indexed, paired-end DNA sequencing libraries were prepared using a TruSeq DNA PCR-free LT sample preparation kit (Illumina) according to the manufacturer's recommendations. The prepared TruSeq PCR-free DNA libraries were sequenced on a MiSeq DNA sequencing system with a 2 x 150 bp setup using a MiSeq Reagent kit V2 (300-cycles) (Illumina).

For DNA samples isolated from Peyer's Patches, library preparation was the same as described above. Clustering was done by "cBot" and samples were sequenced on HiSeq2500 (HiSeq Control Software 2.2.58/RTA 1.18.64) with a 2 x 126 bp setup using "HiSeq SBS Kit v4" chemistry by Science for Life Laboratory (SciLifeLab) and National Genomic Infrastructure Sweden (NGI). The Bcl to FastQ conversion was performed using bcl2fastq-1.8.4 from the CASAVA software suite. The quality scale used is Sanger / phred33 / Illumina 1.8+.

Data analysis and Tracks generation were performed using the CLC Genomic Workbench (CLC bio, Qiagen). Sequences were aligned against *Y. pseudotuberculosis* sequences (NC_010465 for the YPIII chromosome and an in house pIB1 sequence for the plasmid). The plasmid copy number was calculated by dividing the average coverage of plasmid DNA with the average coverage of chromosomal DNA.

Mouse infection and bioluminescent imaging

Eight-week-old female BALB/c mice (Charles river) were allowed to acclimate to the new environment for one week before the experiments. Mice were deprived of food and water for 16 hours prior to oral infection with bioluminescent wild-type YpIII/pIBX, YpIII:(cIBX)_{n=1}, or YpIII:(cIBX)_{n=3} and monitored using IVIS Spectrum (Caliper LifeSciences). Bacteria grown overnight at 26°C were harvested and re-suspended at a concentration of 2 x 10⁸ CFUs/ml in sterile tap-water supplemented with 150 mM NaCl. The bacterial suspensions were provided for 6 hours as drinking water. The infection dose was determined by viable counts and drinking volume. Mice were inspected frequently for signs of infection, and mice showing prominent clinical signs were euthanized promptly to prevent suffering. Mice were monitored for bioluminescent emission using IVIS Spectrum (Caliper LifeSciences). Mice were anesthetized using the XGI-8 gas anesthesia system (Caliper LifeSciences) prior to imaging with 2.5% IsoFluVet in oxygen (Orion Pharma Abbott Laboratories Ltd, Great Britain), and during imaging in 0.5% IsoFluVet. Images were acquired and analyzed using Living Image 4.5 (Caliper LifeSciences). To analyze bacterial localization within organs, mice were euthanized, the intestine, mesenteric lymph nodes, liver, and spleen removed, and the organs imaged by bioluminescent imaging. Bacteria were isolated from infected organs after plating serial, 10-fold dilutions of organ-homogenates in PBS on LA plates with 50 µg/ml Km.

Mice were housed in accordance with the Swedish National Board for Laboratory Animals guidelines. All animal experiments were approved by the Animal Ethics Committee of Umeå University (Dnr A144-12).

RNA isolation from bacterial cultures

Y. pseudotuberculosis strain IP32953 was grown in LB broth to stationary growth phase (16 hours; OD₆₀₀ of approx. 4.0) at 25°C or 37°C. Total bacterial RNA was isolated via a column-free hot phenol RNA extraction protocol (27). Remaining traces of DNA were digested with TURBO DNase (Ambion). The RNA was purified once with phenol:chloroform:isoamyl alcohol and twice with chloroform:isoamyl alcohol and recovered via ethanol precipitation. RNA integrity

was assessed using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). From 8 µg of high quality total RNA (RIN ≥ 9) ribosomal RNA was depleted using the MICROBExpress bacterial mRNA Enrichment Kit (Ambion). 1 µg rRNA depleted RNA was treated with tobacco acid pyrophosphatase (TAP; Epicentre Biotechnologies) as recommended by the manufacturer.

Infection of mice for *in vivo* RNA-seq

Female BALB/c mice aged 7-8 weeks were purchased from Janvier (Le Genest Saint Isle, St Berthevin Cedex, France) and housed in individually ventilated cages under specific pathogen-free conditions. Animal experiments were performed in strict accordance with the German Recommendations of the Society of Laboratory Animal Science (GV-SOLAS) and the European Health Recommendations of the Federation of Laboratory Animal Science Associations (FELASA). The animal protocols were approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit: animal licensing committee permission no. 33.9.42502-04-12/1010. Animals were handled with appropriate care and all efforts were made to minimize suffering. Animals were provided with food and water *ad libitum*; 14 hours prior to infection food was removed and provided again immediately after the infection. *Y. pseudotuberculosis* strain IP32953 was grown 16 hours overnight in LB broth at 25°C, washed and resuspended in 1xPBS. Three groups of five weight-matched mice were infected intragastrically using a gavage needle with approx. 2×10^8 CFUs of *Y. pseudotuberculosis*. All mice were monitored daily to determine the body weight and health status. At day 3 after infection, mice were sacrificed by CO₂ asphyxiation. Peyer's patches were excised aseptically and immediately snap frozen in liquid nitrogen.

RNA isolation from Peyer's Patches

For column-free total RNA isolation from Peyer's patches a modified version of earlier described protocols was applied (28, 29). Snap frozen Peyer's patches were transferred to sterile plastic tubes (Greiner) containing pre-cooled 1.5 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (wt/vol) N-lauroylsarcosine, 0.1 M 2-mercaptoethanol (Solution D) and immediately homogenized on ice at 11,000 rpm for 10 seconds using a Polytron PT 2100 homogenizer (Kinematica, Switzerland). After homogenization, 0.5 ml aliquots of tissue lysate were rapidly transferred to previously cooled 2 ml reaction tubes containing 100 mg of small (100 µm) and big (1 mm) acid washed glass beads (Sigma Aldrich). Bead-beating was performed using a TissueLyser (Qiagen) at fixed speed (30 Hz) for 1 min. Between bead-beating steps, samples were cooled on ice for 1 min. 0.5 ml of lysate was transferred to a new reaction tube prior addition of 50 µl of 2 M sodium acetate pH 4.0 and 500 µl water-saturated phenol and mixed thoroughly by inversion. After addition of 100 µl chloroform:isoamyl alcohol, samples were mixed vigorously by hand, cooled on ice for 15 min and centrifuged at 10,000 g for 20 min at 4°C. The supernatant was washed twice with chloroform:isoamyl alcohol. Total RNA was recovered via ethanol precipitation. DNA was digested using TURBO DNase (Ambion) as described by the manufacturer. The RNA was purified once with phenol:chloroform:isoamyl alcohol and twice with chloroform:isoamyl alcohol and recovered via ethanol precipitation. RNA integrity was assessed using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). High quality RNA (RIN ≥ 9) from Peyer's Patches of 5 mice were combined in equal amounts to one RNA pool. From 3 independent RNA pools mouse rRNA was depleted using the Ribo-Zero Gold rRNA Removal kit (Illumina). Prior TAP-treatment, 1 µl of

either 1:10 diluted ERCC ExFold RNA Spike-In Mix 1 or Mix 2 (Ambion) were added to 1 µg of mouse rRNA depleted RNA. TAP treatment was performed as recommended by the manufacturer.

Strand-specific cDNA library preparation and deep sequencing

RNA adapter based strand-specific cDNA library preparation and deep sequencing (Illumina HiSeq2500) was performed as described earlier (20). Quality of the sequencing output was analyzed using FastQC (Babraham Bioinformatics). Libraries were mapped to the IP32953 genome (NC_006155) and the pYV plasmid (accessions NC_006153) using Bowtie2 (version 2.1.0) with default parameters. After read mapping, SAMtools was employed to filter the resulting bam files for uniquely mapped reads (both strands). The high-throughput read data is deposited at the European Nucleotide Archive (ENA) with the accession no PRJEB14242.

Statistics

All statistical analysis was performed using GraphPad Prism 6. Statistical differences were analysed by Mann-Whitney nonparametric test or Student's unpaired *t*-test as indicated, with the significance set at * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$. Data are presented as the mean $\pm$ S.E.M..

Fig. S1.

Fig. S1. Determination of virulence plasmid copy-numbers in DNA isolated from *Y. pseudotuberculosis* cultures grown under different conditions. **(A)** Virulence plasmid copy-numbers determined by qPCR in DNA isolated from *Y. pseudotuberculosis* YpIII/pIBX grown 3 hours (same samples as in Fig 1A); 26°C (white bar), 37°C under T3SS-repressive (gray bar) and T3SS-inductive (black bar) conditions. Copy-number is defined as the number of plasmid per chromosome. Data represent mean ± S.E.M. of two independent samples quantified with 3 chromosomal primer pairs and 2 virulence plasmid primer pairs, repeated twice (***) $P \leq 0.001$, based on Mann-Whitney *U*-test). **(B)** Representative Southern blot analysis of *Bam*HI digested DNA probed with two radiolabelled plasmid probes (*yopBD* and *yopE*) and a chromosomal probe (*glnA*). 200 ng *Bam*HI digested total DNA isolated from wild-type *Y. pseudotuberculosis* YpIII/pIBX grown 3 hours under indicated conditions was separated on a 1 % agarose gel (Lanes 26°C, 37°C+Ca²⁺ and 37°C-Ca²⁺). Indicated amounts of *Bam*HI digested CsCl₂ purified pIBX (CsCl₂ pIBX) and 200 ng *Bam*HI digested total DNA isolated from a virulence plasmid cured *Y. pseudotuberculosis* strain (YpIII (pIBX⁻)) were loaded as controls. Numbers below the blot: relative *yopBD* and *yopE* signals compared to the 26°C sample, corrected for loading using the *glnA* signals.

Fig. S2

Fig. S2. Overexpression of CopA rescues T3SS-dependent growth restriction under T3SS-induced conditions. Growth of a wild type (YpIII/pIBX, open circles) and a $\Delta yopD$ mutant (YpIII/pIB621, open squares) and corresponding strains overexpressing *copA* under the control of the *yopE* promoter integrated *in cis* into the virulence plasmid (closed symbols). Bacteria were grown at 37°C in BHI supplemented with 20 mM MgCl₂ and 20 mM sodium oxalate (T3SS-induced conditions, -Ca²⁺). Data represent mean of two independent cultures measured in duplicates.

Fig. S3

Fig. S3. Plasmid gene-dose is stable in YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3} grown under different conditions. **(A)** Whole-genome sequencing results showing the ratio of plasmid equivalents compared to chromosome in DNA isolated from YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3} grown 3 hours; 26°C (white bar), 37°C under T3SS-repressive (gray bar) and T3SS-inducing (black bar) conditions. Plasmid gene-dose was calculated as the ratio of the average depth of plasmid DNA coverage/chromosomal DNA coverage (Table S1). **(B)** Ratio of plasmid equivalents compared to chromosome determined by qPCR in whole-cell lysates. Lysates were prepared from *Y. pseudotuberculosis* YpIII:(cIBX)_{n=1} and YpIII:(cIBX)_{n=3} grown 3 hours in different conditions (same as A). Data represent mean ± S.E.M. of three independent experiments.

Fig. S4

Fig. S4. Increased gene-dose of plasmid-encoded genes is essential for *Yersinia* virulence. **(A)** IVIS images showing organs extracted from mice infected with YpIII:(cIBX)_{n=1} (*left*) or YpIII:(cIBX)_{n=3} (*right*) at indicated time-points after infection. Pseudo-colors indicate levels of light emitted by bioluminescent bacteria. Layout of organs is shown in the top-right image: liver (L), mesenteric lymph node (MLN), spleen (S), Peyer's patches (PP) and cecum (C). **(B)** YpIII:(cIBX)_{n=1} (open circles) and YpIII:(cIBX)_{n=3} (solid circles) colonies recovered from Peyer's patches 2 and 5 days after infection. Organs were homogenized and serially diluted before plating on Km selective plates. Data are presented as mean of at least two organs.

Fig. S5

Fig. S5. Growth of a virulence plasmid cured YpIII strain (T3SS⁻, open circles), YpIII:(ciBX)_{n=1} (closed circles), YpIII/piBX (open squares), and YpIII:(ciBX)_{n=3} (closed squares) under T3SS-induced conditions at 37°C. Bacteria were grown at 37°C in BHI supplemented with 20 mM MgCl₂ and 20 mM sodium oxalate (T3SS-induced conditions, -Ca²⁺). Data represent mean of two independent cultures measured in duplicates.

Fig. S6

Fig. S6. The level of CopA decreases after T3SS-induction. **(A)** RPKM values of *repA* mRNA and *copA* antisense RNA from *in vitro* grown bacteria (incubated at 25°C or 37°C) and infected PPs as determined by RNA-seq (Table S2). **(B)** RPKM values of *repA* mRNA and *copA* antisense RNA from *in vitro* grown bacteria (incubated at 25°C or 37°C) and infected PPs corrected for the measured gene-dose (Table S1) in the respective sample (Table S2). Data represent mean $\pm$ S.E.M. (N=3) (Differences were analyzed by unpaired *t* test, * $P \leq 0.05$ and ** $P \leq 0.01$).

Table S1.

Summary of TruSeq DNA sequencing data and plasmid copy-number determination.

Sample	Genome	Genome size	% mapped reads	Mapped reads	Mapped bases (million)	Average coverage	Ratio
26°C (1)	chromosome	4.7Mb	97.94	7.44million	1049	223.63	
	plasmid	66.6kb		107015	15	226.52	1.01
26°C (2)	chromosome	4.7Mb	98.36	8.04million	1133	241.63	
	plasmid	66.6kb		95068	13	201.23	0.83
37°C +Ca²⁺ (1)	chromosome	4.7Mb	97.64	5.12million	722	153.90	
	plasmid	66.6kb		101860	14	215.61	1.40
37°C +Ca²⁺ (2)	chromosome	4.7Mb	98.1	10.41million	1468	313.07	
	plasmid	66.6kb		271730	38	575.18	1.84
37°C -Ca²⁺ (1)	chromosome	4.7Mb	97.3	6.93million	978	208.50	
	plasmid	66.6kb		261704	37	553.96	2.66
37°C -Ca²⁺ (2)	chromosome	4.7Mb	97.38	9.49million	1338	285.24	
	plasmid	66.6kb		395956	56	838.13	2.94
YPIII:(cIBX)_{n=1} 26°C	chromosome	4.7Mb	98.58	1.36million	192	40.95	
	Plasmid (replicon ⁻)	63.2kb		23697	3.34	52.86	1.29
YPIII:(cIBX)_{n=1} 37°C +Ca²⁺	chromosome	4.7Mb	98.38	1.41million	199	42.51	
	Plasmid (replicon ⁻)	63.2kb		21851	3.08	48.74	1.14
YPIII:(cIBX)_{n=1} 37°C -Ca²⁺	chromosome	4.7Mb	98.6	1.28million	180	38.53	
	Plasmid (replicon ⁻)	63.2kb		22474	3.17	50.13	1.30
YPIII:(cIBX)_{n=3} 26°C	chromosome	4.7Mb	97.72	1.59million	225	47.93	
	Plasmid (replicon ⁻)	63.2kb		81959	11.56	182.82	3.81
YPIII:(cIBX)_{n=3} 37°C +Ca²⁺	chromosome	4.7Mb	97.3	2.31million	326	69.46	
	Plasmid (replicon ⁻)	63.2kb		103543	14.60	230.96	3.32
YPIII:(cIBX)_{n=3} 37°C -Ca²⁺	chromosome	4.7Mb	97	2.62million	370	78.89	
	Plasmid (replicon ⁻)	63.2kb		127347	17.96	284.06	3.60
PP (1)	chromosome	4.7Mb	0.07	171389	19.88	4.24	
	plasmid	66.6kb		16631	1.93	28.96	6.83
PP (2)	chromosome	4.7Mb	0.09	239191	27.75	5.92	
	plasmid	66.6kb		18792	2.18	32.72	5.53
PP (3)	chromosome	4.7Mb	0.54	1447136	167.87	35.80	
	plasmid	66.6kb		111981	12.99	195.01	5.45
PP (4)	chromosome	4.7Mb	0.55	1418878	164.59	35.10	
	plasmid	66.6kb		97659	11.34	170.07	4.85

Table S2.*copA* and *repA* transcriptomic profile in response to temperature and during infection.

Condition	Sample	Feature ID*	Region start	Region end	Length	Total reads	RPKM	Plasmid copy-number [†]	Normalized RPKM [‡]	Ratio <i>copA/repA</i> expression
25°C_S	S25_1	<i>copA</i>	5774	5863	90	131	3887.32	0.92	4171	
		<i>repA</i>	4823	5689	867	8	24.33	0.92	26.44	157.75
	S25_2	<i>copA</i>	5774	5863	90	331	7434.95	0.92	8081	
		<i>repA</i>	4823	5689	867	32	74.61	0.92	81.10	99.64
	S25_3	<i>copA</i>	5774	5863	90	371	7176.78	0.92	7800	
		<i>repA</i>	4823	5689	867	39	78.31	0.92	85.12	91.64
37°C_S	S37_1	<i>copA</i>	5774	5863	90	126	2479.62	1.62	1530.63	
		<i>repA</i>	4823	5689	867	58	118.49	1.62	73.14	20.93
	S37_2	<i>copA</i>	5774	5863	90	222	6031.65	1.62	3723.24	
		<i>repA</i>	4823	5689	867	27	76.15	1.62	47	79.21
	S37_3	<i>copA</i>	5774	5863	90	164	4638.37	1.62	2863.19	
		<i>repA</i>	4823	5689	867	27	79.27	1.62	48.93	58.51
<i>in vivo</i>	PP_1	<i>copA</i>	5774	5863	90	46	2235.01	5.67	394.18	
		<i>repA</i>	4823	5689	867	68	342.97	5.67	60.49	6.52
	PP_2	<i>copA</i>	5774	5863	90	108	3295.59	5.67	581.23	
		<i>repA</i>	4823	5689	867	170	538.49	5.67	94.97	6.12
	PP_3	<i>copA</i>	5774	5863	90	60	2909.56	5.67	513.15	
		<i>repA</i>	4823	5689	867	52	261.76	5.67	46.17	11.12

* Genome accession number is NC_006153.

[†] Average plasmid copy number determined by TruSeq (Table S1).[‡] RPKM value normalized against plasmid copy-number.

S25: stationary phase grown bacteria at 25°C, S37: stationary phase grown bacteria at 37°C, PP: bacteria colonizing Peyer's patches of infected BALB/c mice.

Table S3.

Bacterial Strains and Plasmids used in this study.

Strain or plasmid	Relevant phenotype	Reference
<i>E. coli</i>		
TOP10	F ⁻ , <i>mcrA</i> , $\Delta(mrr-hsdRMS-mcrBC)$, $\Phi 80lacZDM15$, <i>DlacX74</i> , <i>recA1</i> , <i>deoR</i> , <i>araD139</i> , $\Delta(ara-leu)7679$, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Str ^R), <i>endA1</i> , <i>nupG</i>	Invitrogen
Sy327 λ -pir	$\Delta(lac-pro)$ <i>nalA</i> <i>recA56</i> <i>araD</i> <i>argE</i> (Am) $\lambda pirR6K$; Sm ^r	(30)
S17-1 λ -pir	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> M ⁺ , Sm ^R , <RP4:2-Tc:Mu:Ku:Tn7>Tp ^R	(31)
<i>Y. pseudotuberculosis</i>		
YpIII	pIB1 cured YpIII strain (T3SS ⁻)	(32)
YpIII/pIBX	YpIII strain with Tn5 <i>luxCDABE</i> inserted in the Tn1000 resolvase homolog in pCD1 (wild type); Km ^r	Caliper Life Sciences, (13)
YpIII/pIB621	In frame <i>yopD</i> deletion; Km ^r	(17)
YpIII/pIB219	In frame <i>yopB</i> and <i>yopD</i> deletion; Km ^r	(33)
YpIII/pIB73	In frame <i>lcrF</i> deletion; Km ^r	(15)
YpIII:(cIBX) _{n=1}	YpIII/pIBX derivative with the pIBX virulence plasmid lacking 3426 bp encoding the IncFII replicon and pNQ705 integrated into <i>YPK_3687</i> in the chromosome; Km ^r , Cm ^r	This study
YpIII:(cIBX) _{n=3}	YpIII:(cIBX) _{n=1} derivative with 4 copies of the integrated IncFII ⁻ pIBX plasmid in the chromosome; Km ^r , Cm ^r	This study
YpIII/pIBX-pyopE- <i>copA</i>	CopA-overexpressing YpIII/pIBX strain with <i>copA</i> under the <i>yopE</i> promoter inserted into pIBX; Km ^r , Cm ^r	This study
YpIII/pIB621-pyopE- <i>copA</i>	CopA-overexpressing $\Delta yopD$ strain (YpIII/pIB621) with <i>copA</i> under the <i>yopE</i> promoter inserted into pIB621; Km ^r , Cm ^r	This study
IP32953	Wild type <i>Y. pseudotuberculosis</i> , pYptb32953	(34)
Plasmids		
Zero blunt/TOPO	Cloning vector	Invitrogen
pUC $\Delta oriR1$ - <i>sacB</i>	pUC 57 derivative containing a 4497 bp <i>SphI</i> fragment for insertion of pIBX into the chromosome, SacB ⁺ ; Cb ^r	This study

pUCpyopE-copA	pUC 57 derivative containing a 860 bp <i>SphI</i> fragment encoding <i>yerA</i> (<i>sycE</i>) and the <i>yopE</i> promoter driving <i>copA</i> transcription, Cb ^r	This study
pNQ705	R6K suicide plasmid; Cm ^r	(30)
pNQ pyopE-copA	pNQ705 derivative containing a 500 bp <i>SphI</i> fragment encoding <i>yerA</i> (<i>sycE</i>) and the <i>yopE</i> promoter driving <i>copA</i> transcription, Cm ^r	This study
pNQΔoriR1-sacB	pNQ705 derivative containing a 4497 bp <i>SphI</i> fragment for insertion of pIBX into the chromosome, SacB ⁺ ; Cm ^r	This study
pNQpyopE-copA	pNQ705 derivative containing a 860 bp <i>SphI</i> fragment encoding <i>yerA</i> (<i>sycE</i>) and the <i>yopE</i> promoter driving <i>copA</i> transcription; Cm ^r	This study
pNQYPK_3687	pNQ705 derivative containing a 831 bp <i>SphI</i> fragment encoding <i>YPK_3687</i> ; Cm ^r	This study
pET101yopH99-bla	pET101 derivative containing a 1210 bp fragment encoding the <i>yopH</i> promoter sequence and amino acids 1-99 of <i>yopH</i> fused to <i>bla</i> (35) lacking the first 23 amino acids; Cb ^r	(22)

Table S4.

Oligonucleotide primers used in this study.

qPCR primers	
Primer name	Sequence (5' to 3')
<i>yopH</i> fw	AATTACCCAAGTGGGACACG
<i>yopH</i> re	TCACTATCACTTCCCCTGCCT
<i>repA</i> fw	TATCAAACCTGAACGTAAGGCTC
<i>repA</i> re	AACGATAGCTTCAATGTCACG
<i>glnA</i> fw	GGCATGGATCCTGAGATGA
<i>glnA</i> re	CGTCAAAACATGTTTCAGCGGAC
<i>rpoB</i> fw	AATCGTCAAGGTGTATCTGG
<i>rpoB</i> re	TCGTAAGGCATATCTTCGAT
<i>YPK_3178</i> fw	ATCCACTGTTACCAGAATGC
<i>YPK_3178</i> re	AGAAGAAACAACGGTCAAAA
Southern blot probes	
<i>yopBD</i> fw	ATGAGTGCGTTGATAACCCATGACCGC
<i>yopBD</i> re	GCGGCTTTCATGGCGTGAGTATGACTGC
<i>yopE</i> fw	GAATTCCCCAACTTTGACACCGATAACCGG
<i>yopE</i> re	GCATGCTAAAAAACGATTGCCCCCTGAAAGATCAG
<i>glnA</i> fw	ATGTCCGCTGAACATGTTTTGACGATGCTG
<i>glnA</i> re	GTAATACAGTTCGAACTCAACAGGATGCGGA