Increased plasmid copy number is essential for *Yersinia* T3SS function and virulence

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Pathogenic bacteria have evolved numerous virulence mechanisms that are essential for establishing infections. The enterobacterium *Yersinia* uses a type III secretion system (T3SS) encoded by a 70-kilobase, low-copy, IncFI1-class virulence plasmid. We report a novel virulence strategy in *Y. pseudotuberculosis* in which this pathogen up-regulates the plasmid copy number during infection. We found that an increased dose of plasmid-encoded genes is indispensable for virulence and substantially elevates the expression and function of the T3SS. Remarkably, we observed 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.

Three human pathogenic *Yersinia* strains—*Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*—share a common 70-kb virulence plasmid (IncFI1) that encodes a set of virulence proteins called Yops (*Yersinia* outer proteins) (1, 2). These bacterial toxins are secreted by a plasmid-encoded organelle, the *ysc/yop* type III secretion system (T3SS) (3–5). The T3SS comprises ~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 increase 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 Ca2+ from the growth medium (11).

Incubation of *Yersinia* at 37°C in Ca2+-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 (22). 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 amounts of virulence plasmid DNA were recovered from wild-type *Y. pseudotuberculosis* cells grown under T3SS-inductive conditions relative to bacteria grown under T3SS-repressive conditions (fig. S1B). Therefore, we hypothesized that *Yersinia* may undergo rapid changes in gene expression by increasing and decreasing virulence plasmid copy numbers. Rapid changes in gene dose 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 number, we first determined plasmid (pIBX) copy numbers in *Y. pseudotuberculosis* YpIII (YP11/pIBX) (13) cultures under different conditions with a polymerase chain reaction (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 chromosome 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-repressive conditions), the copy number increased only modestly (1.6 per chromosomal equivalent). Similar differences in plasmid copy number were observed in other wild-type *Yersinia* strains (fig. S1B). In summary, *Yersinia* is capable of rapidly adjusting gene dose to optimize fitness and the expression of virulence proteins. The increased copy number allows for increased expression of Yops, which is required for intimate contact with the host cell; consequently, the bacterium establishes an intimate contact with the host cell, and Yop secretion increases after contact (15). This process provides a framework for further exploration of the environmental sensing mechanisms of pathogenic bacteria.
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-inductive conditions (Fig. 1B). Bacteria grown at 37°C under T3SS-repressive conditions plateaued at ~2 chromosome equivalents (Fig. 1B). These results confirmed that the plasmid copy number increased in Y. pseudotuberculosis (15, 16). The T3SS transcriptional activator 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 display Ca²⁺-insensitive Yop expression and restricted growth at 37°C, irrespective of Ca²⁺ concentration (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 LcrF mutant (YpIII/pIB79) (Fig. 1C). In contrast, in a yopD mutant (YpIII/pIB621), the plasmid copy number was elevated at 37°C under T3SS-repressive conditions (gray bars, Fig. 1C). Thus, YopD directly or indirectly inhibits 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 whether the IncFII replicon per se is responsible for increased virulence plasmid copy number, we expressed 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-inductive conditions. This construct was integrated in cis into the virulence plasmids of wild-type Yersinia and a ΔyopD mutant. CopA overexpression resulted in reduced plasmid copy numbers under T3SS-inductive 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-inductive conditions (Fig. 1D, inset, and fig. S2). This indicates that Yersinia has evolved a T3SS-dependent mechanism to down-regulate the copy number of its virulence plasmid, and that this mechanism is 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 fig. 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 the wild type (Fig. 2C). Yop translocation experiments showed that YpIII/(cIBX)n=1 could translocate the reporter protein YopH(Glu)-Bla (22) into HeLa cells in a dose-dependent manner, but less than the parental YpIII/pIBX wild-type strain (Fig. 2A). 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 (MLNs; Fig. 3B and fig. S4) but all infected mice survived and recovered their initial body weight (Fig. 3B, inset), showing that the YpIII/(cIBX)n=1 strain is avirulent.

To confirm whether 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 base pairs (bp)] YPK_3687 sequences flanking the integrated IncFII plasmid (Fig. 2A). When YpIII:(cIBX)\(_{n=1}\) was plated on to agar plates with different chloramphenicol concentrations, we recovered clones resistant to chloramphenicol (150 \(\mu\)g/ml) at a frequency of \(1 \times 10^{-4}\) and reverted to the original single-copy genotype at a frequency of \(2 \times 10^{-4}\). Unless specified otherwise, all infection experiments were performed with a frequency of \(1 \times 10^{-1}\) to \(1 \times 10^{-2}\) to minimize the effects of natural variation in the study population. In these experiments, some isolates showed reduced virulence plasmid copy number on agar plates with chloramphenicol (300 \(\mu\)g/ml) at a frequency of \(1 \times 10^{-4}\) in the absence of selective pressure (Fig. 3B). Colors indicate levels of light emitted by bioluminescent bacteria.

**Fig. 3. Increased gene dose of plasmid-encoded genes is essential for Yersinia virulence.** (A) Translocation of YopH\(_{309}\).Bla into HeLa cells after 90 min. Histograms represent mean ± SEM of a representative experiment. Translocation-deficient yopBD mutant (YpIII/pIB219) expressing YopH\(_{309}\).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). Colors indicate levels of light emitted by bioluminescent bacteria. Inset: Changes in average body weight of infected mice over time [open symbols, YpIII:(cIBX)\(_{n=1}\); solid symbols, 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 symbols, YpIII:(cIBX)\(_{n=1}\); solid symbols, YpIII:(cIBX)\(_{n=3}\)]. Data are means ± SEM (**P ≤ 0.001, Mann-Whitney U test).

**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 hours after infection, determined by whole-genome sequencing (TruSeq) (table S1) and by qPCR. Plasmid copy number of infecting inoculum determined by 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. Plasmid copy number of infecting inoculum is shown as control (\(N \geq 3\)). (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 are means ± SEM; **P ≤ 0.05, ***P ≤ 0.001, ****P ≤ 0.0001 (difference between control and the respective conditions; unpaired t test).

**A** clone with three copies of the plasmid [YptIII:(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 fig. S3). YpIII:(cIBX)\(_{n=3}\) showed Yop expression, secretion, and translocation profiles similar to those of the wild-type strain (Fig. 2C and Fig. 3A). Whereas the YpIII:(cIBX)\(_{n=1}\) strain showed a growth rate similar to that of the plasmid-cured YpIII (T3SS) strain, the YpIII:(cIBX)\(_{n=3}\) strain showed reduced growth at 37°C under T3SS-inductive conditions similar to that of the wild-type strain (fig. S5). 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 killed. Bacterial loads 2 days after infection were higher in homogenized PPs from animals infected with virulent YpIII:(cIBX)\(_{n=3}\) than in those infected with avirulent YpIII:(cIBX)\(_{n=3}\) by two orders of magnitude (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 factor of 6 increase in virulence plasmid copy number in the PPs relative to 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.

Reduced amounts of copA antisense RNA relative to repA mRNA have been shown to lead 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 mRNA to repA mRNA decreased significantly during PP colonization relative to bacteria grown in vitro (Fig. 4C), corroborating the findings presented above. Our results show that T3SS-related copy number regulation affects the stability or transcription of both RNA species (table S2 and fig. S6). Taking into account 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 and fig. S6). Such a finding favors a model in which T3SS-related copy number regulation operates via a change in copA antisense RNA levels.

Our results show that an increased 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, there was selective pressure during infection 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-inductive 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 allow pathogens to respond to environmental cues, and plasmid copy number regulation may be important in bacterial antibiotic resistance.

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**SUPPLEMENTAL MATERIALS**

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Materials and Methods

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**EPIGENETICS**

Early-life nutrition modulates the epigenetic state of specific rDNA genetic variants in mice

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A suboptimal early-life environment, due to poor nutrition or stress during pregnancy, can influence lifelong phenotypes in the progeny. Epigenetic factors are thought to be key mediators of these effects. We show that protein restriction in mice from conception until weaning induces a linear correlation between growth restriction and DNA methylation at ribosomal DNA (rDNA). This epigenetic response remains into adulthood and is restricted to rDNA copies associated with a specific genetic variant within the promoter. Related effects are also found in models of maternal high-fat or obesogenic diets. Our work identifies environmentally induced epigenetic dynamics that are dependent on underlying genetic variation and establishes rDNA as a genomic target of nutritional insults.

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