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Calcium-responsive plasmid copy number regulation is dependent on discrete YopD domains in *Yersinia pseudotuberculosis*

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ABSTRACT

Yersinia pathogenicity depends mainly on a Type III Secretion System (T3SS) responsible for translocating effector proteins into the eukaryotic target cell cytosol. The T3SS is encoded on a 70 kb, low copy number virulence plasmid, pYV. A key T3SS regulator, YopD, is a multifunctional protein and consists of discrete modular domains that are essential for pore formation and translocation of Yop effectors. In *Y. pseudotuberculosis,* the temperature-dependent plasmid copy number increase that is essential for elevated T3SS gene dosage and virulence is also affected by YopD. Here, we found that the presence of intracellular YopD results in increased levels of the CopA-RNA and CopB, two inhibitors of plasmid replication. Secretion of YopD leads to decreased expression of *copA* and *copB*, resulting in increased plasmid copy number. Moreover, using a systematic mutagenesis of YopD mutants, we demonstrated that the same discrete modular domains important for YopD translocation are also necessary for both the regulation of plasmid copy number as well as *copA* and *copB* expression. Hence, *Yersinia* has evolved a mechanism coupling active secretion of a plasmid-encoded component of the T3SS, YopD, to the regulation of plasmid replication. Our work provides evidence for the cross-talk between plasmid-encoded functions with the IncFII replicon.

1. Introduction

The *Yersinia* genus includes three human pathogens, *Y. pestis*, *Y. enterocolitica,* and *Y. pseudotuberculosis,* which all share a 70 kb virulence plasmid (Portnoy et al., 1984). This plasmid of *Yersinia* Virulence (pYV) encodes effectors proteins, called *Yersinia* outer proteins (Yops), which are highly conserved among pathogenic *Yersinia* and are able to subvert the host immune response (Chung and Bliska, 2016). The effectors are delivered into the host cell by a molecular apparatus called the Type III Secretion System (T3SS) composed of about 20 pYV-encoded Ysc (*Yersinia* Secretion) proteins (Galán and Wolf-Watz, 2006). They are exclusively expressed at elevated temperature (37 °C), as in the preferred mammalian host, and then assembled into a T3SS. The T3SS spans both inner and outer bacterial membranes and contains a protruding hollow needle-like structure. Upon contact with eukaryotic target cells, this needle complex ('injectisome') is activated to translocate the Yop effectors into the target cell cytoplasm through a pore formed in the target cell plasma membrane. This initial translocation then triggers a feedback loop leading to a massive expression and secretion of effector proteins. The process of T3SS expression, assembly, and Yops deployment causes bacterial growth arrest *in vitro*, likely manifested by acute metabolic burden, and therefore needs to be tightly regulated (Cornelis, 2002; Cornelis, 2006; Plano and Schesser, 2013).

The T3SS is regulated at both the transcriptional and post-transcriptional level by positive and negative loops. Transcriptional regulation involves LcrF, an AraC-like transcriptional activator (Schwiesow et al., 2016). At 37 °C, LcrF accumulates because the

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elevated temperature resolves a temperature-sensitive hairpin-loop structure (RNA thermometer) in the 5'-UTR of the *lcrF* mRNA (Böhme et al., 2012). LcrF, in turn, activates the transcription of *yop/ysc* genes (Schwiesow et al., 2016). Post-transcriptional regulation of the T3SS in *Yersinia* is further dependent on host cell contact which can be mimicked *in vitro* by limiting Ca^{2+}-accessibility (Rosqvist et al., 1994). This so-called low-calcium response (LCR) involves YopD, LcrH and LcrQ (Cornelis et al., 1998). YopD is a bifunctional protein that is both a necessary structural component of the translocation pore and a regulator of the translation of the *yop/ysc* mRNAs by binding to their 5'-UTRs. Both functions require YopD to bind to the cognate chaperone, LcrH, so that the pre-secretory YopD stability is maintained and its eventual secretion is prioritized (Williams and Straley, 1998; Anderson et al., 2002; Chen and Anderson, 2011). The multi-functional nature of YopD is compartmentalized into discrete functional domains (Olsson et al., 2004). The domains were identified by systematic mutagenesis of 13 regions along *yopD*. Some domains have a role in translocation of Yop effectors into target cells and are distinct from other domains responsible for pore formation or T3SS regulation. Upon the LCR activation, T3SS secretion is induced and intracellular YopD concentrations decrease, releasing the YopD translation inhibition of Yop/Ysc. Consequently, a knock-out mutation of y*opD* or *lcrH,* often leads to derepression of T3SS genes expression at 37 °C, irrespective of calcium levels. In these cases, Yops are continuously synthesized and secreted (but not translocated into the host cell), which impairs the growth of a Δ*yopD* or Δ*lcrH* mutant at 37 °C (Schiano and Lathem, 2012).

In addition, *Yersinia* possesses another regulatory mechanism shown to be essential for virulence. Upon entry of the mammalian host, marked by a temperature switch from environmental temperature to 37 °C, a change in T3SS gene dosage is mediated by programmed variations in the pYV plasmid copy number (PCN) (Wang et al., 2016). As a consequence, bacteria are able to trade off the metabolic cost of utilizing the T3SS during infection by modulating PCN (Wang et al., 2016). Bacteria impaired in PCN variation, being fixed at one copy per cell, were attenuated in a mouse model of infection. On the other hand, bacteria with PCN fixed at three copies per cell successfully established an infection in the same mouse model (Wang et al., 2016). The PCN is regulated *in vitro* by temperature and the LCR. At 26 °C, the pYV plasmid is maintained at one copy per cell. The PCN increases to an average of 1.5 when shifted to 37 °C under T3SS repressive conditions (plus Ca^{2+}) and reaches 3 to 4 copies per cell at 37 °C under T3SS-inductive conditions (minus Ca^{2+}). The plasmid replication is under the control of RepA, the plasmid replication initiator, and CopB and CopA, its repressors. CopA, an antisense RNA, interacts with the ribosome binding site upstream of the *repA* transcript, preventing translation, while CopB represses the promoter of *repA*, reducing transcription (Persson et al., 1988; Blomberg et al., 1992; Nordström, 2006). The increased PCN correlated with a decreased ratio of CopA antisense RNA to *repA* mRNA. In addition, Wang et al., identified that a Δ*yopD* mutant had an increased PCN at 37 °C in T3SS repressive conditions (plus Ca^{2+}), while a Δ*lcrF* mutant displayed a wildtype phenotype with respect to PCN. To investigate the role of YopD in the PCN regulation in *Yersinia*, we studied the effect of *yopD* mutations on pYV plasmid replication regulation. The transcription of the genes involved in plasmid replication, *repA*, *copA,* and *copB,* was examined in wildtype bacteria and compared to their transcription in a full-length Δ*yopD* null mutant and in a series of 13 sequential smaller in frame Δ*yopD* deletion mutants lacking previously characterized functional domain. We found that transcription of CopA and CopB RNA was de-regulated in a Δ*yopD* mutant under T3SS repressive conditions at 37 °C. We also show that YopD domains necessary for PCN control are identical to the domains essential for T3SS gene regulation. Hence, we propose that YopD-mediated co-regulation of PCN and T3SS gene expression enables adjustment of the T3SS output in the different phases of infection.

2. Material and methods

2.1. Bacterial strains and growth conditions

The strains used in this study are listed in **Supplementary Table S1**. *Y. pseudotuberculosis* was routinely grown at 26 °C or 37 °C under aerobic conditions in LB (lysogeny broth) solid or liquid media. Antibiotics were used for bacterial selection as follows: kanamycin 50 $\mu g \cdot ml^{-1}$, carbenicillin 50 $\mu g \cdot ml^{-1}$. Unless stated otherwise, cultures were pregrown at 26 °C before being shifted to the desired growth conditions. T3SS-repressing conditions correspond to bacteria grown in normal LB at 37 °C while T3SS-inducing conditions were achieved by growing the bacteria in LB supplemented with 50 mM $Na_2C_2O_4$ and 50 mM $MgCl_2$ at 37 °C.

2.2. Construction of Y. pseudotuberculosis deletion mutants

The different *yopD* mutant constructs from Olsson et al., 2004 (Fig. 2) were re-constructed by homologous recombination using pDM4 as previously described (Olsson et al., 2004). Briefly, 1000 bp DNA fragments encoding 500 bp upstream and downstream sequences from the deletions, were constructed by overlapping PCR. The constructs were cloned into pDM4 using *Xho*I (or *Spe*I) and *Xba*I and electroporated into *E. coli* S17–1 λ-*pir*. The final suicide plasmids were conjugated into YPIII (Xen4) and recombinant clones with the suicide plasmid integrated into the chromosome were recovered on LB agar plates containing 50 $\mu g \cdot ml^{-1}$ Km and 34 $\mu g \cdot ml^{-1}$ Cm. In-frame deletion mutants were recovered after SacB counter-selection on LB agar plates with 10% sucrose and 50 $\mu g \cdot ml^{-1}$ Km. The final mutants were verified by sequencing. In-frame deletions of *yopD* and *yscS* in YPIII were carried out as described previously (Kusmierek et al., 2019).

2.3. Determination of PCN by droplet digital PCR

Bacteria were diluted 1/25 in fresh medium from overnight cultures, grown for 2 h at 26 °C, and then shifted for an additional 6 h to 37 °C with Ca^{2+} (T3SS repressing condition). After incubation, samples were taken, spun down and the supernatants were removed. Whole genome DNA was extracted using the GeneJET Genomic DNA purification Kit (ThermoScientific) following the recommended protocol. The concentration of the eluted DNA was determined using a Qubit 2.0 fluorometer (Thermo Scientific). Droplet digital (dd) PCR was carried out as previously described using one pair of primers for the plasmid and another pair for the chromosome (Schneiders et al., 2021).

2.4. Trans-complementation of the mutants

The *lcrH-yopBD* region was amplified by PCR. Both the amplified genes and the pRS1 vector (Kusmierek et al., 2019) (**Supplementary Table S2**) were digested using *Eco*RI/*Xba*I Fast digest enzymes (New England Biolabs). The inserts were cloned into pRS1 by ligation using T4 DNA ligase. Ligated plasmids were electroporated into *E. coli* DH5α electrocompetent cells and later into the YPIII strain of interest. The final clones were confirmed by PCR using vector-specific primers and subsequently confirmed by Sanger sequencing.

2.5. Construction of lacZ reporter

The different *lacZ* reporter systems used in this study are described in Fig. 1 and **Table S1**. The different inserts were amplified by PCR using primers described in **Supplementary Table S3**. The translational P_{copB}-*copB-lacZ* and the transcriptional P_{repA}-*lacZ* reporter plasmid were constructed by ligating the insert into the *Kpn*I/*Bam*HI site of plasmid pFU67 (Uliczka et al., 2011) (**Table S2**). The transcriptional P_{copA}-*lacZ* reporter was created by ligating the insert into the *Kpn*I/*Bam*HI site of plasmid pFU68 (Uliczka et al., 2011).

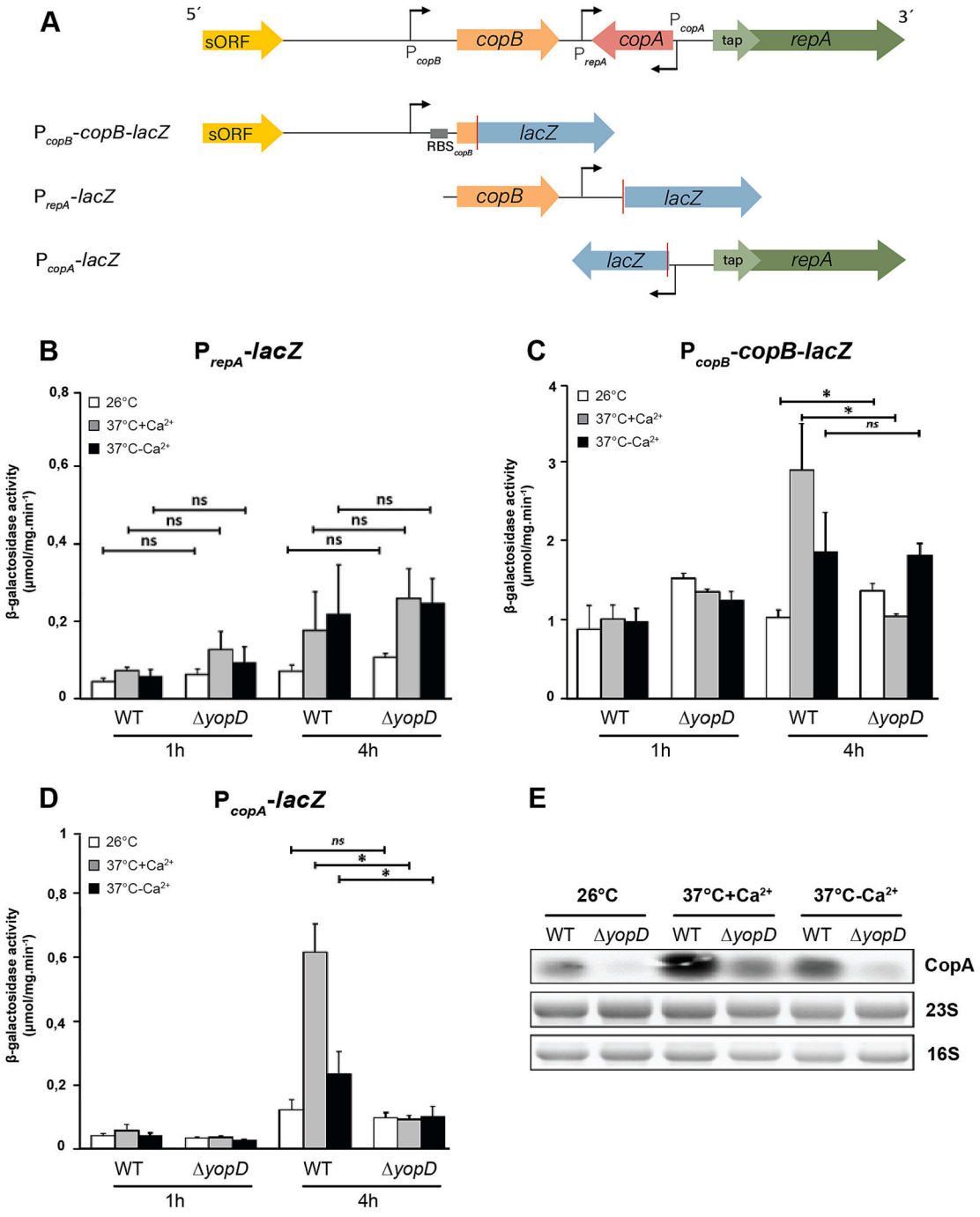

Fig. 1. Deletion of *yopD* leads to reduced level of the CopA-RNA in *Y. pseudotuberculosis*. A. Schematic representation of the *lacZ* reporter systems used. **B-E.** *Y. pseudotuberculosis* was pre-grown in LB medium for 2 h at 26 °C followed by 1 h or 4 h at 26 °C (white bar), at 37 °C under T3SS-repressive (grey bar) or T3SS-inductive (black bar) conditions. **B-D.** β-galactosidase activity of the cells were measured as described in Material and Methods. Data are shown as means ± SD ($n = 3$) and analyzed using Student's *t*-test (*: $p < 0.05$; ns: not significant). **E.** CopA-RNA levels (after a total of 6 h incubation) were analyzed by Northern blotting. Total RNA was prepared, separated on a 1.2% agarose gel, transferred onto a Nylon membrane and probed with Digoxigenin-labelled PCR fragment encoding the *copA* gene. The 16S and 23S rRNAs are shown as RNA loading control.

2.6. RNA isolation and Northern blotting

Bacteria grown under the required growth conditions were pelleted and RNA was isolated using hot phenol-chloroform extraction followed by ethanol precipitation. Total RNA (20 µg) was separated on MOPS agarose gels (1.2%), transferred by vacuum blotting for 1.5 h onto positively charged nylon membranes (Whatman) at 120 V in 10 x SSC buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7) using a semi-dry blotting system. The RNA was crossed-linked using UV exposure. Prehybridization, hybridization to DIG-labelled probes, and membrane washing were conducted using the DIG Luminescent Detection Kit (Roche) according to the manufacturer's instructions. Double-stranded DNA probes for *copB* and *repA* were amplified by PCR exchanging the standard dNTP mix with a 10× DIG DNA labelling Mix (Roche). The single-stranded RNA probe for CopA was first amplified by PCR and later *in vitro* transcribed using the 'Transcript AidTM T7 High Yield Transcription' kit in accordance with the manufacturer's instructions with a 10× DIG RNA labelling Mix (Roche). DNA probes were then denatured

three times at 95 °C for 10 min and incubated on ice for 5 min after the first two heating steps. RNA probes were denatured once at 70 °C for 10 min. All probes were immediately added to the hybridization buffer after the last heating step. After prehybridization, hybridization, blocking, Anti-DIG AP-conjugate incubation, and detection using ChemiDoc XRS + system (Bio-Rad, Hercules, CA) the signal was documented using a CL-XPosure X-ray film (Thermo Scientific).

2.7. Gel electrophoresis and Western blotting

For immunological detection of the YopD protein recovered from equal amounts of bacteria, bacterial cell extracts were prepared and separated on a 15% polyacrylamide SDS gel. Proteins were transferred onto an Immobilon-P membrane (Millipore) and probed with polyclonal antibodies directed against YopD (Davids Biotechnologies, Germany) as described (Heroven et al., 2008).

2.8. β-galactosidase assays

Bacteria harboring *lacZ* reporter fusion plasmids were grown for 2 h at 26 °C and shifted to the desired growth conditions. All the β-galactosidase assays were carried out in 2 technical replicates per 3 biological replicates. β-galactosidase was measured in cell-free extracts as described previously (Nagel et al., 2001). Briefly, cells were lysed using 0.1% SDS and chloroform for 10 min. Next, *Z*-buffer (300 mM Na2HPO4, 100 mM NaH2PO4, 50 mM KCl, 5 mM MgSO4) was added and the reactions were initiated by adding ONPG (4 mg·ml^{-1}). The reactions were stopped by adding 1 M $NaCO_3$ to a final concentration of 0.3 M. The OD was measured using a microplate reader (Tecan) with λ = 655 nm and the color reaction of the assay at λ = 415 nm. The β-galactosidase activity was calculated using the following formula:

$$\beta - \text{galactosidase activity} = \frac{\text{OD}415 \times 6,75}{OD655 \times V \times t}$$

t: time [min].
V: volume [ml].
6,75: extinction coefficient.

2.9. Statistical analysis

All experiments were carried out independently and at least in triplicate. Values are expressed as mean ± standard deviation (SD) of the results of multiple independent experiments. Student's *t*-test were calculated using Microsoft Excel and multiple comparison test were done using Prism 9.

3. Results

The pYV plasmid belongs to the IncFII plasmid family that has been well studied in *E. coli*. The basic replicon of the IncFII plasmid R1 in *E. coli* consists of an origin of replication (*oriR1*), and three regulating genes: a replication initiator (*repA*), a transcriptional inhibitor (*copB*) and a regulatory antisense ncRNA (*copA)* (Rosen et al., 1980). The IncFII replicon organization is the same in pYV. We found that the ratio of CopA antisense RNA to *repA* mRNA decreased *in vivo* during infection, resulting in an elevated PCN (Wang et al., 2016). We also observed that YopD downregulates PCN in T3SS-repressive conditions at 37 °C (Wang et al., 2016). Herein, we wanted to further elucidate the mechanism by which YopD interacts with the pYV regulon and inhibits PCN elevation. Hence, we investigated the transcription of *repA,* CopA, and CopB using transcriptional and translational *lacZ* fusions and monitored the β-galactosidase activity of the wildtype, an in-frame Δ*yopD* full-length deletion mutant and 13 mutants with small internal deletions within *yopD* that lead to different impaired functional domains.

3.1. YopD regulates PCN through replication repressors

Transcription of *repA* is controlled by two promoters. The first promoter (P_{copB}) is located upstream of *copB* and *repA* and transcribes both genes as a polycistronic mRNA. The second promoter (P_{repA}) is situated right upstream of *repA* (Fig. 1A). To measure the transcription of *repA*, we used the transcriptional P_{repA}-*lacZ* fusion to follow the activity of the *repA* promoter under different conditions (Fig. 1A,B**, Supplementary Fig. S1**). This fusion contained the regulatory region of *repA* including the P_{repA} promoter, but not its 3′-end with the P_{copA} promoter encoded on the opposite strand as this would have led to the expression of the *copA* antisense RNA, known to inhibit PCN increase (Wang et al., 2016). The activity of the P_{repA} promoter increased at 37 °C irrespective of Ca^{2+} concentration in both the wildtype and the Δ*yopD* background. Similar to the result found by Wang et al., there was a slightly higher P_{repA} activity in a Δ*yopD* mutant at 37 °C plus Ca^{2+} but this effect was not significant (Fig. 1B). The promoter activity was increased at 37 °C and was higher after 4 h compared to 1 h after the temperature shift, suggesting that the temperature regulation of *repA* transcription already occurs at the transcriptional level and is at least partly mediated by P_{repA}.

To investigate if the control of pYV replication by YopD occurred *via* the modulation of the negative regulators, CopA-RNA and CopB, we constructed a translational P_{copB}-*copB-lacZ* and a transcriptional P_{copA}-*lacZ* fusion (Fig. 1A**, Supplementary Fig. S1**). In the wildtype, the transcription of *copB* and *copA* was increased after 4 h upon the temperature shift from 26 °C to 37 °C at T3SS-repressive conditions (3-fold for *copB*, 7-fold for *copA)*. However, when calcium was depleted and T3SS induced, both *copB* and *copA* transcription was lowered and closer to 26 °C conditions (Fig. 1C-D). As the *copA-lacZ* fusion only reflects the activity of the *copA* promoter but not the amount of the CopA-RNA, which is also subjected to RNase degradation, we investigated the corresponding CopA-RNA levels by Northern blotting with RNA isolated from *Y. pseudotuberculosis* grown under the same conditions. In support of previous results, the CopA-RNA level increased at 37 °C under T3SS-repressive conditions but decreased upon induction of T3SS gene expression (Fig. 1E).

In the Δ*yopD* mutant, *repA* transcription levels remained unchanged compared to the wildtype (Fig. 1B). In contrast, *copA* and *copB* transcription was not increased in the Δ*yopD* mutant at 37 °C in T3SS-repressive conditions after 4 h compared to the wildtype. This was particularly evident for the transcription of the antisense CopA-RNA which was reduced in both T3SS-repressive and inductive conditions (Fig. 1C-D). The amount of CopA remained completely repressed at 37 °C under T3SS-repressive conditions and was also significantly lower at 26 °C and in the absence of calcium (Fig. 1E**, Supplementary Fig. S2**) In summary, the main effect of YopD on PCN was observed at 37 °C in T3SS-repressive conditions and under these conditions the *copA/repA* ratio was much smaller in the Δ*yopD* mutant. Although transcription of *repA*, *copA* and *copB* alone is not sufficient to explain observed PCN changes between 26 °C and 37 °C in T3SS-inductive conditions, it is evident that YopD plays an important role in regulating *copA* and *copB* transcription levels, especially at 37 °C under T3SS-repressive conditions.

3.2. Discrete domains of YopD are essential for PCN control

To investigate the role of different YopD functional domains in PCN regulation, we utilized the pDM4 suicide plasmid used by Olsson et al., 2004 to establish a series of sequential small deletion mutations throughout *yopD* in the YPIII/Xen4 background. All variants were synthesized; Δ3, Δ4 and Δ12 to somewhat reduced levels, whereas the levels of Δ5 and Δ6 were comparable to wildtype (**Supplementary Fig. S3**). We found that Δ3 to Δ6 and Δ12 displayed similar PCN to that seen in the Δ$yopD^{null}$ mutant (Fig. 2). The other deletions showed no significant difference in PCN compared to that of the wildtype. The mutants that resulted in increased PCN, were previously reported to be

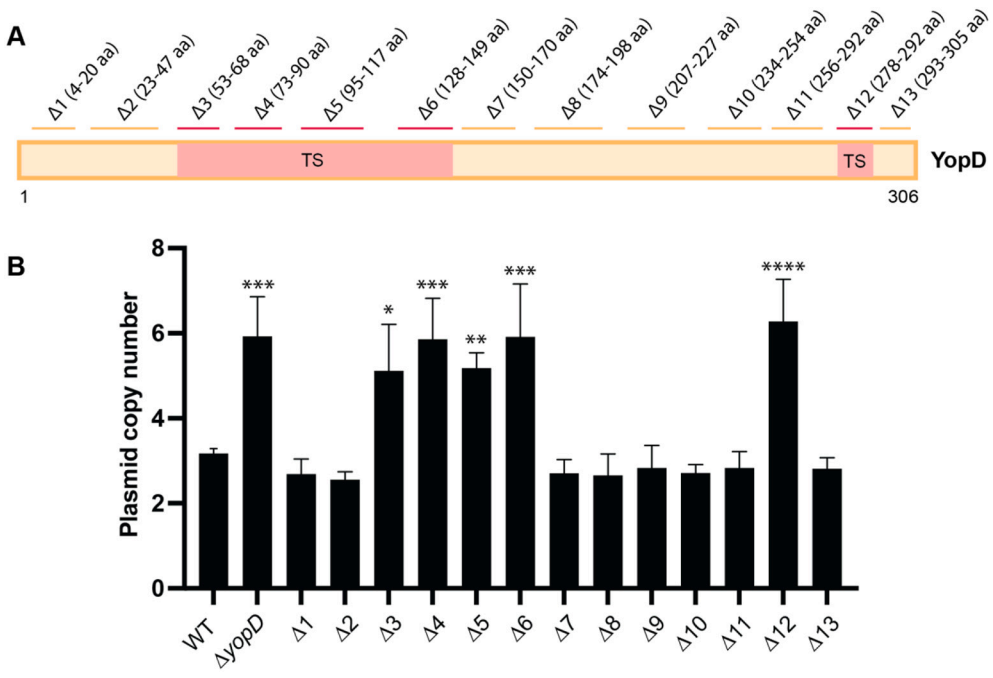

Fig. 2. YopD functional domains important for regulatory function and binding to LcrH are required for PCN regulation. **A.** Features of the different domains of the YopD protein as described in Olsson et al., 2004. YopD is a 306-amino-acid protein. Shown are the locations of the sequential in-frame *yopD* domain deletions used in this study (ex: Δ1, 4–20 aa: deletion of codons encoding the amino acids 4 to 20). Yop synthesis regulation phenotype is indicated. The strains displaying a temperature-sensitive (TS, pink) phenotype, including the full-length Δ*yopD* null mutant, are characterized by a constitutive Yop synthesis at 37 °C, and are growth restricted at elevated temperatures irrespective of the Ca^{2+} levels. The strains displaying wildtype (WT) phenotype (orange) show a normal regulation of Yop synthesis and can grow at 37 °C in presence of Ca^{2+}. YopD domains important for T3SS regulation are identical to domains required for binding the dedicated cognate chaperone LcrH. **B.** *Y. pseudotuberculosis* strains were grown to exponential growth phase (2 h at 26 °C) and then shifted to 37 °C T3SS repressive conditions (+Ca^{2+}) for an additional 6 h. Total DNA was extracted and the PCN was determined using ddPCR. The data are shown as mean ± SD ($n = 3$) and were analyzed using Dunnett's multiple comparison tests comparing each mean to the wildtype (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(i) involved in the temperature-sensitive (TS) phenotype (Olsson et al., 2004), and (ii) are required for binding to LcrH, the cognate chaperone of YopD (Francis et al., 2000).

To further investigate whether the YopD deletion mutants showing increased PCN also had the same effect as the full-length Δ*yopD*^{null} mutant on the pYV replicon, we introduced the same P_{copA}- and P_{copB}-*lacZ* reporters into the different truncated mutant strains. We observed that all truncated YopD mutants that displayed a Δ*yopD*^{null} phenotype for the PCN (Δ3-Δ6, Δ12) also showed a similar phenotype to Δ*yopD*^{null} regarding *copA*, and *copB/repA* transcription levels at 37 °C under T3SS-repressive conditions (Fig. 3).

3.3. Accumulated cytoplasmic YopD increases transcription of copA under T3SS-inductive conditions

It is known that under T3SS-inductive conditions, YopD is secreted outside of the cell through the T3SS. To investigate if the reduced transcription of *copA* under T3SS-inductive conditions is due to a lower concentration of YopD in the cell, we established a T3SS-defective mutant through mutation of *yscS*, a gene encoding an essential component of the T3SS export apparatus (Dewoody et al., 2013). In the Δ*yscS* mutant, where YopD is not secreted, the levels of *copA* transcription were significantly increased compared with wildtype bacteria grown

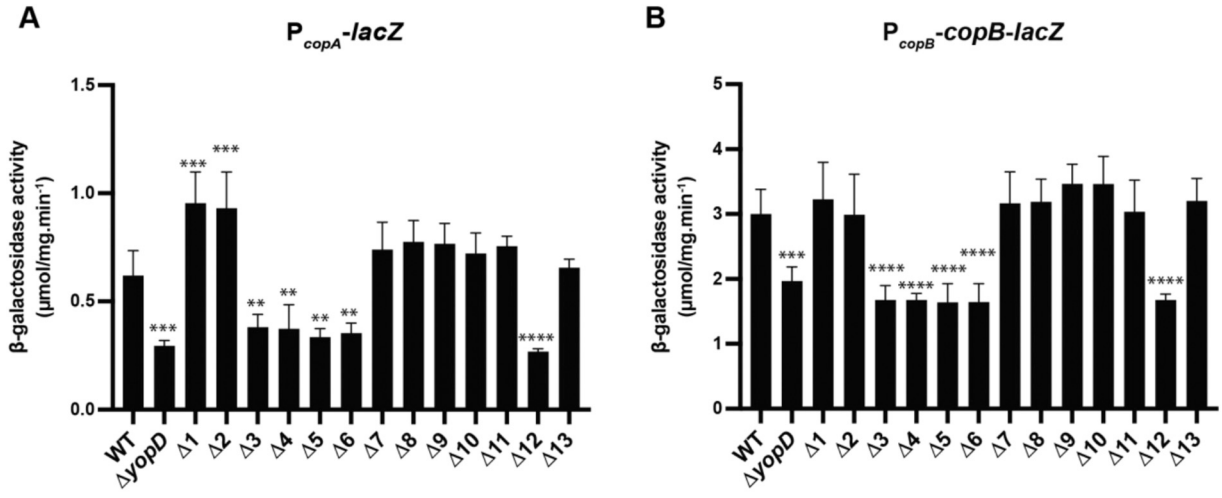

Fig. 3. Elevated PCN correlates with reduced CopA-RNA levels. Expression levels of P_{copA}-*lacZ* (**A**) and P_{copB}-*copB-lacZ* (**B**) were measured using β-galactosidase assay. Different *Y. pseudotuberculosis* strains were grown in LB medium for 2 h at 25 °C and 4 h at 37 °C under T3SS-repressive conditions. The data are shown as mean ± SD (n = 3) and were analyzed using Dunnett's multiple comparison tests comparing each mean to the wildtype (WT) (**: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$).

under the T3SS-inductive condition. However, this effect on *copA* transcription was completely reversible by removing YopD as for the Δ*yscS,* Δ*yopD* double mutant (Fig. 4). Additionally, we over-expressed YopD under T3SS-inductive conditions to re-create a higher concentration of the protein in the cell while keeping an intact T3SS. Under these conditions transcription of *copA* was similar to the Δ*yscS* mutant, *i.e. copA* transcription was much higher when YopD is present in the cell at 37 °C even under T3SS-inductive conditions (Fig. 5). These results indicated that the secretion of YopD is necessary to allow *copA* transcription to decrease under T3SS-inductive conditions.

3.4. Evidence of a YopD-independent plasmid-encoded repressor of copA transcription

The control of T3SS expression involves a complex regulatory network including several genes encoded on the virulence plasmid. YopD is a key regulator of the T3SS and the LCR. To elucidate if PCN control depends mainly on YopD or whether it also involves other components encoded on the plasmid, we determined the activity of the *copA* promoter using our *lacZ* reporter system in strains with or without the pYV plasmid (Fig. 5A). Moreover, we *trans*-complemented *yopD/lcrH* into each strain. Using Western blot analysis with an intracellular protein DnaK as an internal control, we observed an increased level of YopD protein in the strains carrying p*yopD* (Fig. 5B). No significant difference was observed between the wildtype and the pYV-cured strain at 26 °C and at 37 °C under T3SS-repressive conditions. The overexpression of YopD in a wildtype strain results in significant activation of *copA* transcription under T3SS-inductive conditions compared to the non-*trans*-complemented wildtype. In the pYV-cured strain, *copA* transcription follows a pattern similar to what is observed when *yopD* is over-expressed in the wildtype; *i.e. copA* transcription is strongly induced under T3SS-inducing conditions compared to wildtype. This occurred in both the *yopD/lcrH trans*-complemented and the non-*trans*-complemented pYV-cured strains (Fig. 5A). In summary, this shows that *copA* transcription in a plasmid-cured strain is different from a Δ*yopD* mutant indicating that PCN control does not only depend on YopD/LcrH.

Hence, temperature-mediated induction of *copA* transcription is independent of plasmid-borne factors and is either mediated intrinsically by the replicon itself or chromosomal factors. Only the repression under Ca^{2+} depletion requires virulence plasmid-encoded genes. Additionally, YopD and its chaperone, LcrH, are not the only regulators in the control of *copA* transcription; our data indicated the existence of (an)other plasmid-encoded factor(s) repressing *copA* transcription downstream of YopD under T3SS-inductive conditions.

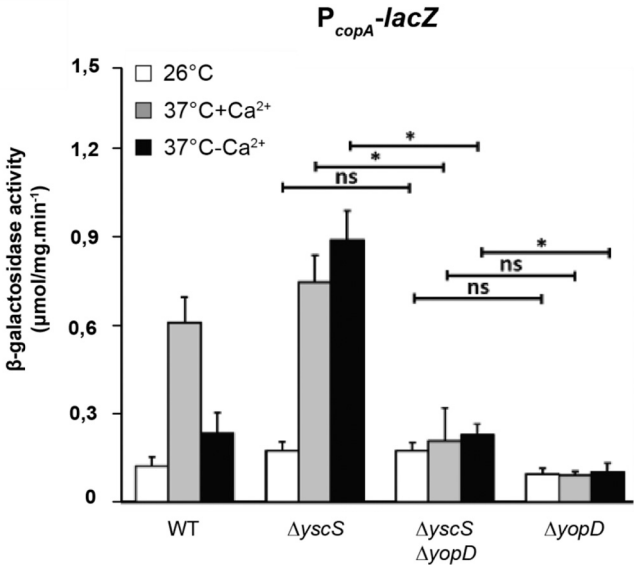

Fig. 4. Lack of YopD secretion induces the expression of the CopA-RNA under T3SS-inductive conditions. Expression levels of P_{copA}-*lacZ* were measured using β-galactosidase assays. Different *Y. pseudotuberculosis* strains were grown in LB medium for 6 h at 26 °C (white), for 2 h at 26 °C and 4 h at 37 °C + Ca^{2+} (grey) or for 2 h at 26 °C and 4 h at 37 °C under T3SS-inductive conditions (black bars). The Δ*yscS* mutants have an impaired Yops secretion. The secretion of YopD through an active T3SS system is necessary to properly control CopA-RNA expression. The data are shown as mean ± SD (n = 3) and were analyzed by Student's *t*-test. (* $p < 0.05$; ns: non-significant).

4. Discussion

Successful infection of mammalian hosts by *Yersinia* is dependent on the T3SS, a protruding needle-like structure that enables the translocation of effector proteins into target cells. A novel mechanism of PCN modulation was recently identified that allows fine-tuning of the T3SS-related metabolic burden (Wang et al., 2016). When over-expressing CopA under the control of the *yopE* promoter during growth in T3SS-inductive conditions, PCN decreased in both a wildtype and Δ*yopD* mutant background. This indicated that CopA is a major repressor of PCN in T3SS inductive conditions and that the IncFII replicon *per se* is essential for PCN increase. Here, we investigated how *Yersinia* regulates PCN *via* CopA-RNA levels and YopD in a T3SS-dependent manner. We show that YopD regulates PCN by controlling *copA* and *copB* transcription levels.

The highest PCN is achieved during infection, when the bacteria sense cell-contact (Wang et al., 2016; Schneiders et al., 2021). This corresponds to the condition when the T3SS is fully induced. This cell contact-dependent activation of T3SS and PCN increase can be mimicked *in vitro* by depletion of Ca^{2+} at 37 °C. This phenotype is dependent on a functional LCR-induced T3SS activation. One major actor of the LCR is YopD (Williams and Straley, 1998; Kusmierek et al., 2019). YopD inhibits Ysc and Yop synthesis until it is secreted upon target cell contact. Subsequent decrease of YopD concentration in the bacterial cytoplasm leads to activation of T3SS gene expression. In this study, we found that a Δ*yopD* mutant displays reduced *copA* and *copB* transcription levels at 37 °C under T3SS-repressive conditions. Moreover, under T3SS-inductive conditions, high levels of *copA* transcription are present in a Δ*yscS* mutant in which YopD cannot be secreted and accumulates in the cytoplasm. The same phenotype was observed in YopD-overexpressing strains. These results indicate that the repression of CopA-RNA under T3SS-inductive conditions is promoted by the secretion-dependent depletion of YopD from the bacterial cytoplasm. Hence, *Y. pseudotuberculosis* has evolved a mechanism to simultaneously trigger PCN increase and LCR activation through YopD secretion. Such coupling of PCN regulation with YopD allows rapid induction of an essential virulence mechanism during host infection, as well as for rapid feedback inhibition and subsequent growth restoration when T3SS is not induced.

YopD is a multifunctional protein involved in the expression, assembly, and function of the T3S machinery in *Yersinia* (Olsson et al., 2004). The different functions are associated with discrete domains of the protein. For example, certain domains of YopD involved in LcrH chaperone-binding are also required to maintain its regulatory function (Olsson et al., 2004). In fact, mutations in LcrH or YopD which prevent the chaperone from binding to YopD also perturbed Yop regulation (Williams and Straley, 1998; Edqvist et al., 2006). The proposed mechanism is that LcrH binding is a prerequisite for the translation repression of T3SS mRNAs (Williams and Straley, 1998; Chen and Anderson, 2011). Here, we found that the YopD functional domains leading to PCN control and *copA* transcription regulation were identical to the domains involved in LcrH chaperone binding and Yop regulation. Therefore, it is likely that the ability to bind to LcrH is required for YopD to regulate *copA* transcription and the PCN. However, the experimental setup did not allow us to distinguish whether the effects, *e.g.* seen in Δ3

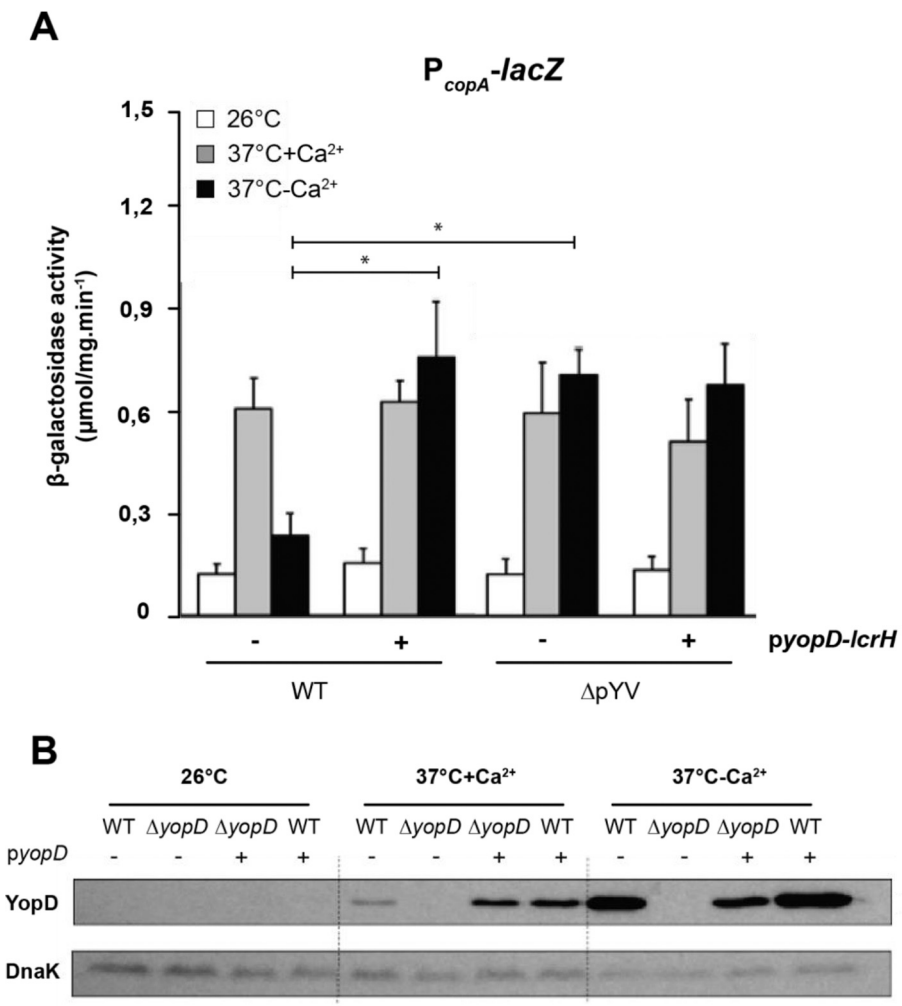

Fig. 5. An additional plasmid-encoded factor(s) other than YopD overexpression is also required for regulating CopA-RNA levels. Different *Y. pseudotuberculosis* strains harboring pYV were grown in LB medium for 6 h at 26 °C (white), for 2 h at 26 °C and 4 h at 37 °C + Ca^{2+} (grey) or for 2 h at 26 °C and 4 h at 37 °C under T3SS-inductive conditions (black bars). YopD/LcrH was overexpressed in different strains (indicated as p*yopD* +). **A.** Expression levels of P_{copA}-*lacZ* were measured using a β-galactosidase assay. The ΔpYV strain has been cured of the virulence plasmid. The data are shown as mean ± SD (n = 3) and were analyzed by Student's t-test. (* $p < 0.05$). B. Western blot detection of YopD expression in different strains, with DnaK as an internal control.

and Δ4, Δ5, Δ6 which are equally expressed and secreted as YopD wildtype, are due to a direct mechanism mediated by YopD. The same *yopD* in-frame deletions that resulted in an increase in pYV copy number and decreased *copA/copB* transcription also caused T3SS-activity independent of Ca^{2+}-depletion. Hence, the phenotypes seen could in fact be due to the secretion of a *copA/copB* regulator under T3SS-repressive conditions in those mutants. This hypothesis is further supported by the mechanism in which YopD interacts with its target genes to regulate the T3SS.

YopD regulates T3SS by direct binding to *ysc/yops* mRNAs (Chen and Anderson, 2011). However, several parameters indicate that the regulation of *copA* transcription by YopD is indirect. Firstly, since our reporter system only includes the *copA* promoter and the first nucleotides of the CopA-RNA, it is unlikely that YopD regulates CopA-RNA levels through direct interaction. Secondly, a plasmid-cured strain that lacks the T3SS, including YopD, still displayed increased *copA* transcription at 37 °C under T3SS-inductive conditions, while *copA* induction was not observed in the plasmid-containing singular Δ*yopD* mutant. Altogether, these results suggest that YopD inhibits at least another plasmid-encoded factor, which itself inhibits the induction of *copA* transcription. This factor being plasmid encoded is strongly supported by the fact that YopD-mediated control of *copA* transcription under T3SS-inductive conditions was lost in the absence of pYV.

In response to temperature, some chromosomally-encoded proteins, such as RovA, YmoA, and H-NS are putative regulators of PCN (Cornelis et al., 1991; Cathelyn et al., 2007). However, an alternative non-proteinaceous mechanism could be the structural rearrangement of the nucleic acid in response to temperature, such as hairpin-like structures that act as biological RNA thermometers. This notion results from the fact that the DNA recognition site of CopB, the entire CopA-RNA, its cognate target mRNA locus CopT and the 5′- UTR of the *repA* gene, all contain single or multiple imperfect secondary structures in the R1 replicon (Riise and Molin, 1986; Öhman and Wagner, 1989; Persson et al., 1990; Blomberg et al., 1992). This hypothesis is even further supported by the role of RNA hybridization for the inhibitory function of CopA and the essential resolution of a stem-loop for *repA* translation (Blomberg et al., 1992).

In summary, our results indicate that the domains of YopD necessary for T3SS gene regulation also contribute to PCN control. Also, the mechanism by which Yop/Ysc expression is induced under T3SS-permissive conditions, which involves YopD depletion through secretion, is also essential for increased PCN. However, the YopD effect on PCN occurs through *copA* and *copB* transcription regulation while T3SS is regulated by direct binding to *ysc/yops* mRNA. Furthermore, YopD-mediated induction of *copA* transcription seems to involve (an) additional factor(s) encoded on the virulence plasmid and it is the identification of these that will be the subject of future studies. In conclusion, *Yersinia* has evolved a regulatory mechanism by which T3SS and PCN regulation are coupled through YopD secretion in reaction to the LCR.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plasmid.2023.102683.

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