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UNIVERSITY OF CALIFORNIA
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**MAINTENANCE AND REGULATION OF THE pYV VIRULENCE PLASMID
ENCODING THE TYPE III SECRETION SYSTEM IN PATHOGENIC *YERSINIA***

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MOLECULAR, CELL AND DEVELOPMENTAL BIOLOGY

with an emphasis in MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

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March 2024

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Table of Contents

List of Figures.....	v
List of Tables.....	vii
Abstract.....	ix
Dedication.....	xiii
Acknowledgements.....	xiv
Chapters	
Chapter 1	1
<u>The <i>Yersinia</i> type III secretion system as a tool for studying cytosolic innate immune surveillance</u>	
By: Katherine Schubert, Yue Xu, Feng Shao, Victoria Auerbuch	
Abstract.....	2
1. Introduction.....	4
2. NFκB and MAP kinase host signaling and the <i>Yersinia</i> T3SS.....	6
2.1 The host Alpk1/TIFA pathway.....	6
2.2 <i>Yersinia</i> YopJ inhibition of NFκB and MAP kinase signaling.....	12
3. The <i>Yersinia</i> T3SS interactions with inflammasomes.....	14
3.1 Naip recognition of T3SS structural components.....	16
3.2 Pyrin sensing of <i>Yersinia</i> RhoGTPase-targeting T3SS effectors.....	17
3.3 YopM T3SS effector inhibition of pyrin.....	18
3.4 Regulation of Yop translocation by YopK and Nlrp3 activation....	23
3.5 YopJ-dependent host cell death occurs through a novel caspase-8-GSDMD axis.....	27
4. Conclusions.....	34

Acknowledgements.....	35
References.....	35

Chapter 2.....70

Characterization of a novel locus affecting *Y. pseudotuberculosis*

virulence plasmid copy number, T3SS activity, and fitness.

By: Katherine Schubert, Micah Braly, Diana Hooker Romero, Derfel Terciano,
Henry Moore, Hanh Lam, Helen Wang, Victoria Auerbuch

Introduction.....	71
Results.....	76
Discussion.....	100
Materials and Methods.....	105
Acknowledgements.....	122
References.....	122

Chapter 3.....134

The polyadenylase PAP I is required for *Y. pseudotuberculosis*

virulence plasmid maintenance.

By: Katherine Schubert, Micah Braly, Jessica Zhang, Hanh Lam, Christine
Jacobs-Wagner, Helen Wang, Victoria Auerbuch

Abstract.....	135
Introduction.....	136
Results.....	139

Discussion.....	161
Materials and Methods.....	169
Acknowledgements.....	187
References.....	187

List of Figures

Chapter 1

<u>Figure 1.</u> T3SS-dependent triggering of the ALPK1–TIFA–NF- κ B pathway by heptose- containing metabolites during <i>Yersinia</i> infection.....	10
<u>Figure 2.</u> YopM inhibits inflammasome activation and induces anti-inflammatory IL- 10 production	20
<u>Figure 3.</u> Caspase-8 activation in response to NF- κ B/MAPK blocking by YopJ.....	30

Chapter 2

<u>Figure 1.</u> Identification of the previously unannotated plasmid copy number inhibitory locus (<i>pil</i>) near the <i>cop-rep</i> locus of the <i>Y. pseudotuberculosis</i> pYV plasmid.....	77
<u>Figure 2.</u> Disruption of <i>pil</i> drives increased pYV PCN, T3SS expression and activity, and a severe growth defect at 37°C.....	79
<u>Figure 3.</u> Deletion of <i>pil</i> has no effect on pYV PCN, T3SS activity, or <i>Y.</i> <i>pseudotuberculosis</i> growth.....	83

<u>Figure 4.</u> Insertion into <i>pil</i> does not affect <i>Y. pseudotuberculosis</i> virulence in a mouse bread feeding model of infection.....	85
<u>Figure 5.</u> Ectopic over-expression of <i>pil</i> decreases pYV PCN, T3SS activity, and improves <i>Yersinia</i> growth under T3SS-inducing conditions.....	87
<u>Figure 6.</u> Ectopic over-expression of a Pil(1-99) allele drives down pYV PCN and produces little to no protein under T3SS-inuducing conditions.	89
<u>Figure 7.</u> Bioinformatically predicted promoters within the <i>pil</i> locus show no apparent activity at 26°C or 37°C.....	92
<u>Figure 8.</u> The <i>pil::pNQ</i> mutant readily produces suppressor mutations affecting T3SS activity and/or pYV PCN.....	94
<u>Figure 9.</u> The <i>pil::Tn</i> mutant globally over-expresses pYV-encoded genes at 26°C and 37°C.....	99

Chapter 3

<u>Figure 1.</u> <i>Y. pseudotuberculosis</i> pYV plasmid copy number (PCN) dynamically changes in response to temperature.....	141
<u>Figure S1.</u> A transposon insertion into <i>pil</i> leads to a constitutively high pYV PCN, elevated T3SS expression, and a severe growth defect under T3SS-inducing conditions.....	144
<u>Figure 2.</u> Disruption of the <i>pcnB</i> gene encoding poly (A) polymerase PAP I decreases pYV PCN and T3SS expression and activity.	146

<u>Figure 3.</u> The <i>pcnB</i> gene is required for maintenance of the pYV plasmid.....	149
<u>Figure 4.</u> PAP I point mutations leads to a decrease in pYV PCN, plasmid stability, and T3SS activity.....	154
<u>Figure 5.</u> The <i>pcnB</i> gene is required for plasmid-mediated antibiotic resistance in <i>Y. pseudotuberculosis</i>	156
<u>Figure 6.</u> The <i>pcnB</i> gene is required for normal <i>Y. pseudotuberculosis</i> virulence in a mouse IP infection model.....	158
<u>Figure 7.</u> Loss of PAP I does not affect <i>Y. pseudotuberculosis</i> growth following stress exposure.....	160

List of Tables

Chapter 2

<u>Table 1.</u> Summary of <i>pil</i> alleles tested by over expression assays.....	90
<u>Table 2.</u> Summary of suppressor isolates and identified mutations.....	95
<u>Table 3.</u> <i>Y. pseudotuberculosis</i> strains used in this study.....	117
<u>Table 4.</u> Primers used in this study.....	118
<u>Table 5.</u> Plasmids used in this study.....	121

Chapter 3

<u>Table S1.</u> Phenotypic categories and mutations identified in suppressor screen_...	143
<u>Table 1.</u> PAP I is required for plasmid maintenance in <i>Y. pseudotuberculosis</i>	150
<u>Table 2.</u> Important PAP I residues are required for stability of the pYV plasmid.....	153
<u>Table 3.</u> <i>Y. pseudotuberculosis</i> strains used in this study.....	182
<u>Table 4.</u> Primers used in this study.....	183
<u>Table 5.</u> Plasmids used in this study.....	186

Abstract

MAINTENANCE AND REGULATION OF THE pYV VIRULENCE PLASMID ENCODING THE TYPE III SECRETION SYSTEM IN PATHOGENIC *YERSINIA*

Kate Schubert

The current rate of acquired antibiotic resistance (AR) in bacterial pathogens is projected to cause 10 million deaths per year and result in a global cost of up to 100 trillion USD by 2050. It is therefore urgent that we develop new therapies that target pathogen-specific virulence factors (*i.e.*-microbial genes required to cause disease). However, our limited understanding of how these virulence factors are regulated is a major obstacle in the field, and studies exploring how these pathogens coordinate expression of virulence genes are desperately needed in general. Many Gram negative pathogens harbor large extrachromosomal plasmids encoding genes important for virulence and/or antimicrobial resistance, many of which belong to the IncF family of plasmids. Understanding how these plasmids are regulated and maintained is critical for addressing the threat posed by AR pathogens, and yet the underlying biology regulating many of these plasmids remains poorly understood. Using the Gram negative enteric pathogen *Y. pseudotuberculosis* as a model organism, the aim of this research is to broaden our understanding of how facultative pathogens like *Yersinia* regulate virulence gene expression at the level of virulence plasmid copy number (PCN) and plasmid maintenance. *Y. pseudotuberculosis* is closely related to *Y. pestis*, the causative agent of the plague. Both *Y. pseudotuberculosis* and *Y. pestis* harbor an IncFII, ~70kb virulence plasmid known as pYV that encodes a major *Yersinia* virulence factor known as the type III secretion system (T3SS). The T3SS is a specialized injectisome used by many species of

Gram negative pathogens to inject effector proteins into the host cell cytosol, interfering with host innate immunity signaling pathways and ultimately dampening host defense mechanisms. Despite being critical for *Yersinia* virulence, expression of the T3SS is energetically costly and must be tightly controlled to achieve optimal fitness, and so expression and activity of the T3SS is restricted to host temperatures of 37°C where it is needed for virulence. It was recently discovered that expression and activity of the T3SS is dynamically regulated via changes in PCN, or the number of pYV plasmids per *Y. pseudotuberculosis* cell. At environmental temperatures of 26°C, where the T3SS is not needed, pYV PCN is kept low to about one copy per cell. However, upon entering the mammalian host (37°C), pYV PCN is quickly and specifically increased to 5-12 copies per cell. This increase in pYV PCN is required for *Y. pseudotuberculosis* virulence in mice, and yet the mechanism by which pYV PCN is kept low at 26°C and rapidly increased at 37°C is completely unknown. The first chapter of this dissertation reviews how the *Yersinia* T3SS has served as a tool for discovering and elucidating host immune pathways that have evolved through extensive host-pathogen interactions with T3SS-utilizing bacteria. The rest of this work focuses on the discovery of two factors that impact pYV PCN and/or maintenance in *Y. pseudotuberculosis*. In chapter 2, we demonstrate that a transposon (Tn) insertion in a previously unannotated locus near the pYV IncF replicon drives abnormally high pYV PCN at environmental temperatures of 26°C, resulting in a hyperactive T3SS phenotype at 37°C and a severe growth defect under T3SS-inducing conditions. This locus, which we will call *plasmid copy number inhibitory locus* or *pil*, encodes a predicted ORF that is conserved in some other species of Gram negative bacteria. By taking advantage of the severe growth defect

of this strain, we further demonstrate that *pil* insertion mutants can be used as genetic tools for identifying novel factors affecting pYV PCN, T3SS activity, and *Yersinia* growth in a suppressor screen. A pilot suppressor screen identified specific residues in factors that impacts pYV PCN and T3SS activity in various ways, and we propose that *pil* insertion mutants will serve as a powerful tool for identifying additional factors in future studies. Chapter 3 of this dissertation focuses on one of the hits arising from the *pil* suppressor screen, which identified the *pcnB* gene encoding the polyadenylase poly (A) polymerase I (PAP I) as a novel regulator of pYV maintenance in pathogenic *Yersinia*. We show that PAP I is essential for normal pYV PCN, T3SS activity, and *Yersinia* virulence in a mouse infection model. In addition, we report that PAP I is required for *Y. pseudotuberculosis* maintenance of sRNA-regulated plasmids that harbor AR genes in general, suggesting that PAP I is important for the maintenance of both virulence and AR plasmids by pathogenic bacteria. PAP I homologs are widespread in bacteria but have only been studied in a small handful of species to date. Importantly, this is the first time PAP I has been linked to regulation of a virulence plasmid in the natural pathogenic host for that plasmid. To our knowledge, this is also the first direct evidence that a bacterial polyadenylase impacts virulence of a bacterial pathogen. Overall, this work broadens our understanding of how IncF plasmids are regulated to balance virulence and bacterial fitness by pathogens like *Y. pseudotuberculosis*. Given that PAP I homologs are widespread and many AR plasmids and nearly all virulence plasmids belong to the IncF family, this work likely applies to other species of Gram negative pathogens as well. Finally, this work highlights that there are many additional levels of gene expression regulation to consider when it comes to addressing the threat

posed by bacterial pathogens, including changes in gene dosage via changes in PCN of extrachromosomal plasmids and the potential contribution of polyadenylation to regulation of virulence gene expression.

Dedication

I dedicate this dissertation to my late father Professor Richard E. Schubert, who was a kind parent, an inspiring philosopher, and a great friend. Thank you for being my first and most influential teacher in life.

This dissertation is also dedicated to Nemo Black, my partner in life and best friend.

This work would not have been possible without the endless emotional and intellectual support you have given and continue to give me.

Finally, I dedicate this dissertation to my beloved lab mates. From technical advice on experiments to much needed comedic relief, you have all helped me so much throughout my time in graduate school.

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During the second half of my graduate career my life has been heavily impacted by the COVID-19 pandemic, economic instability, climate change, and the loss of a parent. I am grateful to have had a kind and understanding supervisor and mentor during some of my most challenging times.

I would also like to acknowledge that this dissertation would not have been possible without the training, support, and general advice given by former and current lab members in the Auerbuch Stone lab. This work has also been supported in many ways by several collaborating scientists from different national and international groups. I would like to thank everyone who was involved in this interesting and exciting work. Contributors are either listed as authors or recognized in the acknowledgement section of the relevant chapter. Specific contributions to each chapter are also listed below.

Chapter 1:

This chapter is a reprint of a previously published paper in *Annual Review of Microbiology* in July 2020. I am responsible for the majority of writing and all figures. The discussion on how the caspase-8-GSDMD axis drives inflammatory cell death in section 3.5 was written by Yue Xu and Feng Shao.

Chapter 2:

I am responsible for all writing and the majority of figures. Diana Hooker Romero constructed the original *pil::Tn* mutant in the IP1666pIB1 background and initiated the project. Micah Braly assisted with *pil* over expression assays in Table 1 and Hanh Lam assisted with bioinformatic analysis of *pil* suppressor isolates. Derfel Terciano and Henry Moore assisted with processing of RNAseq data. Helen Wang constructed the original *pil::pNQ* YPIII/pIBX strain.

Chapter 3:

I am responsible for the majority of writing and figures. Micah Braly assisted with spot tests, Western blot analyses, *in vitro* secretion assays, and pGFPuv time courses. Microscopy and quantification of pYV puncta was performed by Jessica Zhang. Hanh Lam assisted with identification of mutations arising from the *pil::pNQ* suppressor screen. Derfel Terciano and Henry Moore assisted with processing of RNAseq reads and Helen Wang generated the original *pil::pNQ* YPIII/pIBX mutant.

Chapter 1.

The *Yersinia* type III secretion system as a tool for studying cytosolic innate immune surveillance

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Abstract

Microbial pathogens have evolved complex mechanisms to interface with host cells in order to evade host defenses and replicate. However, mammalian innate immune receptors detect the presence of molecules unique to the microbial world or sense the activity of virulence factors, activating antimicrobial and inflammatory pathways. We focus here on how studying the major virulence factor of one group of microbial pathogens, the type III secretion system (T3SS) of human pathogenic *Yersinia*, has shed light on these important innate immune responses. *Yersinia* are largely extracellular pathogens yet insert T3SS cargo into target host cells that modulate the activity of cytosolic innate immune receptors. This review covers both the host pathways that detect the *Yersinia* T3SS and the effector proteins used by *Yersinia* to manipulate innate immune signaling.

Contents

- 1 Introduction
- 2 NF κ B and MAP kinase host signaling and the *Yersinia* T3SS
 - 2.1 The host Alpk1/TIFA pathway
 - 2.2 *Yersinia* YopJ inhibition of NF κ B and MAP kinase signaling
- 3 The *Yersinia* T3SS interactions with inflammasomes
 - 3.1 Naip recognition of T3SS structural components
 - 3.2 Pyrin sensing of *Yersinia* RhoGTPase-targeting T3SS effectors
 - 3.3 YopM T3SS effector inhibition of pyrin
 - 3.4 Regulation of Yop translocation by YopK and Nlrp3 activation
 - 3.5 YopJ-dependent host cell death occurs through a novel caspase-8-GSDMD axis
- 4 Conclusions

1. Introduction

Mammals express an arsenal of pattern recognition receptors (PRR) that can detect a variety of so-called pathogen associated molecular patterns (PAMPs) as well as “patterns of pathogenesis” [1, 2]. PAMPs are molecules unique to the microbial world that can trigger innate immune signaling pathways. For example, lipopolysaccharide (LPS) lipid A is a PAMP produced by many Gram-negative bacteria that activates Toll-like receptor 4 (TLR4) on the host cell plasma membrane, leading to signaling through the NF κ B, MAP kinase, and type I interferon pathways involved in host defense, inflammation, and tissue repair [3]. In contrast, “patterns of pathogenesis” are alterations to host pathways induced by microbial virulence factors and sensed by mammalian host cells. For example, pore-forming toxin activity leads to activation of the Nlrp3 inflammasome, leading to pyroptosis, a form of inflammatory programmed cell death [4]. Because mammals and microbial pathogens have co-evolved, studying microbial virulence mechanisms points us to the most critical innate immune pressures shaping the host-pathogen interaction.

Microbial pathogens occupy a number of different compartments within mammalian host cells; some microbes replicate extracellularly whereas others replicate in the host cell vacuole or cytosol. In addition, certain virulence factors allow microbially-derived molecules to access the host cytosol in the absence of microbial intracellular replication. For example, pathogenic *Yersinia* replicate largely extracellularly, but introduce bacterially-derived molecules to the mammalian host cytosol through a type III secretion system (T3SS), a nano-syringe expressed by dozens of plant and animal Gram-negative pathogens to inject effector proteins and possibly other types of molecules into target host cells [5]. Importantly, PRRs can be

found in the plasma membrane, vacuolar membrane, and cytosol of mammalian cells. Therefore, both the identity of the ligands recognized by each PRR, as well as their subcellular localization, dictate the types of microbes they can recognize. Given that most strains of *Yersinia* that have been studied cannot replicate inside mammalian cells, yet activate cytosolic PRRs through their T3SS, dissecting the way in which the *Yersinia* T3SS interacts with the innate immune system provides a unique perspective on cytosolic PRRs and their role in host defense.

The overarching theme of T3SS effector proteins in the dozens of T3SS-expressing organisms studied to date is manipulation of host defenses to enable pathogen survival and growth [6]. The Ysc T3SS translocates up to seven known effector proteins called Yops (for *Yersinia* outer proteins) into cells of the innate immune system during infection with the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* as well as *Y. pestis*, the causative agent of bubonic and pneumonic plague. Four of these Yops inhibit phagocytosis, enabling *Yersinia* to remain largely extracellular in the host [7]. However, mammalian cytosolic PRRs have evolved the ability to recognize the presence of a functional T3SS and launch protective responses. Interestingly, three other Yops injected into host cells via the T3SS inhibit inflammatory pathways that counteract T3SS innate immune recognition. In this review, we describe both sides of this host-pathogen battle for survival centered around use of this ancient, widely-used bacterial virulence mechanism.

2. NFκB and MAP kinase host signaling and the *Yersinia* T3SS

2.1. The host ALPK1-TIFA pathway

Despite replicating largely extracellularly, *Yersinia* molecules are inserted into target host cells via the T3SS, exposing *Yersinia* to detection by cytosolic innate immune receptors. Indeed, *Y. pseudotuberculosis* induced secretion of the neutrophil chemokine IL-8 from HeLa cells, dependent on bacterial expression of the T3SS translocon protein YopB [8]. Furthermore, bone marrow macrophages derived from mice lacking TLR signaling (MyD88^{-/-}/Trif^{-/-}) still launched a robust response against *Y. pseudotuberculosis* expressing a functional T3SS [9]. This response included NFκB activation, which could also be detected in a human fibroblast cell line independently of the Nod1 and Nod2 cytosolic PRRs [9, 10], but was found to involve the NFκB-inducing kinase, NIK, depending on the presence of actin cytoskeleton-targeting T3SS effectors [11]. This NFκB response was only induced following infection with *Yersinia* strains capable of translocating T3SS cargo into host cells, but did not require six of the known T3SS effectors YopHEMOJT [8, 9, 12]. However, the host receptor and bacterial ligand responsible were unclear.

Recently, *Y. pseudotuberculosis* with specific defects in the LPS biosynthesis pathway were found to induce less NFκB activation in the human fibroblast cell line HEK293T [10]. Furthermore, alpha protein kinase 1 (ALPK1) was identified as the cytosolic PRR responsible for inducing NFκB in response to the *Yersinia* T3SS in HEK293T cells [10]. ALPK1 had been previously implicated in signaling through TRAF-interacting protein with forkhead-associated domain (TIFA) to activate NFκB in response to a metabolic intermediate of the LPS pathway [13, 14]. TIFA signaling is

activated in various cell types during infection with Gram-negative pathogens, including *Helicobacter pylori* and *Shigella flexneri* [14-17]. Heptose-containing intermediates are generated in a highly conserved LPS biosynthesis pathway in Gram negative bacteria [18]. Specifically, the heptose-containing intermediates that have been implicated in the ALPK1-TIFA signaling axis include D- β -D-heptose 1-P (HBP) and ADP-L- β -D-heptose (ADP-heptose) [10, 13, 14, 17]. Binding of ADP-heptose to ALPK1 induces phosphorylation of TIFA at threonine 9 (Thr9), leading to its activation and oligomerization [10, 13, 14, 19, 20]. Phosphorylated TIFA can directly bind TRAF6, promoting its oligomerization and downstream phosphorylation of TAK1 and IKK [14, 21, 22]. Phosphorylation of IKK, in turn, leads to phosphorylation of I κ B α , entry of NF κ B into the nucleus, and expression of NF κ B-responsive genes such as chemokines and proinflammatory cytokines [23]. In addition to NF κ B, TIFA can promote AP-1 activation through phosphorylation and activation of c-Jun N-terminal kinase (JNK) [21]. Activated TIFA can also directly interact with TRAF2 and this interaction may be necessary to drive activation of NF κ B and AP-1 [22, 24]. Taken together, these data suggest that heptose-containing metabolites of the LPS biosynthesis pathway from *Y. pseudotuberculosis* induce the ALPK1-TIFA signaling pathway leading to activation of NF κ B and MAP kinases. However, whether this ALPK1-TIFA are also involved in detecting *Y. enterocolitica* or *Y. pestis* remains unclear.

While both ADP-heptose and HBP are capable of activating the ALPK1-TIFA signaling pathway, HBP is not membrane permeable [10, 13-17, 20]. However, when purified HBP is transfected into mammalian cells, it triggers NF κ B activation in a TIFA-dependent manner [13-16]. ADP-heptose, which is membrane permeable, can

trigger the same response in the absence of a mechanism of cytosolic entry such as transfection [10]. Recently, it was shown that ADP-heptose, but not HBP, can directly bind ALPK1, suggesting that ADP-heptose is the *bona fide* PAMP for ALPK1 [10]. Because of the slower kinetics of HBP induction of NF κ B, Garcia-Weber et al proposed that HBP is processed in host cells prior to activation of innate immune signaling [17]. Indeed, human HEK293T cells express one or more unknown adenylyltransferases that can convert HBP into ADP-heptose, triggering ALPK1 activation [10]. Therefore, HBP introduced into the host cytosol may be converted to ADP-heptose via one or more adenylyltransferases, inducing ALPK1-TIFA signaling and NF κ B activation (Figure 1).

An outstanding question still remaining is how heptose-containing metabolites access the host cytosol to activate the ALPK1-TIFA pathway. *Yersinia* must express a functional T3SS to induce NF κ B activation independently of TLRs [9, 10]. Likewise, TIFA-dependent NF κ B production in response to *H. pylori* requires an intact cag4 T4SS [15, 16]. However, *C. rodentium* can activate the ALPK1-TIFA signaling cascade independently of the *Citrobacter* T3SS [25]. Activation of the heptose metabolite-ALPK1-TIFA-NF κ B pathway also enables recognition of *Shigella* and *Salmonella* [14, 17]. With these intracellular pathogens, it is challenging to assess the contribution of their T3SSs, as these are required for bacterial uptake or intracellular replication. One possibility is that heptose-containing metabolites are injected into host cells via the T3SS or T4SS channel. Another possibility is that secretion system activity on host cell membranes mediates entry of bacterially-released heptose-containing metabolites into the host cytosol [14]. While the involvement of ALPK1-TIFA in the HeLa cell response to *Y. pseudotuberculosis* has

not been determined, the translocon protein YopB, but not YopD, has been implicated in induction of IL-8 secretion in this cell type [8]. Indeed, NFκB activation was observed following transfection or scrape-loading of *Yersinia* lysates into HEK293T cells but not *Yersinia* supernatants containing T3SS cargo [9]. This suggests that intimate bacterial-host cell interaction, perhaps more specifically YopB insertion into the host cell membrane, may be necessary for heptose-metabolite induction of NFκB.

ADP-heptose is a critical component of the LPS core oligosaccharide [18, 26], and disruption of the heptose biosynthesis pathway is associated with altered LPS and O-antigen structures [27]. Changes in these structures can affect *Yersinia* membrane integrity and virulence; for example, mutations in LPS synthesis genes have been shown to result in increased binding of the antimicrobial chemokines CCL25 and CCL28 to the surface of *Y. pseudotuberculosis*, leading to increased killing of the bacteria [28]. In the case of *Y. enterocolitica*, it has been shown that the LPS outer core enforces membrane integrity and is required for normal virulence [29]. Thus, *Yersinia* is highly limited in its ability to evolve mechanisms for modifying LPS biosynthesis genes. One interesting possibility is that *Yersinia* attempts to limit detection of heptose-containing metabolites by halting growth during active type III secretion. It has long been observed that upon induction of the T3SS, *Yersinia* undergo growth arrest. It is possible that *Yersinia* shut down certain metabolic pathways during active type III secretion to slow production of heptose-containing metabolites under conditions where they are most at risk of encountering host cells and triggering NFκB activation.

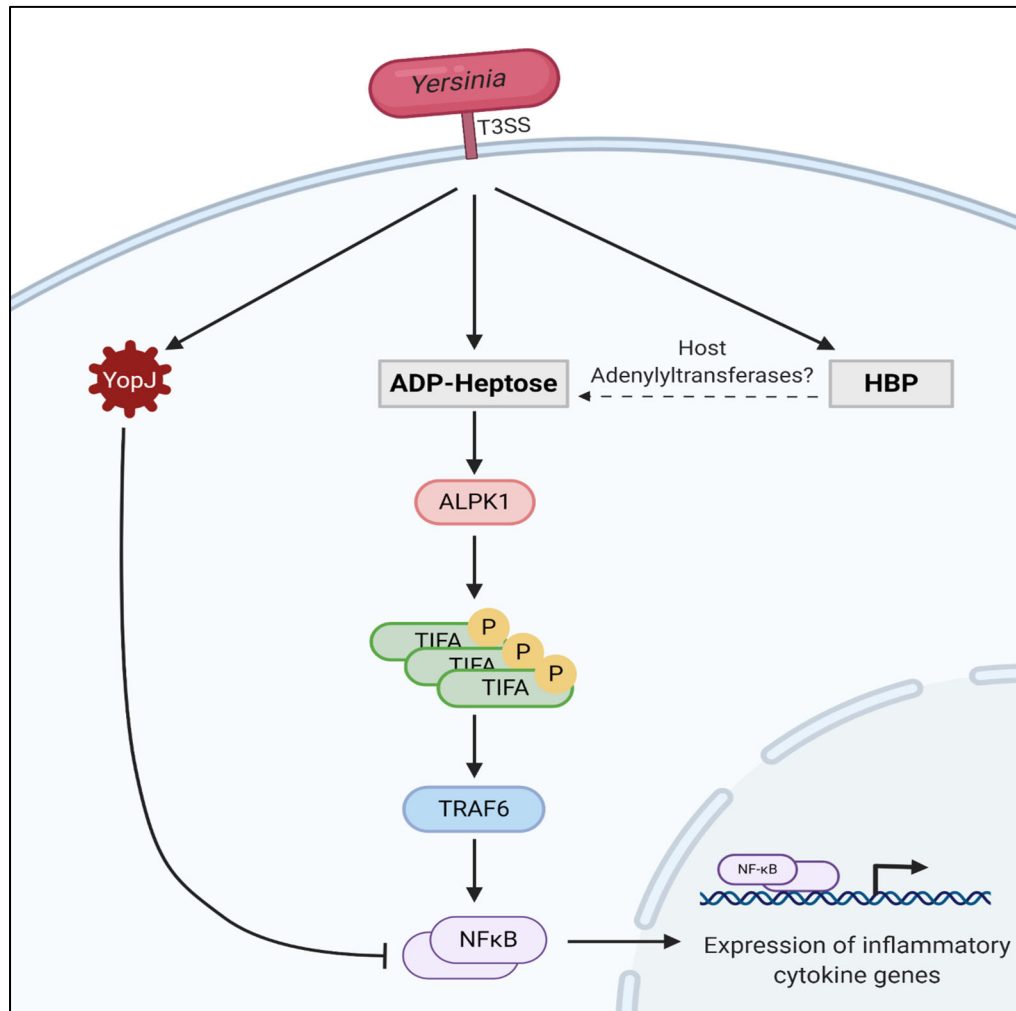


Figure 1. T3SS-dependent triggering of the Alpk1-TIFA-NFκB pathway by heptose-containing metabolites during *Yersinia* infection.

ADP-heptose and heptose bis-phosphate (HBP) are both produced by Gram-negative bacteria during lipopolysaccharide biosynthesis. Both molecules have been suggested to gain access to the host cytoplasm in the presence of a functional T3SS through an unknown mechanism. ADP-heptose can bind the ALPK1 host kinase, which triggers phosphorylation and activation of TIFA. TIFA oligomers signal through TRAF6 to activate NFκB for inflammatory cytokine gene expression. HBP does not seem to bind ALPK1, but HBP may be converted to ADP-heptose by as yet unidentified host adenylyltransferases, ultimately leading to TIFA activation. *Yersinia* YopJ has long been known to inhibit NFκB signaling downstream of TLR and cytokine receptor signaling. However, YopJ may also be important in blocking NFκB activation downstream of the ALPK1-TIFA axis.

TIFA has been linked to increased expression of pro-caspase-1, pro-IL-1 β , and pro-IL-18 and is thought to promote NLRP3 inflammasome priming and activation in vascular endothelial cells [30]. Thus, ALPK1-TIFA-NF κ B activation might prime cells for inflammasome activation in response to other innate immune stimuli (see below). Interestingly, AKT serine/threonine kinase, which can be activated by several stimuli including reactive oxygen species (ROS), has been shown to phosphorylate TIFA at Thr9 *in vitro*, suggesting that ALPK1 is not the only kinase upstream of TIFA [30]. TIFA signaling dynamics during *Yersinia* infection may be additionally influenced by a known TIFA inhibitor called TIFAB. TIFAB can bind TIFA and block its oligomerization and negatively regulates TRAF6-dependent activation of B cell and macrophage functions [31, 32]. In addition, bone marrow derived macrophages lacking TIFAB are hyper-responsive to LPS, suggesting that TIFAB may be involved in modulating multiple signaling pathways of the innate immune system [33]. However, TIFAB expression and activity has not been studied in the context of infection, so any contribution it may have during infection with *Yersinia* remains to be seen.

In summary, the *Yersinia* T3SS enables recognition of an LPS metabolic intermediate containing heptose by an ALPK1-TIFA pathway leading to NF κ B activation. However, the physiological consequence of the heptose metabolite-ALPK1-TIFA-NF κ B pathway in *Yersinia* pathogenesis or host resistance to *Yersinia* remains unclear.

2.2. *Yersinia* YopJ inhibition of NFκB and MAP kinase signaling

Yersinia YopJ (referred to as YopP in *Y. enterocolitica*) is a T3SS effector that enzymatically targets several innate immune signaling proteins and has complex effects on mammalian cells. YopJ is an acetyltransferase that dampens the activation of NFκB and MAP kinase signaling pathways that would otherwise be triggered downstream of PRR recognition of *Yersinia* PAMPs or through cytokine-induced signaling. YopJ is an acetyltransferase but has been ascribed other catalytic functions, including deubiquitinase and cysteine protease activities. The variety of YopJ activities and molecular targets is a reflection of its unique properties, including a catalytic core that is reminiscent of a ubiquitin-like protease, as opposed to a conventional acetylase [34-37]. YopJ preferentially targets serine and threonine residues for substrate acetylation [34, 38], thereby inhibiting substrate activation by blocking key phosphorylation sites [39]. YopJ acetyltransferase activity is reported to be activated in response to inositol hexakisphosphate (IP₆), indicating that YopJ becomes catalytically active upon entering the cytosol of mammalian cells [40].

YopJ dampens signaling through MAPKs and NFκB in infected cells (Figure 1) [34, 41]. MAPKs and NFκB are attractive targets for disarming immune cells due to their central role in mediating inflammatory responses during infection. While PRRs recognize a wide variety of PAMPs through distinct mechanisms, many of them converge onto activation of MAPK and/or NFκB pathways. For instance, activated TLRs signal through IL-1R-associated kinases (IRAKs) and TNFR-associated factor 6 (TRAF6) to activate TGFβ-activated kinase 1 (TAK1). TAK1 can then phosphorylate both MAPKs and components of the IKK complex, leading to activation of the AP-1 and NFκB transcription factors, respectively. These

transcription factors subsequently promote expression of many genes important for immunity including cytokine and inflammasome components. *Yersinia* YopJ has been shown to acetylate and inhibit activation of multiple MAPKs [38, 39, 42]. YopJ additionally acetylates TAK1 to block downstream immune signaling [43, 44]. YopJ can also inhibit NFκB more directly by preventing phosphorylation of IκB [38]. Thus, *Yersinia* YopJ functions as a potent inhibitor of MAPK and NFκB signaling, significantly dampening the consequences of PAMP recognition through multiple PRRs.

YopJ has also been reported to promote deubiquitylation of several host proteins [45, 46]. Ubiquitin plays an important role in immune signaling pathways; for example, activated TRAF proteins can mediate their own autoubiquitination, promoting oligomerization and formation of an active signaling complex [47, 48]. In addition, ubiquitination of IKK leads to NFκB activation by signaling IKK degradation and dissociation from NFκB. TRAF2, TRAF3, and TRAF6 have been reported to be less ubiquitinated in the presence of YopJ in HEK293T cells, and this response depends on YopJ catalytic activity [45, 46]. YopJ has also been proposed to remove ubiquitin from IKK components [45]. Deubiquitylation of TRAF2, TRAF3, TRAF6, and IKK by *Yersinia* YopJ correlates with a loss of downstream MAPK and NFκB activation [45, 46].

Where and when YopJ inhibition of NFκB and MAP kinases might be important during *Yersinia* infection is unclear. YopJ has been implicated in intestinal barrier dysfunction through a poorly characterized pathway involving Nod2 [43]. In addition, while YopJ clearly inhibits the production of inflammatory cytokines such as tumor necrosis factor TNF-α in cultured phagocytic cells infected with *Yersinia* [49-

52], the importance of this has not been demonstrated *in vivo*. Indeed, no difference in TNF- α levels were found in the tissues of mice intraperitoneally or orally infected with *Y. pseudotuberculosis* [53, 54]. However, it may be that the “pattern of pathogenesis” response of the host against YopJ enzymatic activity (see below) masks the potent NF κ B and MAP kinase inhibitory activity seen in cultured cells. With the recent discovery that the *Yersinia* T3SS induces the ALPK1-TIFA-NF κ B pathway, it is tempting to speculate that YopJ may have evolved specifically to block the consequences of ALPK1-TIFA activation. Indeed, ALPK1 is enriched in neutrophils, a key target of *Yersinia* Yop translocation [55].

3. The *Yersinia* T3SS interactions with inflammasomes

Programmed cell death is another important mechanism by which the host immune system resists pathogenic invaders. Intricate molecular networks determine different types of programmed cell death, including apoptosis, pyroptosis, and necroptosis. Apoptosis, the most widely studied form of cell death, packages cellular contents into apoptotic bodies, which are then removed by phagocytes, resulting in immunological silence. Conversely, pyroptosis and necroptosis are characterized by membrane rupture and release of cellular danger signals, or alarmins, thus leading to inflammation [56]. The complex interplay between pathogenic *Yersinia* and cell death has provided crucial insight on the molecular basis of these dynamic and intertwined pathways.

Inflammatory cell death is driven by inflammasomes, which are multimeric protein complexes that form in the cytosol of mammalian cells in response to PAMPs or DAMPs (danger associated molecular patterns). Inflammasome activation

requires two signals; signal 1 primes cells with expression of inflammasome components and inactive inflammasome targets, and signal 2 triggers assembly and activation of the mature inflammasome. The signal 1 derives from activation of PRRs like TLRs and/or cytokine receptors that drive expression of proinflammatory genes, leading to production of pro-caspases, pro-IL-1 β , and pro-IL-18. Signal 2 is provided when cytosolic PRRs associated with the inflammasome bind their corresponding ligand. Upon ligand binding, PRRs form a complex with their adapter proteins, such as ASC (apoptosis-associated speck-like protein containing a CARD) or NLRC4 (NLR and caspase recruitment domain containing receptor 4), that acts as a scaffold for pro-caspase-1 nucleation and activation. Active caspase-1 then cleaves and activates gasdermin D (GSDMD) as well as pro-IL-1 β and pro-IL-18. Several copies of active GSDMD come together to form a pore in the host cell membrane, and the addition of several of these pores disrupts membrane integrity and culminates in a form of inflammatory cell death known as pyroptosis. Mature IL-1 β and IL-18 are released through the pores formed by GSDMD, increasing local inflammation. Inflammasome-associated PRRs include Naips (NLR family apoptosis inhibitory proteins), pyrin, and NLRP3 (NLR and pyrin domain containing receptor 3). Caspase11 (caspase-11 in mice, caspase-4/5 in humans) can also function as an inflammasome receptor in the non-canonical pathway for NLRP3 inflammasome activation [57].

In a lung infection model, *Y. pestis* expressing its T3SS induced early expression of IL-1 β , and this was dependent on caspase-1 and NLRC4, but not NLRP3 [58]. This IL-1 β expression was correlated with inflammation and tissue pathology, although not with bacterial burden or host survival. However, *Y. pestis*

also induced expression of the IL-1 receptor antagonist IL-1RA and neutralization of this protein led to increased inflammatory cytokine production [58]. These data suggest that NLRC4 inflammasome recognition of the *Y. pestis* T3SS leads to IL-1 β release that affects early pneumonic plague. However, *Y. pestis* also antagonizes IL-1R signaling in the lung to dampen this response. Similar to infection with *Y. pestis*, mice lacking caspase-1 did not demonstrate altered bacterial burden or host survival in response to intraperitoneal infection with wildtype *Y. pseudotuberculosis* [59, 60]. However, several studies using *Yersinia* lacking certain T3SS effector proteins demonstrated that inflammasome signaling can be induced in response to, and has the potential to protect against, *Yersinia* oral infection, but this is blocked by effector protein targeting of different inflammasome components (see below) [59, 61, 62].

3.1. Naip recognition of T3SS structural components

The NLR family of apoptosis inhibitory proteins (NAIPs) are a group of PRRs that associate with the NLRC4 adapter protein. Mice express several different Naips, while humans only express a single functional Naip [63-65]. In mice, Naip1 responds to needle proteins of the T3SS (*Yersinia* orthologue, YscF; universal name, SctF), Naip2 binds the T3SS inner rod component (YscI/SctI), while Naip5 and 6 recognize flagellin [66-69]. Unlike mouse Naips that recognize single ligands, human Naip responds to multiple ligands, including SctF, SctI, and flagellin [68, 70, 71]. Ligand-Naip binding recruits NLRC4 and drives inflammasome formation and activation. While enteropathogenic *Yersinia* flagellin can induce Naip5 activation if present in the host cell cytosol [67, 72], enteropathogenic *Yersinia* escape Naip5 detection by down-regulating expression of flagella at host temperatures (169). Furthermore, *Y.*

pestis has lost the ability to express flagellin under any condition [73]. While *Yersinia* YscI contains the sequence necessary for triggering activation of NLRC4 inflammasomes (Figure 2) [74], not all SctI orthologues are recognized by Naip5 [75].

3.2. Pyrin sensing of *Yersinia* RhoGTPase-targeting T3SS effectors

Pyrin is an inflammasome NLR that is highly expressed in neutrophils, monocytes, and eosinophils [170, 171]. Active pyrin recruits the adapter protein ASC and pro-caspase-1, leading to inflammasome formation and subsequent cytokine release and pyroptosis [76, 77]. Pyrin activation is regulated by RhoA-dependent phosphorylation; under homeostatic conditions, normal RhoA GTPase activity promotes activity of the kinase PRK [78]. PRK phosphorylates pyrin, allowing 14-3-3 proteins to bind pyrin and blocks its activation [79-82]. Pyrin can become activated when Rho GTPase activity is disturbed by microbial secreted proteins [78, 82]. Therefore, pyrin detects pathogen-mediated disruption of RhoGTPases as a “pattern of pathogenesis”, as many pathogens have been shown to target RhoGTPases to promote their virulence [2]. Indeed, *Yersinia* T3SS effectors YopE and YopT inhibit Rho GTPase activity to block phagocytosis. Specifically, YopE functions as a GTPase activating protein (GAP) for the Rho family of GTPases [83, 84]. YopE has been shown to target the RhoGTPases Rac1, RhoA, and Cdc42 for GTP hydrolysis, leaving them in their inactive GDP-bound form [83]. YopT is a cysteine protease that primarily targets RhoA [85-88]. YopT inactivates RhoA by cleaving the prenyl group, thereby removing active RhoA from the plasma membrane [88]. Together, YopE and YopT cause dephosphorylation and activation of pyrin by disrupting RhoA GTPase activity (Figure 2) [61, 62, 89]. It is noteworthy that serotype O3 strains of *Y.*

pseudotuberculosis naturally lack YopT, although this loss may not significantly affect *Yersinia* virulence due to the overlapping roles of YopT and YopE [7]. The importance of the pyrin inflammasome in protecting the host against *Yersinia* infection is evidenced by the presence of another T3SS effector protein called YopM that blocks pyrin activation and is essential for *Yersinia* virulence (discussed in detail in the next section).

3.3. YopM T3SS effector inhibition of pyrin

YopM is a leucine rich repeat (LRR) effector protein that is secreted by all three strains of human pathogenic *Yersinia*. Loss of *Yersinia* YopM ($\Delta yopM$) results in reduced colonization, increased inflammatory and decreased anti-inflammatory cytokine production, and improved host survival [62, 90-94]. *Yersinia*-infected macrophages display significantly higher caspase-1 activation, IL-1 β secretion, and host cell death in response $\Delta yopM$ strains compared to infection with wild type strains, suggesting that YopM inhibits host cell death [60, 62]. The presence of caspase-1 is protective against *Yersinia* infection [59, 60], and deletion of YopM enhances this protection. Note that a caspase-11 carrier mutation in caspase-1^{-/-} mice was identified in 2011 [95], so caspase-11 may instead play the main role or an additional role in protection against *Yersinia*. Together, these data support the hypothesis that YopM prevents caspase-1/11-mediated host defense. In addition to its role in inhibiting inflammasome signaling, the presence of YopM correlates with production of the anti-inflammatory cytokine IL-10 and decreased production of the pro-inflammatory cytokine IFN- γ [93, 94, 96]. Lastly, *Y. pestis* YopM has been suggested to promote neutrophil survival in the lung dependent on YopM expression

[97]. Interestingly, neutrophil NETosis has been found to involve GSDM-D, NLRP3, caspase-11, and IL-18 [98-100], suggesting that YopM might block neutrophil cell death that leads to NETosis. Overall, YopM is an important *Yersinia* effector that dampens inflammation.

YopM does not contain any catalytic domains, suggesting that it relies on protein-protein interactions to inhibit caspase-1 activation [37, 101, 102]. YopM from *Y. pseudotuberculosis* YPIII and *Y. pestis* CO92 have been shown to directly bind caspase-1 *in vitro*, preventing caspase-1 from being recruited to NLRP3 complexes for inflammasome activation [60]. The YopM isoforms in these strains contain the consensus caspase-1 cleavage motif Tyr-Leu-Thr-Asp (YLTD) where the aspartic acid residue in position 271 is essential for direct caspase-1 inhibition [60]. The YopM YLTD motif is required for normal *Yersinia* virulence in mice, indicating that YopM-caspase-1 binding contributes to *Yersinia* virulence *in vivo* [60, 103, 104]. However, YopM alleles lacking this caspase-1 inhibitory motif are still capable of inhibiting caspase-1 activation, suggesting that YopM can target inflammasome signaling through another mechanism [62, 102].

YopM has been shown to inhibit caspase-1 activation indirectly by binding the host kinases PRK and RSK, which normally repress activation of the pyrin inflammasome (see above; Figure 2) [61, 62, 82, 89, 105]. In the absence of YopM, decreased RhoGTPase activity in response to YopE and YopT triggers pyrin activation through loss of PRK and RSK activity [62, 82]. Upon binding, YopM mediates activation of PRK and RSK such that 14-3-3 proteins phosphorylate pyrin [62, 106]. Phosphorylated pyrin fails to recruit ASC for caspase-1 nucleation and

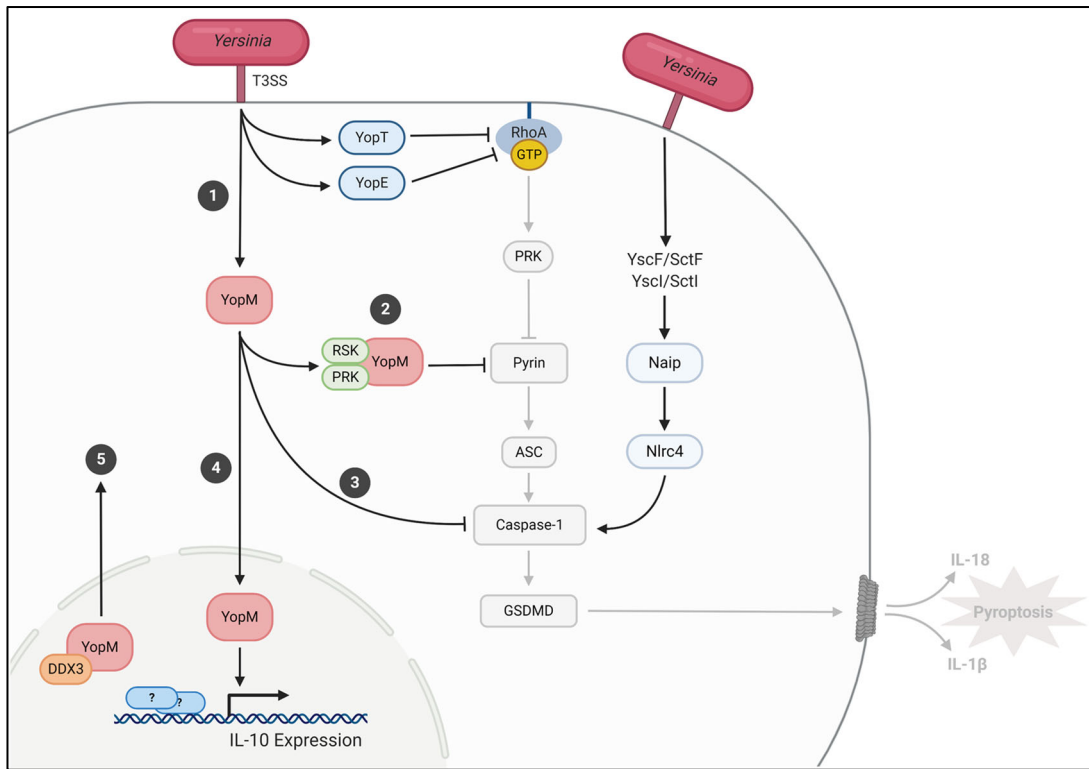


Figure 2. YopM inhibits inflammasome activation and induces anti-inflammatory IL-10 production.

(1) YopE and YopT block RhoA-dependent phosphorylation of PRK by inactivating and removing RhoA from the membrane, leading to activation of pyrin. This is an example of “pattern of pathogenesis” innate immune detection.

(2) However, YopM recruits RSK and PRK to phosphorylate 14-3-3 proteins (not shown), which in turn phosphorylate and inhibit pyrin activity.

(3) Some alleles of YopM contain a caspase-1 pseudosubstrate motif (YLTD) that allows direct inhibition of caspase-1, blocking inflammasome signaling downstream of various NLRs. Several alleles of YopM lack the YLTD motif but still block caspase-1 activation, presumably through pyrin inhibition. Therefore, the importance of the YopM YLTD motif in blocking inflammasome activation through other NLRs like NLRC4 remains unclear.

(4) YopM from *Y. pseudotuberculosis* can additionally enter the nucleus and drive IL-10 expression through activation of an unknown transcription factor.

(5) YopM binding to DDX3 in the nucleus facilitates its nuclear export back to the cytosol. Basal rates of YopM nuclear import and export may help YopM toggle between its various nuclear and cytosolic functions.

activation, preventing the cell from responding to YopE and YopT inhibition of RhoGTPases. This allows *Yersinia* Yops to block phagocytosis with little consequence. YopM is critical for inhibiting pyrin inflammasome activation in professional phagocytes, as macrophages infected with $\Delta yopM$ *Yersinia* display a robust pyrin inflammasome response. Importantly, YopM binds RSK through a C-terminal domain that is distinct from the YLTD motif that mediates direct caspase-1 binding [62, 92, 93]. YopM has therefore evolved to inhibit caspase-1 activation through multiple mechanisms, and the ability of YopM to inhibit the NLRP3/NLRC4 and pyrin inflammasomes can be potentially uncoupled in different YopM isoforms.

Wildtype mice infected with $\Delta yopM$ *Yersinia* have increased survival and reduced bacterial loads compared to infection with wildtype *Yersinia* [62, 91]. In contrast, mice lacking pyrin have increased susceptibility to $\Delta yopM$ *Yersinia* compared to wildtype mice infected with $\Delta yopM$ *Yersinia*, indicating that the pyrin inflammasome limits dissemination and promotes clearance of *Yersinia*, but YopM counters this protective response [62]. YopM mutants have revealed that activation of the pyrin inflammasome and subsequent pyroptosis of host cells can serve as an important protective response against microbial infections.

YopM inhibition of inflammasome activity is thought to occur primarily in the cytosol of the host cell, but YopM has been shown to be additionally targeted to the nucleus [96, 103, 104, 107]. Despite lacking a nuclear localization signal (NLS), the C-terminal region of YopM facilitates YopM nuclear trafficking [107]. This C-terminal region seems to be sufficient for YopM nuclear entry, which is thought to occur at a basal rate without specific interaction with additional host factors [96]. However, there is evidence that at least some *Y. pestis* YopM isoforms rely on the vesicle-

associated pathway of mammalian cells to transit from the cytosol to the nucleus [104]. In contrast to nuclear import, nuclear export of YopM requires direct interaction with a host DEAD box helicase called DDX3. Interaction with DDX3 facilitates nuclear export of YopM via the exportin CRM1 [96]. Interestingly, YopM can interact with RSK both in the cytosol and the nucleus, influencing the relative amount of phosphorylated RSK in these distinct cellular compartments. YopM can also interact with PRK in the nucleus and cytosol. In the nucleus, YopM binding of DDX3 and PRK is mutually exclusive, so YopM forms either a YopM/RSK/PRK complex or a YopM/RSK/DDX3 complex. The YopM/RSK/DDX3 complex is associated with nuclear export of YopM, returning it to the cytosol where it can presumably carry out its roles in blocking inflammasome activation. In contrast, nuclear YopM/RSK/PRK is associated with RSK hyperphosphorylation and increased expression of the anti-inflammatory cytokine IL-10. It is hypothesized that hyperphosphorylated RSK triggers activation of an unknown transcription factor that directs IL-10 expression [96]. Increased IL-10 secretion is thought to be beneficial to *Yersinia*, suggesting that YopM has evolved to upregulate IL-10 expression as part of the *Yersinia* virulence program [93, 94, 96]. Thus, the role of YopM depends both on the identity of its interacting partner and the cellular location where this interaction occurs. However, there are still several aspects of the YopM nuclear transport pathway that remain unclear. While YopM is thought to mediate its own entry into the nucleus, active transport by a second factor of host or microbial origin has not been ruled out. In addition, the mechanism that drives RSK hyperphosphorylation in the nucleus is unknown, and the transcription factor that drives IL-10 expression downstream of nuclear YopM/RSK/PRK has yet to be identified. Finally, the *in vivo*

kinetics of these distinct YopM activities has not been worked out. It is possible that YopM can transit between the nucleus and cytosol to toggle between its two activities of inflammasome inhibition and IL-10 expression under different conditions.

3.4. Regulation of Yop translocation by YopK and NLRP3 activation

NLRP3 is an inflammasome NLR that is robustly expressed in immune cells such as monocytes, macrophages, neutrophils, and conventional dendritic cells [108]. Activation of the NLRP3 inflammasome can occur through two pathways: canonical and non-canonical. Several stimuli can provide signal 2 for canonical NLRP3 inflammasome activation, including ROS, pore-forming toxins, cytosolic dsRNA, and rupture of phagolysosomes or vacuoles in the cell [109-114]. During canonical activation, NLRP3 oligomers recruit the adapter protein ASC, allowing for caspase-1 activation [115]. Active caspase-1 then cleaves pro-IL-1 β , pro-IL-18, and pro-pyroptotic GSDMD, resulting in pyroptosis and release of inflammatory signals. Non-canonical activation of the NLRP3 inflammasome is mediated by caspase-11. Caspase-11 directly binds intracellular LPS lipid A, triggering its own oligomerization and activation [116, 117]. Active caspase-11 can directly cleave and activate GSDMD, promoting progression to pyroptosis. Caspase-11 also promotes activation of the NLRP3 inflammasome through an incompletely understood mechanism, activating caspase-1 for IL-1 β , IL-18, and additional GSDMD activation [118, 119]. Inflammasome activation in response to *Yersinia* infection can occur through both canonical and non-canonical NLRP3 pathways [59, 111, 112, 120].

Activation of the NLRP3 inflammasome is partially blocked by the *Yersinia* T3SS effector protein YopK (YopQ in *Y. enterocolitica*). YopK is a regulatory protein that controls translocation of other Yops into the host cell and is required for *Yersinia* virulence [121-126]. Importantly, YopK prevents hyper-translocation of the T3SS pore-forming proteins YopB and YopD into host cells, although this interaction is not sufficient to prevent entry of low basal levels of YopB and YopD [111, 126]. YopB and YopD can be translocated into host cells from extracellular host cell-attached *Yersinia* as well as internalized, vacuolar *Yersinia*, and this YopB and YopD translocation is associated with NLRP3 inflammasome activation. Indeed, the magnitude of inflammasome activation appears to be a function of the relative amount of YopB and YopD introduced into the host cell, as increasing levels of YopD in the host cytosol correlates with larger caspase-1 oligomers [111]. YopK affects the size of pores induced on the host cell membrane by the T3SS, where loss of YopK results in an abnormally large translocation pore [122]. Cells infected with *Yersinia yopK* mutants display robust ASC-dependent NLRP3 inflammasome activation compared to cells infected with wildtype bacteria that is dependent on caspase-1 and caspase-11 [59, 120]. Therefore, YopK has an indirect but critical role in preventing NLRP3/ASC/caspase-1/caspase-11 inflammasome activation via limitation of YopB and YopD injection. Interestingly, while all organisms that encode a T3SS have YopB and YopD orthologs, no known YopK ortholog has been described outside the *Yersinia* genus. However, as *Yersinia yopK* mutants hyper-translocating YopB and YopD have been shown to induce robust NLRP3 activation, they have been used as tools to study NLRP3 inflammasome activation.

Studies using *Yersinia yopK* mutants have identified members of the galectin and guanylate binding protein (GBP) families as key mediators of NLRP3 inflammasome activation in response to *Yersinia* [127]. Galectins are carbohydrate-binding proteins that respond to membrane damage and vacuolar instability [128, 129]. GBPs co-localize with pathogens in the cytosol and pathogen-containing vacuoles, where they facilitate lysis of the vacuolar membrane to destroy the growth niche of intracellular bacteria and may directly target microbial membranes as well [112, 130-133]. Galectin-3 is thought to be recruited to *Yersinia* YopB and YopD-containing vacuoles due to the ability of these amphipathic proteins to insert into the vacuolar membrane and disrupt membrane integrity [112, 133]. Disruption of the vacuolar membrane exposes glycans, which are normally confined to the inner leaflet of the vacuole but can be bound by galectins when exposed to the cytosol [129, 133]. Indeed, YopD was shown to co-localize with Galectin-3, GBP2, and NLRP3 inflammasome components [112]. Similarly, secretion of YopB and YopD from vacuolar *Yersinia* recruits Galectin-3 and, in turn, GBP2 [133]. Recruitment of Galectin-3 results in activation of the canonical and non-canonical NLRP3 inflammasomes [112]. Zwack et al 2017 hypothesize that during hyper-injection of YopB and YopD, LPS can access caspase-11 in the cytosol [112]. Indeed, caspase-11 is known to become activated following rupture of pathogen-containing vacuoles [134], and Galectin-3 and GBPs may promote NLRP3 inflammasome activation by making LPS components more accessible for caspase-11 binding. A strain of *Y. pseudotuberculosis* lacking all effector Yops as well as YopK induced host cell lysis and IL-1 β release that was dependent on both caspase-1 and caspase-11 [120]. TIFA signaling has also been implicated in mediating NLRP3 inflammasome

activation, so ALPK1 binding to ADP-heptose may contribute to activation of the NLRP3 inflammasome under these conditions [30].

YopK is essential for *Yersinia* pathogenesis; enteropathogenic *Yersinia yopK* mutants are able to initially colonize the PPs, but fail to cause systemic infection following oral or intraperitoneal inoculation [121]. Furthermore, mice infected with *Y. pestis yopK* mutants mount an unusually early inflammatory response that prevents dissemination to the spleen and liver [135-137]. Lastly, *Y. pseudotuberculosis* expressing YopK but lacking large portions of the pYV virulence plasmid that encodes the T3SS (lacking *yopHEMOJ* and *yadA*, among other genes) colonize mouse spleens better following intraperitoneal infection when they ectopically express YopK [59]. Importantly, this boost in colonization imparted by bacterial YopK expression is eliminated by lack of caspase-1, but not NLRP3. The contribution of caspase-11 was not tested in this study. While the ability of YopK to mitigate caspase-1-driven host protection against *Yersinia* appears to be important, the contribution of NLRP3 and caspase-11 to host protection against *Yersinia* expressing or lacking YopK remains unclear. One complication to future studies on YopK is its role in regulating translocation of other Yops [122], several of which have their own effects on inflammasome signaling. However, studies of YopK have made clear the importance of bacterial virulence factor regulation to mitigate triggering T3SS-induced innate immune signaling. This indicates that these immune detection pathways impart sufficient selective pressure on pathogens to drive pathogen evolution.

In summary, the *Yersinia* T3SS can lead to inflammasome activation as a result of PAMP or “pattern of pathogenesis” recognition by distinct inflammasome-

associated PRRs. Naips recognize inadvertent delivery of T3SS structural proteins into the host cytosol during type III secretion, activating NLRC4-containing inflammasomes. Injection of YopB and YopD T3SS translocon proteins into host cells, normally minimized by the unique *Yersinia* effector protein YopK, may lead to vacuolar disruption and subsequent canonical and non-canonical NLRP3 inflammasome activation through a Galectin-3/GBP pathway. In addition, inhibition of RhoA GTPase activity by the T3SS effector proteins YopE and YopT is recognized as a “pattern of pathogenesis” through a pyrin/14-3-3/PRK mechanism surveillance mechanism. Yet pyrin activation is blocked by the T3SS effector protein YopM. As most of the immune responses triggered by the *Yersinia* T3SS are inhibited by T3SS effector proteins, the question remains of whether the *Yersinia* T3SS triggers a net immune response that is protective for the host.

3.5 YopJ-dependent host cell death occurs through a novel caspase-8-GSDMD axis

In 1997, three groups found that infection of murine macrophages with *Y. enterocolitica* or *Y. pseudotuberculosis* resulted in cell death. They attributed this cell death response to apoptosis based on cell morphology and DNA fragmentation, which is considered to be a hallmark of apoptosis [138-140]. This was initially supported by other studies that reported tBid cleavage, cytochrome c release, and pro-caspase-3/7 activation in *Yersinia*-infected macrophages [141]. However, while this cell death response was alleviated by treating cells with a pan-caspase inhibitor (z-VAD-fmk), a caspase-1 inhibitor (Ac-YVAD-cmk) failed to block cell death in response to *Yersinia* [138, 141]. This was in contrast to what was observed for two

other Gram-negative bacteria, *Shigella* and *Salmonella*, both of which failed to induce cell death when caspase-1 was blocked [142-144]. In addition, while blocking of caspase-8/FADD reduced *Yersinia*-mediated cell death [145], inhibition of the critical apoptotic mediators caspase-3/7 were found to have little effect on the cell death response to *Yersinia* [141], an observation that was neglected at the time. Together, these early findings suggested that wildtype *Yersinia* was somehow activating a novel cell death pathway that was distinct from the conventional apoptotic signaling pathway.

Given that YopJ can effectively block NF κ B and MAPK signaling, targeting of immune cells by the T3SS might be expected to render these cells ineffectual in responding to *Yersinia* infection. However, this is not the case; instead, YopJ blocking of MAPK and NF κ B signaling trips a host “pattern of pathogenesis” alarm, triggering a unique cell death response in infected macrophages [140, 146]. Importantly, YopJ is indispensable for *Yersinia*-induced rapid killing of macrophages [139, 140]. This YopJ-dependent cell death requires the host proteins RIPK1, caspase-8, and FADD, but not RIPK3 [147-150]. This is reminiscent of the well-characterized “death switch” downstream of tumor necrosis factor receptor (TNFR1), where following TNFR1 stimulation, TRADD recruits RIPK1, TRAF2/5, and cIAP1/2 to form complex I, leading to activation of the NF κ B/MAPK signaling pathways. When NF κ B/MAPK signaling is uninhibited, signaling through these pathways promotes expression of pro-inflammatory and pro-survival genes, enforcing cell survival during infection (Figure 3A). One of these upregulated genes encodes the caspase-8 inhibitor cFLIP [151] which binds caspase-8 and prevents it from autoprocessing such that it remains in a caspase-8/cFLIP heterodimer. Caspase-8/cFLIP

heterodimers also promote cell survival by blocking RIPK3-mediated necroptosis. These caspase-8/cFLIP heterodimers can additionally promote expression of inflammatory genes through a less defined pathway [152]. Thus, in the absence of NFκB/MAPK blockade, caspase-8/cFLIP heterodimers enforce cell survival and contribute to expression of pro-inflammatory genes during infection.

In the case where NFκB/MAPK signaling pathways are blocked, such as by YopJ enzymatic activity, RIPK1 dissociates from complex I and recruits FADD and caspase-8 to form complex II, consisting of RIPK1/FADD/caspase-8 (Figure 3B) [153, 154]. No longer bound to the inhibitory cFLIP, caspase-8 is free to undergo autoprocessing, producing active caspase-8 homodimers. The formation of the RIPK1/FADD/caspase-8 complex II in response to NFκB/MAPK inhibition is driven both by dissociation of RIPK1 from receptors like TNFR1 and by the drop in cFLIP expression that occurs when NFκB/MAPK signaling is lost. The RIPK1/FADD/caspase-8 complex can then cleave caspase-3/7/9 to promote apoptosis [155-158]. However, attempts to better elucidate this pathway in the context of *Yersinia* YopJ-induced cell death have revealed a novel role for caspase-8 in driving pyroptosis.

Contrary to early observations, recent reports have found that *Yersinia*-infected macrophages display a morphology that is indicative of pyroptosis, rather than apoptosis [159]. Pyroptosis occurs when pore forming proteins belonging to the gasdermin (GSDM) family become cleaved and nucleate to form a pore in the host cell plasma membrane [160]. Gasdermin D (GSDMD) is the only GSDM family member that is known to initiate pyroptosis downstream of caspase-1 and/or

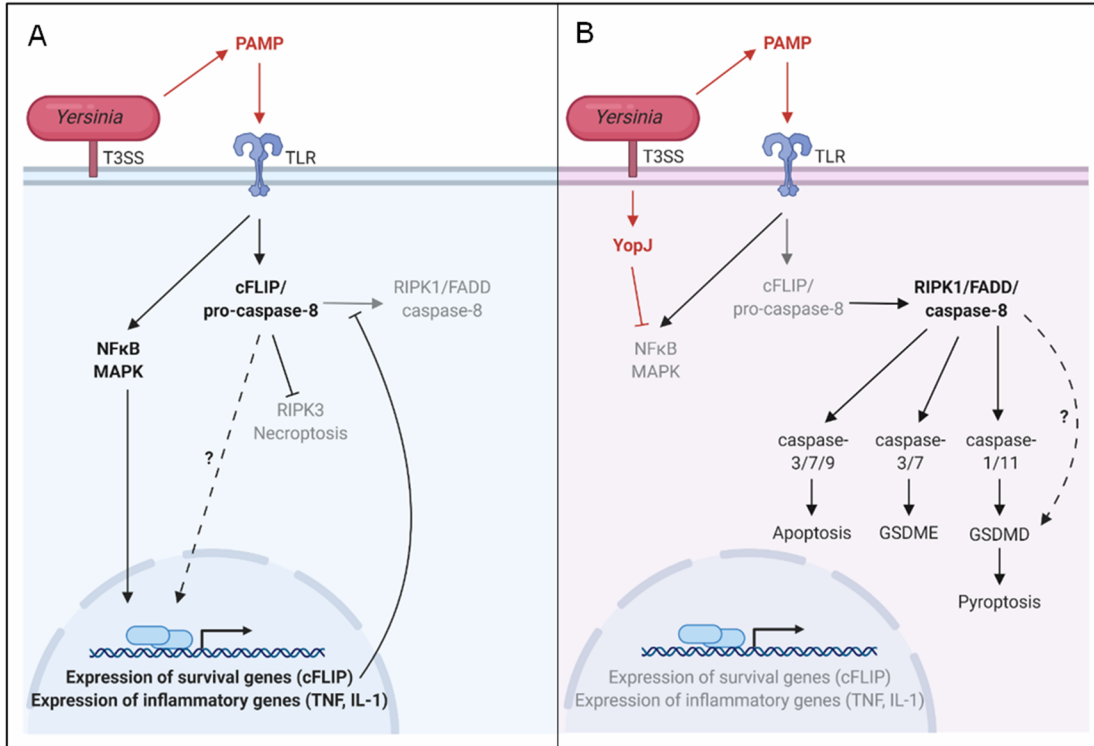


Figure 3. Caspase-8 activation in response to NF-κB/MAPK blocking by YopJ

(A) In the absence of YopJ: Signaling downstream of PAMP detection triggers activation of NFκB/MAPK signaling to promote expression of inflammatory and cell survival genes. Increased expression of the caspase-8 inhibitor cFLIP promotes formation of an inactive cFLIP/caspase-8 complex. The cFLIP/caspase complex represses RIPK3-dependent necroptosis and enforces cell survival. cFLIP/caspase-8 also promote expression of pro-inflammatory genes through an undefined mechanism.

(B) In the presence of YopJ: NFκB/MAPK blockade prevents expression of inflammatory and cell survival genes, reducing the amount of inhibitory cFLIP in the cell. At the same time, RIPK1 promotes formation of a RIPK1/FADD/caspase-8 complex where caspase-8 is cleaved to become fully active. Caspase-8 processing of caspase-3/7/9 promotes apoptosis and GSDME cleavage. Caspase-8 processing of caspase-1/11 leads to cleavage and activation of GSDMD for pyroptosis. Caspase-8 may also directly cleave and activate GSDMD, although the physiological relevance and importance of this direct cleavage remains unclear.

caspase-11 (caspase-4/5 in humans) [118, 119]. Importantly, caspase-8 is essential for GSDMD processing by caspase-1/11 during *Yersinia* infection. When NFkB and/or MAPK pathways are blocked, caspase-8 can also directly cleave and activate GSDMD, albeit it at a lower rate and efficiency compared to GSDMD cleavage by caspase-1/11 [150]. In addition, simultaneous stimulation of TLR4 and inhibition of TAK1 in macrophages recapitulates the cell death response to *Yersinia*, suggesting that caspase-8 activation occurs when NFkB/MAPK signaling is blocked downstream of PRRs such as TLRs [150]. Gasdermin E (GSDME), which is linked to necroptosis but may also contribute to pyroptosis, is also cleaved by caspase-3/7 following caspase-8 activation in response to *Yersinia* [150, 159].

Studies in mice have revealed that YopJ-dependent cell death may contribute to the host immune response to *Yersinia* infection. Some studies reported that *Y. pseudotuberculosis* YopJ (or *Y. enterocolitica* YopP) is necessary for full virulence following oral infection, while other studies using the oral or the intraperitoneal infection models showed no role for YopJ in bacterial virulence as measured by tissue colonization or mouse survival [43, 53, 91, 161, 162]. However, *Y. pseudotuberculosis* lacking YopJ catalytic activity induced less serum IL-18 and IFN- γ as well as decreased splenic neutrophil recruitment compared to wildtype bacteria following oral infection [54]. Furthermore, while *Yersinia*-infected wildtype and RIPK3 deficient mice (RIPK3^{-/-}) are comparable in terms of bacterial burden, mortality, and recruitment of inflammatory monocytes to infected tissues [147], mice deficient in both RIPK3 and caspase-8 (RIPK3^{-/-}/CASP8^{-/-}) have increased bacterial burdens, higher mortality, and significantly fewer inflammatory monocytes in infected tissues [147]. Together, these results indicate that YopJ-dependent cell death and the

ensuing inflammatory response is protective against *Yersinia* infection, while RIPK3-mediated necroptosis is dispensable for this cell death response. In keeping with this notion, mice expressing a catalytically dead version of RIPK1 (RIPK1^{KD}) fail to carry out a cell death response during infection with wildtype *Yersinia*. Similar to RIPK3^{-/-}/CASP8^{-/-} mice, RIPK1^{KD} mice are also subject to increased bacterial burdens and decreased survival when infected with wildtype *Yersinia*. Furthermore, RIPK1 expression in hematopoietic cells is specifically required for tissue TNF- α and IL-12 and host resistance to *Y. pseudotuberculosis* oral infection [148]. Interestingly, RIPK1^{KD} mice infected with $\Delta yopJ$ *Yersinia* have reduced colonization in the spleen and liver and led to higher survival rates than RIPK1^{KD} mice infected with wildtype *Yersinia* [172]. The reduced survival of RIPK1^{KD} mice infected with wildtype *Yersinia* supports the notion that a host cell death response to YopJ enzymatic activity is protective against *Yersinia* infection, yet YopJ activity promotes virulence in the absence of RIPK1 immune sensing. These results help us pull apart the two consequences of YopJ in the cell, one being inhibitory acetylation of NF κ B and MAPK signaling pathway components as a *Yersinia* virulence mechanism, and the second being activation of caspase-8-dependent cell death through RIPK1 as a “pattern of pathogenesis” host protective response.

Given that the cell death response to YopJ is protective against *Yersinia* infection, *Yersinia* may be under strong selective pressure to prevent caspase-8 activation. However, direct blocking of caspase-8 activity leads to necroptosis via RIPK3-MLKL pathway [163-166]. Indeed, caspase-8 blockade during *Yersinia* infection switches the cell death to necroptosis [147, 149]. Thus, direct blocking of caspase-8 by *Yersinia* would be expected to alter, but not subvert, the host cell death

response. Interestingly, while *Y. enterocolitica* YopP is 94% identical to *Y. pseudotuberculosis* and *Y. pestis* YopJ, it is more cytotoxic than YopJ isoforms, at least in part because of a higher rate of translocation into host cells [167, 168]. Expression of YopP in *Y. pseudotuberculosis* instead of YopJ also leads to attenuated virulence [168], suggesting that there is a fitness cost for high levels of YopJ activity in the host cell during *Yersinia* infection. This fitness cost, which is likely due to caspase-8 activation in response to complete or near complete blockade of NFkB/MAPK signaling, may be offset by YopJ-dependent inhibition of pro-inflammatory cytokines and chemokines during infection. Therefore, YopJ/P may exhibit a Goldilocks effect: some is needed for countering innate immune signaling but too much triggers a protective cell death response through caspase-8.

In summary, blockage of NFkB and MAPKs by YopJ is counteracted by an intact caspase-8/RIPK1/FADD pathway. Yet, loss of YopJ is associated with attenuated virulence in the absence of caspase-8 signaling, perhaps explaining why *Yersinia* have not lost YopJ expression all together. In addition, *Yersinia* is not likely to evolve a mechanism for directly inhibiting caspase-8 activation, since inhibition of caspase activity triggers activation of RIPK3-dependent necroptosis through RIPK1 [163-166]. Instead, *Yersinia* YopJ evolution seems to be aimed at fine-tuning the amount of YopJ that is translocated into the host cell, as both *Y. pseudotuberculosis* and *Y. pestis* have been found to translocate lower levels of YopJ [168]. *Yersinia* evolution may therefore be moving towards dampening, but not completely blocking, NFkB/MAPK signaling to decrease expression of proinflammatory genes without triggering extensive caspase-8 activation.

4. Conclusions

The last several decades of *Yersinia* T3SS research has revealed a complex web of host-pathogen interactions. These have been shaped by both the fitness benefits imparted by T3SS effector proteins that inhibit phagocyte effector functions such as phagocytosis, and by the inflammatory responses induced by cytosolic innate immune receptor recognition of the T3SS. During type III secretion, heptose-containing metabolites as well as T3SS needle structural proteins enter the host cytosol to trigger the ALPK1-TIFA-NFκB and the NLRC4 inflammasome pathways. While entry of the YopB and YopD T3SS translocon proteins into host cells also activates the NLRP3 inflammasome, YopB and YopD translocation is kept to a minimum by the unique T3SS protein YopK. In addition to these PAMPs gaining access to the host cell cytosol during type III secretion, T3SS effector proteins also enter the host cell to target RhoGTPases, inhibiting phagocytosis but triggering the pyrin inflammasome. This “pattern of pathogenesis” recognition by the host cell is masked by the YopM effector protein, which downregulates pyrin activation and what would otherwise be a protective response against *Yersinia*. Lastly, the T3SS effector YopJ inhibits NFκB and MAP kinase signaling activated by TLR, cytokine receptor, and perhaps ALPK1, but this “pattern of pathogenesis” triggers host cell death and inflammatory cytokine release through RIPK1 and caspase-8, leading to a protective response. The intricate layers of bacterial virulence factor and host immune system response demonstrates the importance of these cytosolic innate immune receptors in defending the host against pathogens.

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Chapter 2.

Characterization of a novel locus affecting *Y. pseudotuberculosis* virulence plasmid copy number, T3SS activity, and fitness.

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Introduction

Many species of pathogenic bacteria harbor extrachromosomal virulence and/or antimicrobial resistance plasmids. Replication of such plasmids is tightly controlled to maintain a specific plasmid copy number (PCN), or the number of plasmid copies per cell or chromosome equivalent. The facultative mammalian pathogen *Yersinia pseudotuberculosis* harbors an IncFII, 70kb virulence plasmid known as pYV which is highly similar to pCD1 from *Y. pestis*, the causative agent of the plague [1]. The pYV plasmid is known to be essential for normal *Y. pseudotuberculosis* and *Y. pestis* virulence in mouse infection models as it encodes several crucial virulence factors, including the type III secretion system (T3SS) [1, 2]. The T3SS is a large injectisome used by human pathogenic *Yersinia* species to translocate a series of effector proteins known as *Yersinia* outer proteins, or Yops, directly into the cytoplasm of host innate immune cells. In short, these Yops target various pathways in the cell in such a way that dampens the host immune response and promotes *Yersinia* virulence and dissemination to deeper tissues [3-5]. Expression of the T3SS is thought to be energetically costly to the bacterium, and so expression and activity of the T3SS is limited to host conditions (i.e. within the mammalian host *in vivo* or at 37°C in low calcium media *in vitro*) [6-8].

Previous work in the field has elucidated several pathways that work together to limit T3SS expression to host conditions, including transcriptional and translational regulation of T3SS genes [9-11]. For example, the transcription factor LcrF, which is the master regulator of T3SS gene expression and is encoded on the pYV plasmid, is differentially expressed in a temperature-dependent manner [12]. An RNA hairpin structure in the *lcrF* mRNA transcript ensures that LcrF is only translated at 37°C and

as a result, T3SS genes are preferentially transcribed at 37°C [12, 13]. An additional RNA thermometer also controls translation of YopN, a regulatory protein that controls secretion of T3SS effector proteins [10].

In addition to regulation of T3SS gene expression, unique regulation of T3SS gene dosage was previously uncovered in *Yersinia*. *Y. pseudotuberculosis* maintains a low pYV PCN of about 1-2 copies per cell at environmental temperatures of 26°C and specifically upregulates pYV PCN to 3+ copies per cell at host temperatures of 37°C [14]. This increase in T3SS gene dosage is required for robust T3SS activity and is essential for normal *Y. pseudotuberculosis* virulence in mice [14]. This suggests a model where *Y. pseudotuberculosis* has evolved to maintain low pYV PCN outside the host when the metabolically costly T3SS is not needed and specifically upregulates pYV PCN under conditions where T3SS expression is required for bacterial virulence and fitness in the host. However, how these dynamic changes in pYV PCN are achieved in response to temperature remains poorly understood.

The pYV/pCD1 plasmids of *Y. pseudotuberculosis* and *Y. pestis* belong to the IncFII family of plasmids [15-17]. Much of what is known about IncFII plasmids comes from studies on the IncFII plasmid R1, which was originally isolated from a clinical sample of *Salmonella enterica* and subsequently extensively studied in *E. coli* [18-20]. Studies in *E. coli* defined the minimal locus required for replication and copy number maintenance of plasmid R1, which we will refer to as the *cop-rep* locus [20-22]. The *cop-rep* locus includes the origin of replication (Ori) and encodes the essential replication initiation factor RepA. When RepA is expressed at adequate levels, it binds specific sequences in the Ori and promotes duplex melting for

initiation of plasmid R1 replication, which then proceeds via the Theta mode of plasmid replication [20, 23-25].

In addition to RepA, the *cop-rep* locus of plasmid R1 also encodes two inhibitors of plasmid replication: the CopB protein and a small RNA (sRNA) known as CopA [20]. The *repA* mRNA transcript can be transcribed from two different promoters: *pcopB* or *prepA*. Transcription from *prepA* produces a monocistronic *repA* mRNA that, in the absence of CopA sRNA, is translated to produce enough RepA to drive plasmid replication and increased PCN. Transcription from *pcopB* on the other hand produces a *copB-repA* polycistronic mRNA that favors translation of CopB but produces little RepA. Under normal conditions, the *prepA* promoter is significantly stronger than *pcopB*; however, when CopB is expressed, it inhibits expression by repressing *prepA* [20, 26, 27]. RepA expression is also regulated at the post-transcriptional level by CopA sRNA, which is an antisense RNA complimentary to the so-called CopT region of the *repA* and *copB-repA* mRNA transcripts. When CopA sRNA levels are high, CopA binds the complimentary CopT sequence in the *repA* and *copB-repA* transcripts and forms a kissing complex that greatly reduces translation of *repA*, reducing plasmid replication and copy number [20, 28-30]. The inhibitory activities of CopB and CopA are thought to enforce R1 PCN homeostasis by correcting deviations from a given “set point.” The CopA sRNA from plasmid R1 is constitutively expressed from its own *pcopA* promoter in the *cop-rep* locus. In addition, CopA is unstable and subject to degradation by RNases, and so CopA sRNA levels are proportional to R1 PCN [20]. Since the *pcopA* promoter is stronger than *pcopB*, CopA sRNA levels are more responsive to PCN deviations that increase PCN and thus CopA gene dosage [20, 31]. When R1 PCN exceeds its setpoint, the

high levels of CopA gene dosage and CopA sRNA inhibit translation of RepA, leading to decreased plasmid replication rate and lowering R1 PCN back towards its homeostatic set point. When R1 PCN drops below the set point, CopA sRNA and CopB protein levels also drop, with a significant reduction in CopA levels since it is naturally unstable. Under this condition, lower levels of CopB protein allows for derepression of *prepA*, leading to increased RepA levels and increased PCN. In summary, when R1 PCN is too high, CopA sRNA promotes reduced plasmid replication through inhibition of RepA translation whereas when R1 PCN is too low, derepression of *prepA* by CopB allows for an increase in *repA* transcription to promote RepA expression and plasmid replication.

The *cop-rep* locus that was elucidated for plasmid R1 is highly conserved in pYV/pCD1, suggesting conserved RepA, CopB, and CopA sRNA function. It is important to note that most R1 studies were conducted in *E. coli*, which is not the natural host of plasmid R1. To our knowledge, plasmid R1 has never been shown to undergo temperature dependent shifts in PCN and the *cop-rep* locus of pYV/pCD1 has not been extensively studied. Thus, it remains unclear how components of *cop-rep* locus are regulated to allow for dynamic changes in PCN “set points” at different temperatures in *Y. pseudotuberculosis*.

While carrying out a transposon mutagenesis screen to identify genes affecting T3SS function, we identified a *Y. pseudotuberculosis* mutant that induced a significantly higher NFκB response from infected HEK293T cells (for more information on the genetic screen, see Miller et al 2014) [32]. This mutant was found to harbor a transposon (Tn) insertion in a previously unannotated locus adjacent to the highly conserved *cop-rep* locus on the pYV plasmid, which we will refer to as the

plasmid copy number inhibitory locus (pil). Bioinformatic analysis indicates that the *pil* locus encodes a hypothetical protein containing a single copy of a highly conserved phospholipase D (PLD) motif, indicative of EDTA-resistant bacterial endonucleases [33, 34]. The *pil::Tn* mutant was found to have significantly higher pYV PCN at 26°C and immediately after shifting to 37°C, resulting in a continuously high pYV PCN that is temperature-independent. The increased pYV PCN in the *pil::Tn* mutant results in increased expression of T3SS genes at 26°C and 37°C and increased T3SS activity specifically at 37°C, which is likely the underlying cause of the increased NFκB response from infected HEK293T cells that originally identified the locus.

Here, we report that insertion into, but not deletion of, the previously unannotated *pil* locus drives increased pYV PCN at 26°C, increased T3SS expression and activity upon a shift to 37°C, and imparts a significant growth defect especially under T3SS-inducing conditions. We show that ectopic over-expression of specific regions of the *pil* locus drives down pYV PCN specifically at 37°C independently of PLD protein function or expression. Finally, we provide proof-of-concept that the *pil::Tn* high pYV PCN mutant can be used a genetic tool to identify factors affecting *Y. pseudotuberculosis* growth, T3SS function, and pYV PCN.

Results

Discovery of the *plasmid copy number inhibitory locus (pil)* of *Y. pseudotuberculosis* pYV.

To identify *Y. pseudotuberculosis* genes important for T3SS activity and host-pathogen interactions, our lab previously carried out an NFκB-based luciferase reporter screen in HEK293T cells [32]. HEK293T cells were infected with a library of *Y. pseudotuberculosis* transposon mutants in the Δyop6 genetic background lacking the YopHEMOJT effectors. Through this screen, we identified a Tn5 mutant (823B) that induced two-fold NFκB activity in HEK293T cells compared to the Δyop6 parental control (Fig. 1A), suggesting that this isolate harbors a Tn5 insertion in or near a locus important for regulating T3SS expression and/or activity. The Tn5 insertion from the 823B mutant was mapped to a previously unannotated locus ~123 nucleotides away from the pYV *copB* gene (Fig. 1B). The Tn5 insertion disrupts a putative open reading frame predicted to encode a 122 amino acid protein with a single copy of a highly conserved HxKx(4)Dx(6)GSxN phospholipase D (PLD) motif (Fig. 1B-D), indicative of bacterial EDTA-resistant endonucleases.

Protein alignment of the predicted *pil* ORF protein products from all *Y. pseudotuberculosis* and *Y. pestis* reveals 100% conservation, but significant divergence in the N-terminal portion of the predicted *Y. enterocolitica* protein (Fig. 1C). Additional *pil* ORF orthologues were identified in other Gram negative pathogenic species using the Basic Local Alignment Search Tool (BLAST). In general, the C terminus and putative PLD domain is moderately to highly conserved in species such as *E. coli*, *Salmonella enterica*, and *Klebsiella pneumoniae* (Fig. 1D).

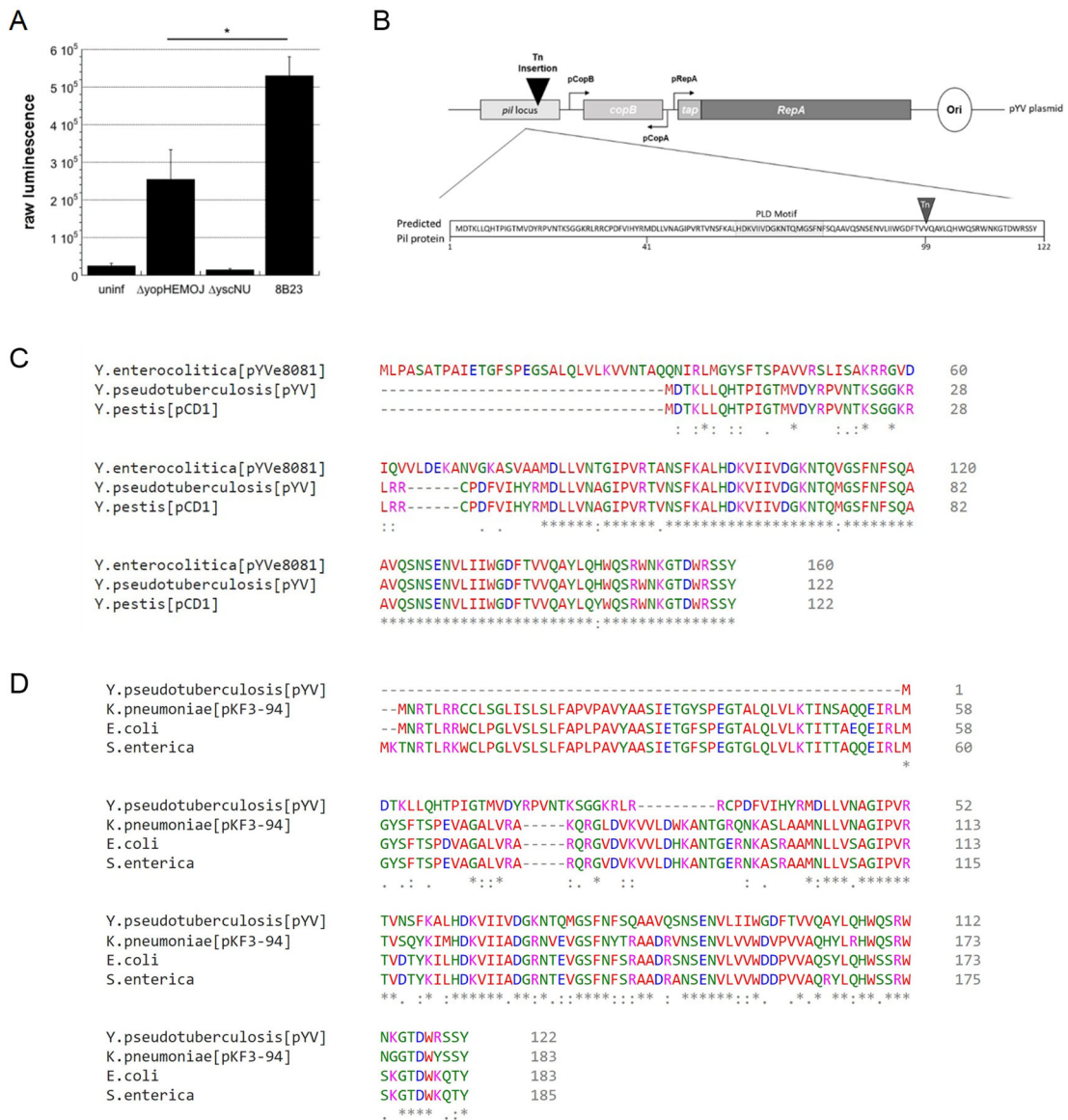


Figure 1. Identification of the previously unannotated plasmid copy number inhibitory locus (*pil*) near the *cop-rep* locus of the *Y. pseudotuberculosis* pYV plasmid.

(A) Raw luminescence from HEK293T cells transfected with an NFκB luciferase reporter following infection with Δyop6 (ΔyopHEMOJ), a T3SS deficient strain (ΔyscNU), the original 823B strain harboring a Tn insertion in *pil* compared to an uninfected (uninf) control. (B) Top: schematic displaying the location of the previously unannotated *pil* locus relative to the *cop-rep* locus. Bottom: Schematic representing the amino acid sequence of the full length putative Pil protein with the site of Tn insertion indicated with a black triangle. (C) Multiple sequence alignment of Pil homologs from the virulence plasmids of mammalian pathogenic *Yersinia* species (D) Multiple sequence alignment comparing Pil orthologues from different species of Gram negative bacteria.

At least some *pil* ORF orthologues are plasmid encoded and located near the plasmid *cop-rep* locus (data not shown). The *pil* ORF from *K. pneumoniae* shares many conserved residues with the *Yersinia pil* and a single copy of the PLD motif (Fig. 1D). Further analysis of the *pil* locus in both *Y. pseudotuberculosis* and *Y. pestis* shows that a region upstream of the *pil* ORF and the predicted first ~13 amino acids of the *pil* ORF is duplicated in the pYV plasmid, suggesting that this locus may have been involved in a recombination event (data not shown).

A *pil::Tn* *Y. pseudotuberculosis* mutant has elevated T3SS activity and pYV plasmid copy number (PCN).

To understand why the *pil::Tn* mutant induced more NFkB activity, we reconstituted the Tn5 insertion in a wild type background of *Y. pseudotuberculosis* IP2666pIB1 to generate a *pil::Tn* strain that has intact YopHEMOJT. Note that in this strain, pYV is referred to as pIB1. Independently, a *pil::pNQ* insertional mutant was constructed in *Y. pseudotuberculosis* YPIII/pIBX, which carries a pYV-encoded *lux* luciferase reporter. Both the *pil::Tn* and *pil::pNQ* mutants were used to study the impact of *pil* insertion on *Y. pseudotuberculosis*.

Since increased NFkB activity is suggestive of increased T3SS activity, we tested T3SS activity in the *pil::Tn* strain. We found that the *pil::Tn* mutant displays significantly increased T3SS activity after 1.5 hours in T3SS-inducing conditions (37°C/-Ca²⁺) (Fig. 2A). In contrast, there is no detectable secretion of Yops through the T3SS at 26°C or in the presence of high calcium in wildtype or *pil::Tn* *Y. pseudotuberculosis* (Fig. 2A). To determine if the *pil::Tn* mutant over-expresses T3SS genes, we carried out western blot analysis to measure levels of the T3SS

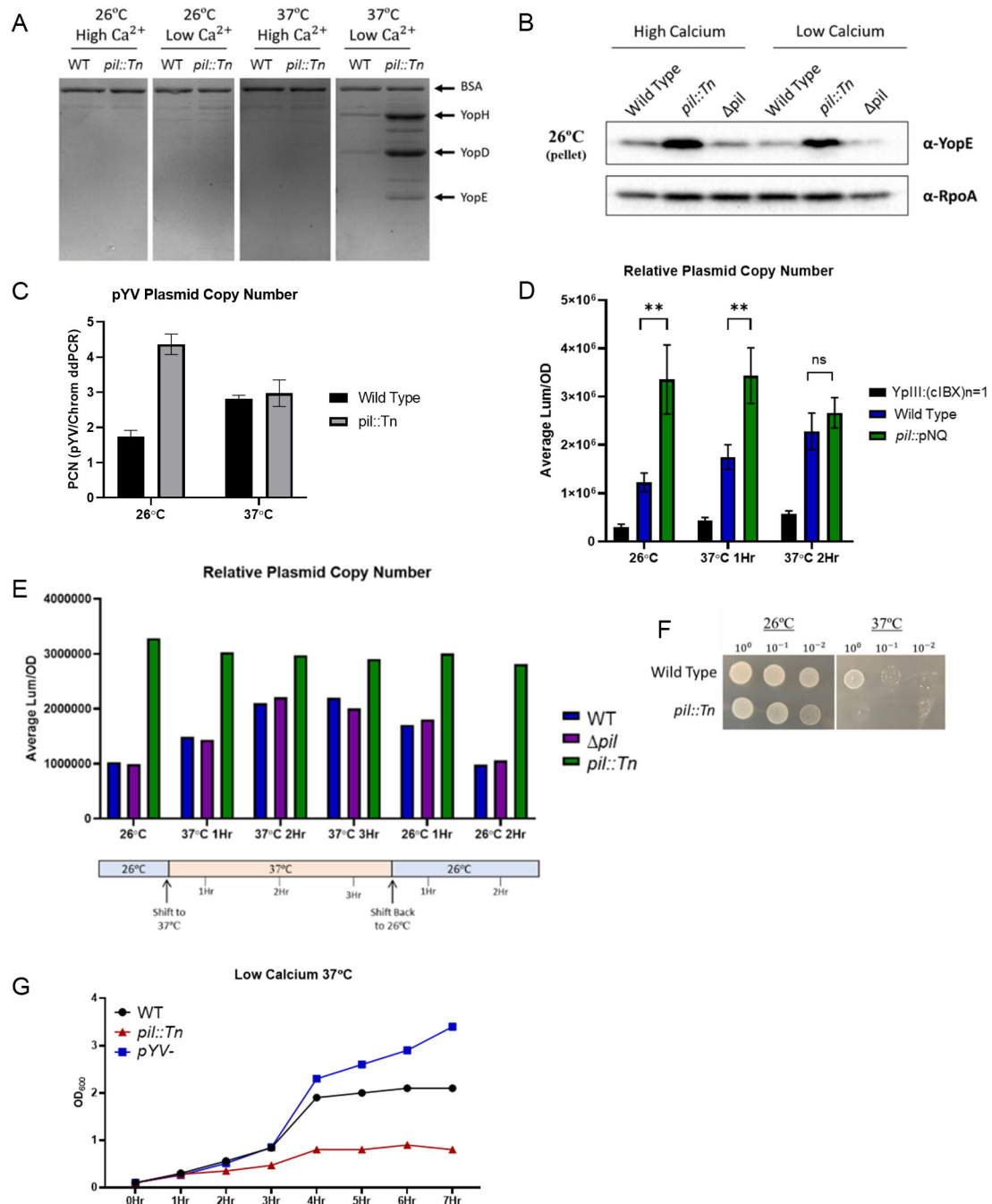


Figure 2. Disruption of *pil* drives increased pYV PCN, T3SS expression and activity, and a severe growth defect at 37°C.

Yersinia strains were grown in regular or low calcium media at 26°C or at 37°C to induce T3SS activity. **(A)** Strains in the IP2666pIB1 background were grown at the indicated

temperature/condition and secreted proteins (Yops) were precipitated and visualized by SDS-Page gel separation and Coomassie blue staining. Bovine serum albumin (BSA) was added as a loading and precipitation control. Data shown is representative data of three biological replicates. **(B)** Strains in the IP2666pIB1 background were grown at 26°C before collecting bacterial pellets for western blot analysis probing against YopE. Samples run on the same gel were also probed for RpoA expression as a loading control. Data shown is representative of two biological replicates. **(C)** Absolute pYV plasmid copy number (PCN) was estimated for strains in the IP2666pIB1 background using ddPCR with primers that amplify pYV-specific DNA normalized to chromosomal equivalents from the same sample. Data shown is the average of three biological replicates. **(D)** Relative pYV PCN was estimated for strains in the YPIII/pIBX background of *Y. pseudotuberculosis* using a luciferase PCN assay. For each timepoint, luminescence from the pYV plasmid was measured and normalized to optical density. Data shown is the average of three biological replicates. **(E)** Relative pYV PCN was estimated for YPIII/pIBX strains after shifting from 26°C to 37°C and then returning to 26°C. Data shown is one biological replicate. **(F)** Spot dilutions for strains in the IP2666pIB1 background were plated onto NaOX (low calcium) plates and grown at 26°C or 37°C to induce T3SS expression for ~16 hours. Data shown is representative of three or more biological replicates. **(G)** Growth curve of wild type, *pil::Tn*, and plasmid cured (pYV-) IP2666pIB1 in low calcium media at 37°C. Data shown is representative of at least two biological replicates.

effector protein YopE. Interestingly, we found that the *pil::Tn* mutant expresses YopE at 26°C regardless of calcium levels despite showing no T3SS activity under these conditions. These data indicate that while the regulatory mechanisms preventing T3SS activity at 26°C remains intact in the *pil::Tn* mutant, LcrF activity at 26°C is not fully repressed in this mutant. The *pil::pNQ* mutant was confirmed to have a similar secretion profile (data not shown). Subsequent RNA-seq analysis of *pil::Tn* IP2666pIB1 compared to the parental wildtype strain confirmed that the mutant over-expresses nearly all T3SS genes at 26°C and 37°C/-Ca²⁺ (Fig. 9A-B).

Given that the *pil* locus is near the *cop-rep* locus on the pYV plasmid, and changes in pYV PCN are known to impact T3SS gene dosage and expression, we sought to determine if the *pil::Tn* mutant has altered pYV PCN. To test this in the IP2666pIB1 background, we used droplet digital (dd)PCR to determine the relative

abundance of pYV per chromosomal equivalent [35]. The *pil::Tn* mutant displayed a ~2 fold increase ($p < 0.0001$) in pYV PCN at 26°C compared to wildtype; however, after 1.5 hours at 37°C/-Ca²⁺ *pil::Tn* pYV PCN was comparable to wildtype (Fig. 2C). To confirm these results, we took advantage of the YPIII/pIBX strain of *Y. pseudotuberculosis* that harbors a *lux* operon stably incorporated into pYV. Relative abundance of pYV can be measured by bioluminescence normalized to cell density. The *pil::pNQ* mutant displays significantly higher luminescence at 26°C and after shifting to 37°C compared to the parental strain, while luminescence was comparable between wildtype and *pil::pNQ* at later timepoints at 37°C (Fig. 2D). Wildtype *Y. pseudotuberculosis* rapidly reduces luminescence upon return to ambient temperatures after 37°C incubation, while the *pil::pNQ* mutant maintains elevated PCN across all temperature shifts (Fig. 2E).

Since induction of the T3SS at 37°C/-Ca²⁺ is known to induce growth arrest *in vitro*, we tested if the high pYV PCN and elevated T3SS activity in the *pil::Tn* mutant affected *Yersinia* growth under various conditions. We found that the *pil::Tn* mutant grows similar to wildtype at 26°C in a spot test assay (Fig. 2F) and this was recapitulated in growth curves carried out in low calcium media at 26°C (data not shown). In contrast, the *pil::Tn* mutant displays a >10-fold decrease in growth at 37°C when the T3SS is (Fig. 2F). A growth curve comparing wildtype and *pil::Tn* at 37°C/-Ca²⁺ shows that the *pil::Tn* mutant enters growth arrest almost immediately following the shift to 37°C while wildtype enters growth arrest approximately 4 hours after shifting to 37°C (Fig. 2G). In contrast, a plasmid cured strain of wildtype *Y. pseudotuberculosis* (pYV-) grows well under these conditions, as this strain lacks the T3SS and thus is not subject to a T3SS-related metabolic burden.

Together, this suggests that the *pil::Tn* mutant has elevated PCN at environmental temperatures where PCN is normally kept low in wildtype *Y. pseudotuberculosis*. This in turn leads to increased expression and activity of the T3SS upon shift to 37°C and a significant growth defect in T3SS-inducing conditions.

In-frame deletion of the entire *pil* locus does not impact *Y. pseudotuberculosis*.

Since the *pil* locus is predicted to encode a protein that should be disrupted in the *pil::Tn* and *pil::pNQ* mutants, we tested if loss of the putative *pil* ORF recapitulates the phenotypes of *pil* insertional mutants. To do this we generated a clean in-frame deletion of the entire *pil* ORF (Δpil) in both the YPIII/pIBX and IP2666pIB1 backgrounds of *Y. pseudotuberculosis*. We found that loss of *pil* had no significant effect on relative pYV PCN at 26°C or 37°C/-Ca²⁺ (Fig. 3A). Deletion of the *pil* locus also had no effect on relative PCN when shifting back to 26°C from 37°C (Fig. 2E). Consistent with this, the Δpil mutant was found to have normal T3SS activity relative to wildtype at 37°C/-Ca²⁺ (Fig. 3B). Lastly, the Δpil mutant grew similarly to wildtype *Y. pseudotuberculosis* at 26°C and 37°C (Fig. 3C). Since loss of the entire *pil* locus failed to recapitulate *pil::Tn* and *pil::pNQ* phenotypes, this suggests that loss of expression from the *pil* locus is not responsible for the elevated PCN and T3SS activity of these insertion mutants.

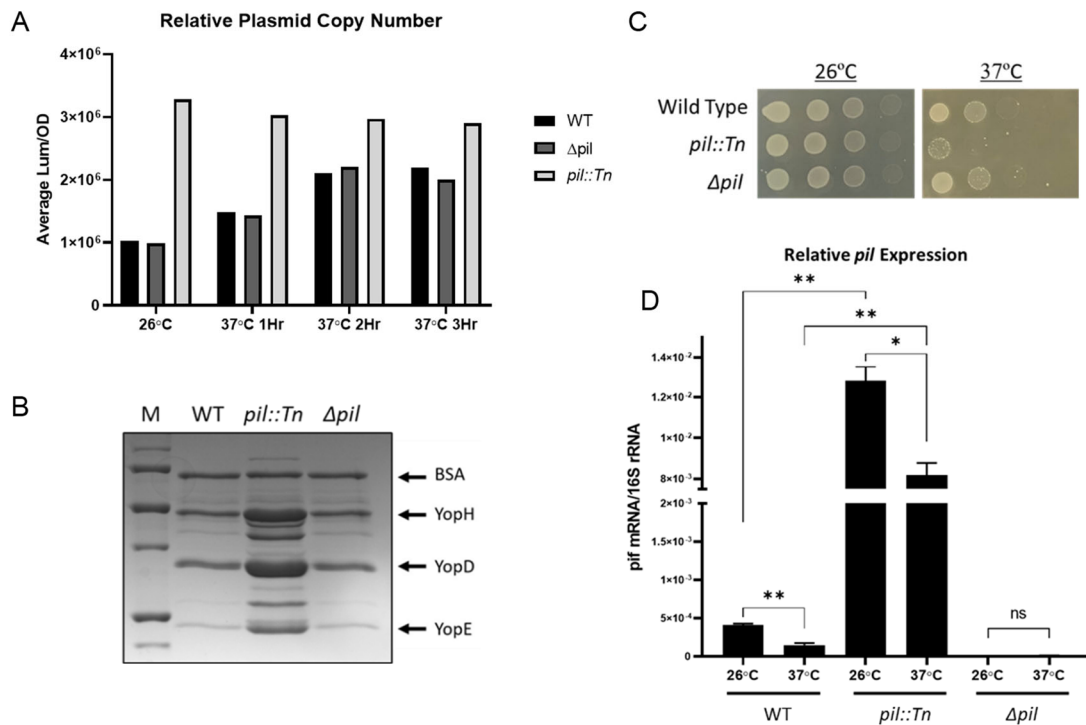


Figure 3. Deletion of the entire *pil* locus has no effect on pYV PCN, T3SS activity, or *Y. pseudotuberculosis* growth

(A) Relative PCN was estimated for strains in the YPIII/pIBX background using a luciferase PCN assay. Data shown is representative of three or more biological replicates. **(B)** Strains in the IP2666pIB1 background were grown at 37°C in low calcium media and secreted proteins (Yops) were precipitated and visualized by SDS-Page gel separation and Coomassie blue staining. Bovine serum albumin (BSA) was added as a loading and precipitation control. Data shown is representative data of three biological replicates. **(C)** Spot dilutions for strains in the IP2666pIB1 background were plated onto NaOX (low calcium) plates and grown at 26°C or 37°C to induce T3SS expression for ~16 hours. Data shown is representative of three or more biological replicates. **(D)** The relative amount of *pil* RNA at 26°C and 37°C was quantified for strains in the IP2666pIB1 background using RT-qPCR and normalizing against 16S rRNA.

The Tn and pNQ insertions are predicted to truncate the putative Pil protein immediately after amino acid 99 in the *pil* ORF, creating an early stop codon predicted to remove the last 23 amino acids of the 122 amino acid protein (Fig. 1B). To test if the *pil* ORF is transcribed in the *pil::Tn* mutant, we measured *pil* mRNA by using qPCR primers that anneal upstream of the insertion site. We found that the *pil::Tn* mutant had significantly more *pil* mRNA compared to wildtype *Y. pseudotuberculosis* (Fig. 3D). In keeping with this, RNA-seq analysis found that the wildtype strain contains very few *pil* reads. However, the *pil::Tn* mutant contained very high *pil* RNA-seq reads, with the *pil* mRNA being the most upregulated gene compared to wildtype at both 26°C and 37°C (Fig. 7C and Supplementary Dataset 1). This confirmed that the mutant phenotypes of the *pil::Tn* and *pil::pNQ* mutants is not due to a simple loss of expression from the *pil* locus and may instead be driven by over-expression of the *pil* and *cop-rep* locus and/or aberrant activity of a truncated *pil* ORF protein.

Disruption of *pil* does not affect *Y. pseudotuberculosis* virulence in a mouse oral infection model.

Since disruption of *pil* in the *pil::Tn* mutant results in increased T3SS activity and enhanced growth arrest under 37°C/low calcium conditions, we hypothesized that *pil::Tn* would display altered virulence in a mammalian host. To test this hypothesis, we orally infected mice with wildtype, *pil::Tn*, or Δpil *Y. pseudotuberculosis* via bread feeding [36]. We found no significant difference in colonization of any tissues by the

pil::Tn or Δpil strains relative to wildtype (Fig. 4A-D). These data suggest that growth arrest induced by T3SS activity may not play a significant role in tissue colonization.

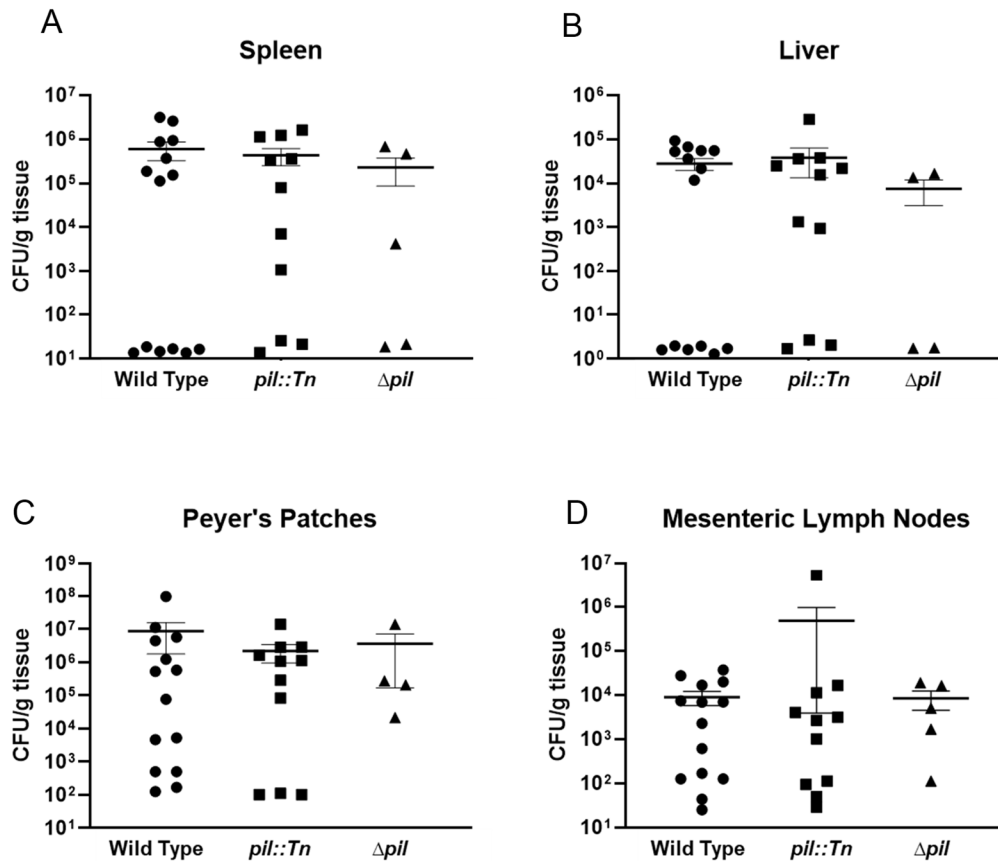


Figure 4. Insertion into the *pil* locus does not affect *Y. pseudotuberculosis* virulence in a mouse bread feeding model.

Mice were infected with $\sim 2 \times 10^8$ *Y. pseudotuberculosis* using a bread feeding model for oral infection. Mice were sacrificed 5 days post infection and organs were collected, homogenized, and plated to determine bacterial load as the CFU per gram (CFU/g) tissue. Shown is the CFU/g tissue with means depicted as horizontal bars and SEMs depicted as vertical bars for (A) spleens, (B) livers, (C) Peyer's patches, and (D) mesenteric lymph nodes of infected mice.

Ectopic expression of the *pil* ORF drives down pYV PCN and T3SS activity.

Since deletion of the *pil* ORF had no effect on the T3SS but insertional mutations did, we next sought to determine how ectopic expression of the native *pil*(1-122) ORF encoding all 122 amino acids of the predicted *pil* protein impacts pYV PCN and T3SS activity. IPTG-inducible induction of *pil* had no effect on pYV PCN at 26°C for either strain (Fig. 5A). In contrast, over-expression of *pil* at 37°C significantly reduced luminescence in both the wildtype and *pil::pNQ* *Y. pseudotuberculosis* strains, suggesting that high levels of *pil* expression drives down relative pYV PCN (Fig. 5A). Likewise, over-expression of the *pil* ORF at 37°C/-Ca²⁺ also led to loss of T3SS activity (Fig. 5B). In addition, *pil* over-expression had no impact on growth at 26°C, but led to enhanced growth at 37°C (Fig. 5C-D). This is consistent with decreased pYV PCN and T3SS activity at 37°C/-Ca²⁺ (Fig. 5A-B).

To determine if the effect of *pil* over-expression was due to expression of the putative *pil* ORF, we over-expressed a C-terminal pTrc-*pil*-6xHis construct. Induction of this construct with IPTG was found to drive down pYV PCN, consistent with what was previously observed for the untagged construct (Fig. 5B). A single band of the expected size for the full length (122 amino acid) Pil-6xHis protein was faintly detectable for the 26°C samples, while bands were much more intense for all 37°C samples, suggesting that Pil-6xHis steady-state levels are higher at 37°C compared to 26°C despite being exposed to IPTG for the same amount of time (Fig. 5E). When the same samples were probed for cytoplasmic YopE levels, we observed that YopE levels accumulate over the same time period when T3SS activity is decreased in response to *pil*-6xHis over expression (Fig. 5F).

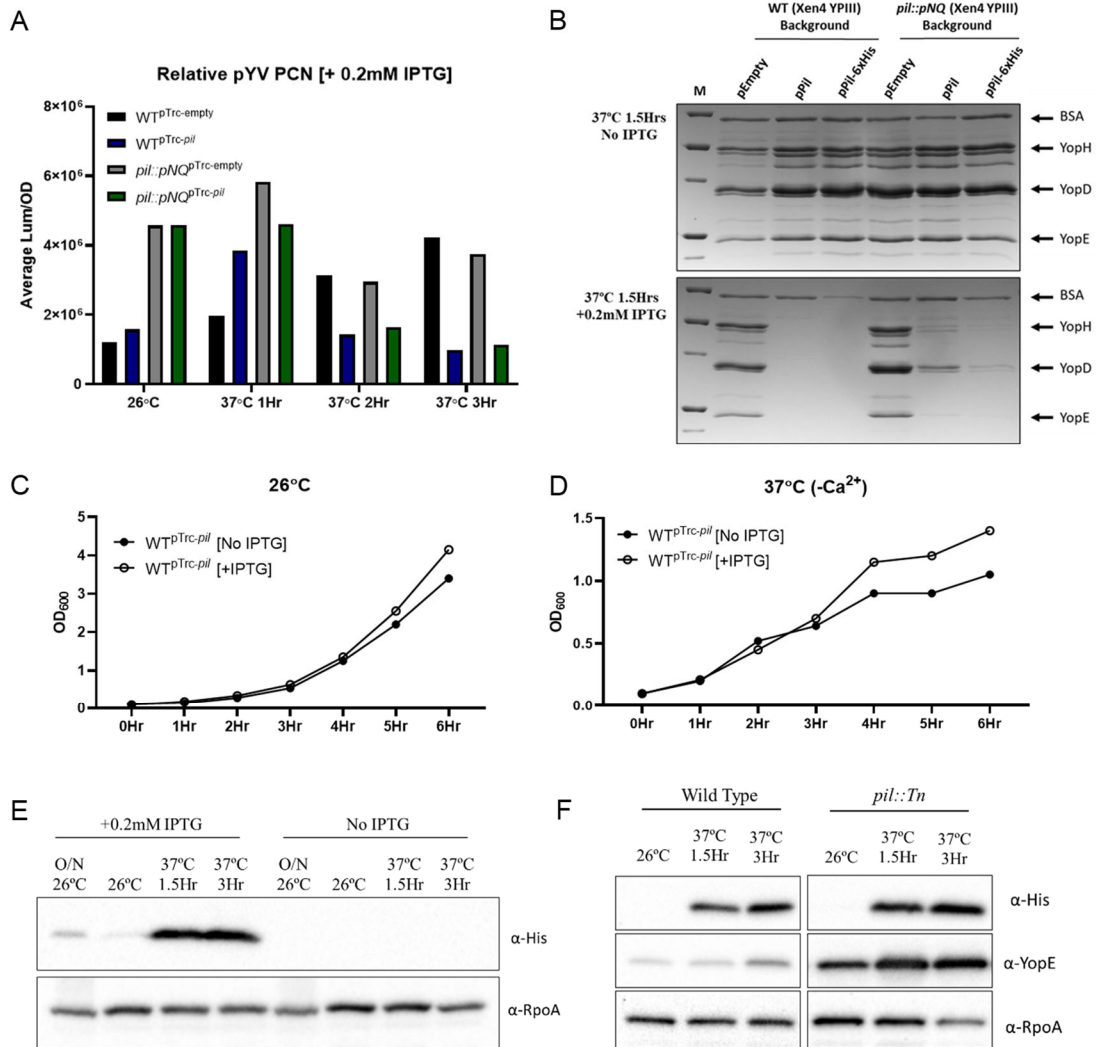


Figure 5. Ectopic over-expression of the *pil* locus decreases pYV PCN, T3SS activity, and improves *Yersinia* growth under T3SS-inducing conditions.

Wildtype and *pil::pNQ* cells were electroporated with an pTrc-empty vector control or pTrc-*pil*. Overexpression of *pil* was induced with addition of 0.2mM IPTG. **(A)** Relative luciferase PCN with or without *pil* overexpression in YPIII/pIBX strains. Data shown is representative of three biological replicates. **(B)** YPIII/pIBX strains were grown at 37°C/low calcium in the presence or absence of IPTG. Secreted proteins (Yops) were visualized using an *in vitro* secretion assay. Data shown is representative data of three biological replicates. **(C-D)** IP2666pIB1 strains were grown in plain 2xYT media at 26°C (C) or 2xYT low calcium media at 37°C (D). Data is representative of one biological replicate. **(E)** IP2666pIB1 strains were grown in low calcium media with or without IPTG before carrying out western blot analysis. Overnight samples (O/N) represent stationary phase at 26°C. Images shown represent a single blot that was cut and probed for Pil-6xHis and an RpoA loading control and is representative of three biological replicates. **(F)** IP2666pIB1 strains were grown in low calcium media at the indicated temperatures before analyzing by western blot. Images shown represent a single blot that was cut and probed for expression of Pil-6xHis, YopE, and an RpoA loading control and is representative of two biological replicates.

This suggests that over-expression of *pil* inhibits secretion of effector proteins, allowing them to build up in the cytoplasm. Since the *pil::Tn* mutant displays elevated pYV PCN (Fig. 2D) and over-expresses the *pil* locus (Fig. 3D), it was surprising that ectopic *pil* over expression drives down pYV PCN. Furthermore, it was unexpected that the hypothetical Pil protein is detected at 37°C given that the elevated PCN phenotype of the *pil::Tn* mutant occurs at 26°C.

We next expressed a series of *pil* ORF mutants in *Y. pseudotuberculosis* to determine the minimal region required for the phenotypes associated with Pil overexpression (Fig. 6A). All of the various mutations and truncations tested are summarized in Figure 6A and Table 2. The putative *pil* ORF has three possible start codons (M1, M14, M41) (Fig. 6A). We constructed *pil* over expression vectors that should begin translation at M14 or M42. Over expression of either the Pil(M14) or Pil(M42) constructs had no impact on pYV PCN or T3SS activity (Table 2). This suggests that M1 likely represents the start codon of the *pil* ORF and that the first 41 amino acids are important for the phenotypes observed when the *pil* ORF is overexpressed. However, overexpression of just the first 40 amino acids of the *pil* ORF had no impact on pYV PCN or T3SS activity (Table 1). In addition, truncation of the predicted Pil protein at amino acid 57 or 66 abolished any effect on PCN or T3SS activity when overexpressed, indicating that the C-terminal portion of the predicted protein also contributed to activity. In contrast, overexpression of a Pil construct with an early stop codon at position 99, Pil(1-99), drove down pYV PCN and T3SS activity similar to wildtype Pil(1-122) levels (Table 1 and Fig. 6B). Surprisingly, very little Pil(1-99) protein could be detected (Fig 6C). This finding suggests that either a very small amount of the Pil protein can decrease pYV PCN and T3SS activity, or that

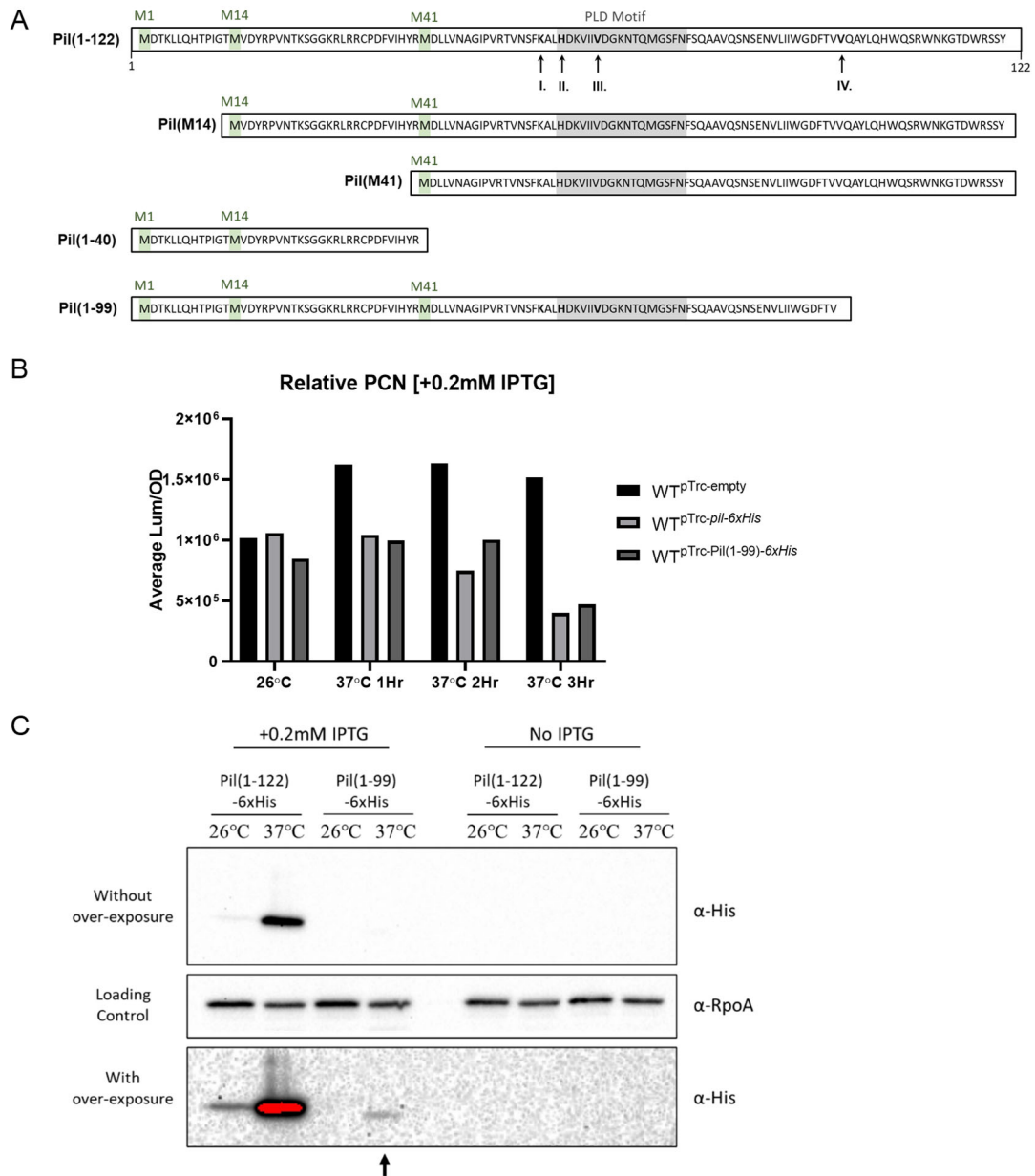


Figure 6. Ectopic over-expression of a *Pil*(1-99) allele drives down pYV PCN and produces little to no protein under T3SS-inducing conditions.

(A) Schematic showing a subset of the *pil* alleles tested in over-expression experiments **(B)** Wildtype YPIII/pIBX was electroporated with pTrc-pil, pTrc-Pil(1-99)-6xHis, or an empty vector control and relative PCN was estimated using the luciferase PCN assay. **(C)** Wildtype IP2666pIB1 was electroporated with pTrc99a containing full length Pil(1-122)-6xHis or truncated Pil(1-99)-6xHis. Strains were grown in low calcium media with or without IPTG before analyzing by western blot. Images shown represent a single blot that was cut and independently probed for expression of Pil-6xHis or RpoA as a loading control. The blot at the bottom represents a ~6minute over-exposure required to visualize Pil(1-99)-6xHis. All data shown in this figure is representative of at least two biological replicates.

Table 1. Summary of *pil* alleles tested by overexpression assays

<i>pil</i> allele:	PCN ¹ at 26°C	PCN ¹ at 37°C	Type III secretion ² at 37°C?	Protein ³ at 37°C?	Pil protein being expressed:
Pil	No Effect	Decreases PCN	Decreases T3S	Yes	Full length Pil (1-122 a.a.)
Pil-6xHis	No Effect	Decreases PCN	Decreases T3S	Yes	Full length Pil with a C terminal 6xHis tag.
Pil(H61A)	No Effect	Decreases PCN	Decreases T3S	N.D.	Full length Pil with an H61A missense mutation in the PLD motif.
Pil(M14)	No Effect	No Effect	No Effect	N.D.	Region encoding the first 13 a.a. is deleted so only M14-122 is expressed.
Pil(M41)	No Effect	No Effect	No Effect	N.D.	Region encoding the first 40 a.a. is deleted so only M41-122 is expressed.
Pil(1-40)	No Effect	No Effect	No Effect	N.D.	Contains the region encoding the first 40 a.a. of the Pil protein.
Pil(57STOP)	No Effect	No Effect	No Effect	N.D.	Full length Pil with a nonsense mutation introduced at position 58
Pil(66STOP)	No Effect	No Effect	No Effect	N.D.	Full length Pil with a nonsense mutation introduced at position 67
Pil(90STOP)	No Effect	Decreases PCN	Decreases T3S	N.D.	Full length Pil with a nonsense mutation introduced at position 90
Pil(1-99)-6xHis	No Effect	Decreases PCN	Decreases T3S	No ⁵	First 99 a.a. of Pil followed by a C terminal 6xHis tag.

¹Relative PCN was determined at 26°C and 37°C using a luciferase PCN assay

²Activity of the T3SS was determined using an in vitro secretion assay

³Protein expression for alleles with C-6xHis tags was determined by western blot analysis

⁴N.D. for has not been determined

⁵Produces nearly undetectable amounts of Pil protein

overexpression of the *pil* mRNA alone confers these phenotypes. Consistent with both of these possibilities, mutation of the predicted PLD motif had no effect on the ability of *pil* overexpression to decrease pYV PCN and T3SS activity. Histidine 61 in the HxKx(4)Dx(6)GSxN phospholipase D (PLD) motif has been shown to be essential for PLD endonuclease activity in Nuc from *E. coli* [37]. Over-expression of pTrc::Pil^{H61A} did not affect the phenotypes caused by Pil overexpression, suggesting that catalytic activity of the hypothetical Pil protein is not necessary to affect pYV PCN (Table 1).

Transcription of the *pil* locus in *Y. pseudotuberculosis*.

The *pil* locus is very poorly expressed in wildtype *Y. pseudotuberculosis*, although we detect more mRNA at 26°C compared to 37°C (Fig. 3F, Fig. 7C, and Supplementary Dataset 1). In addition, over-expression data suggests that while the *pil* locus can produce a 14 kDa protein at 37°C, production of this protein is not necessarily linked to the effect on pYV PCN and T3SS activity (Fig. 5 and 6). It is possible that the *pil* locus encodes a sRNA that is not captured by the RNA extraction method used for qPCR and RNA-seq analysis. To better understand what transcripts might be produced from this locus, we used bioinformatic analysis (BPPROM Softberry) to identify three putative s-70 promoters on the coding strand for the putative Pil protein (p1, p2 and p3) and four putative s-70 promoters encoded on the antisense strand but overlapping with the *pil* locus (pR1, pR2, pR3, and pR4 (Fig. 7A). We examined the ability of each promoter to induce a LacZ reporter. We found no significant β -gal activity for promoters p1 p2, p3, or pR1-pR4 (Fig 7B and data not

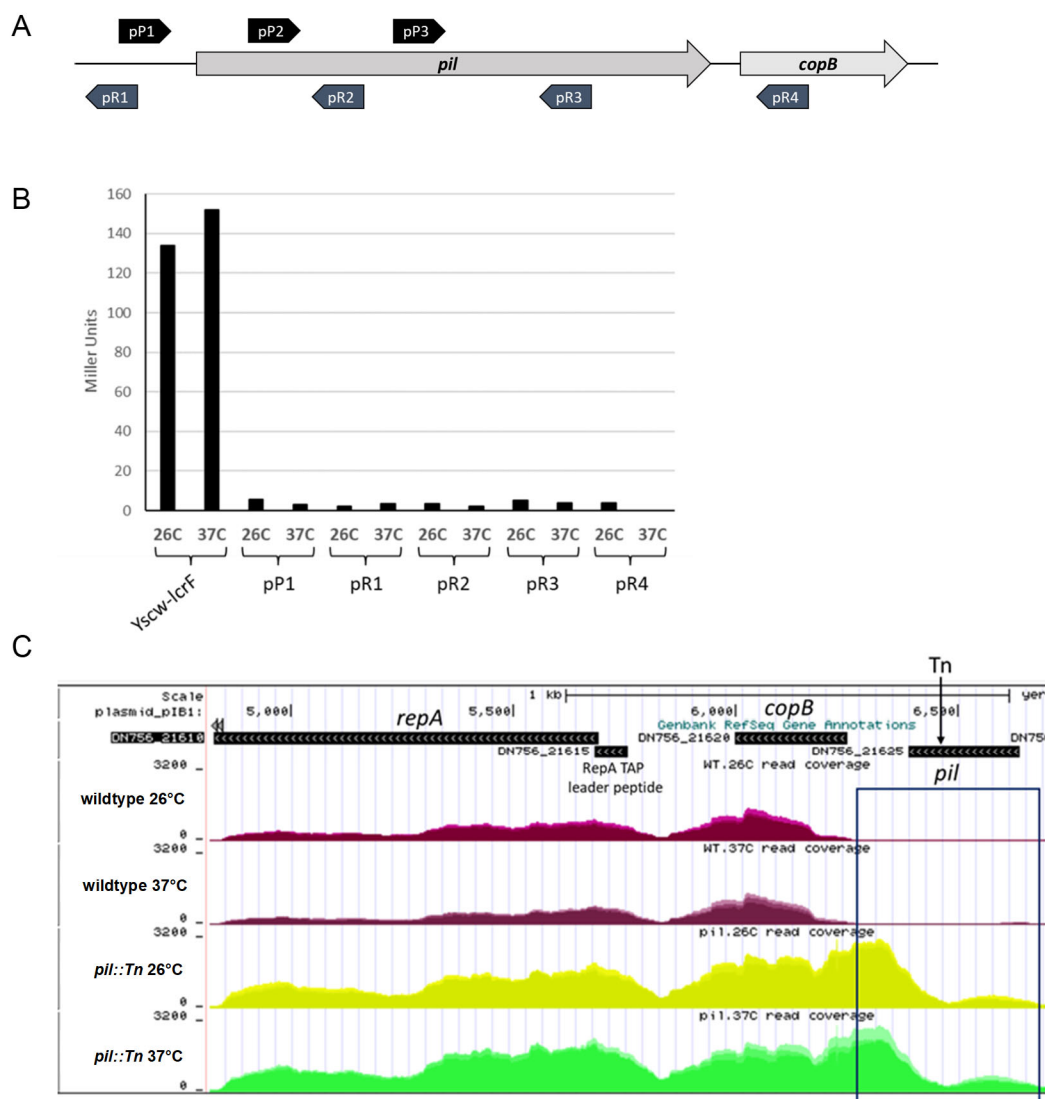


Figure 7. Bioinformatically predicted promoters within the *pil* locus show no apparent activity at 26°C or 37°C.

(A) Schematic showing the approximate locations of three predicted promoters in the sense direction (pP1, pP2, and pP3) and four predicted promoters encoded in the antisense direction (pR1, pR2, pR3, and pR4) in or near the *pil* locus. Putative Sigma7 promoters were predicted using Softberry BPRM analysis. **(B)** Strains in the IP2666pIB1 background were grown in low calcium media at 26°C or 37°C and promoter activity was determined as a function of Beta galactosidase activity which was measured in Miller units. A construct containing the yscW-lcrF promoter was included as a control. Data shown is representative of two biological replicates. **(C)** Reads from RNA-seq analysis mapped to the *cop-rep* locus and adjacent *pil* locus from wildtype and *pil::Tn* (*pil*) *Y. pseudotuberculosis* IP2666pIB1 grown at 26°C or 37°C (low calcium). The full length putative *pil* locus is indicated by a black box and the approximate location where the Tn insertion occurred is indicated with an arrow.

shown). Interestingly, the pattern of mapped reads from RNA-seq analysis is altered around the *pil* locus for *pil::Tn* relative to wildtype and irrespective of temperature (Fig. 7C). It is possible that *pil* is transcribed from a unique promoter in the *pil::Tn* mutant, giving rise to the over-expression of *pil* RNA observed for this strain (Fig. 3D and Supplemental Data File 1).

The *pil::Tn* mutant can be used as a tool for identifying factors impacting pYV PCN and T3SS activity in *Y. pseudotuberculosis*.

Insertion into the *pil* locus results in a significant growth defect under T3SS-inducing conditions (Fig. 2F-G). We observed that after prolonged incubation in T3SS-inducing conditions (~16 hours) *pil::Tn* and *pil::pNQ* *Y. pseudotuberculosis* mutants readily accumulate secondary mutations that allow some CFU to break growth arrest (Fig. 8A). Preliminary analysis of ~50 of these CFU found that many had acquired mutation(s) suppressing one or more of the parental phenotypes: poor growth at 37°C/-Ca²⁺, increased T3SS activity, and increased pYV PCN. We therefore decided to take advantage of the tendency of this mutant to generate suppressor mutations in order to identify other factors involved in regulation of *Y. pseudotuberculosis* pYV PCN. We carried out this screen in the *pil::pNQ* YPIII/pIBX background as it harbors a kanamycin resistance cassette that allows us to easily identify CFU that have lost the pYV plasmid during the selection process. Phenotypic screening of ~50 *pil::pNQ* suppressor mutant isolates revealed a variety of suppressor phenotypes with some affecting only growth, some affecting growth and secretion, and others affecting growth, secretion, and pYV PCN (Table 2). Suppressor mutants with differences in

pYV PCN and T3SS compared to the parental strain were subjected to whole genome sequencing to identify mutation(s) (Fig. 8A).

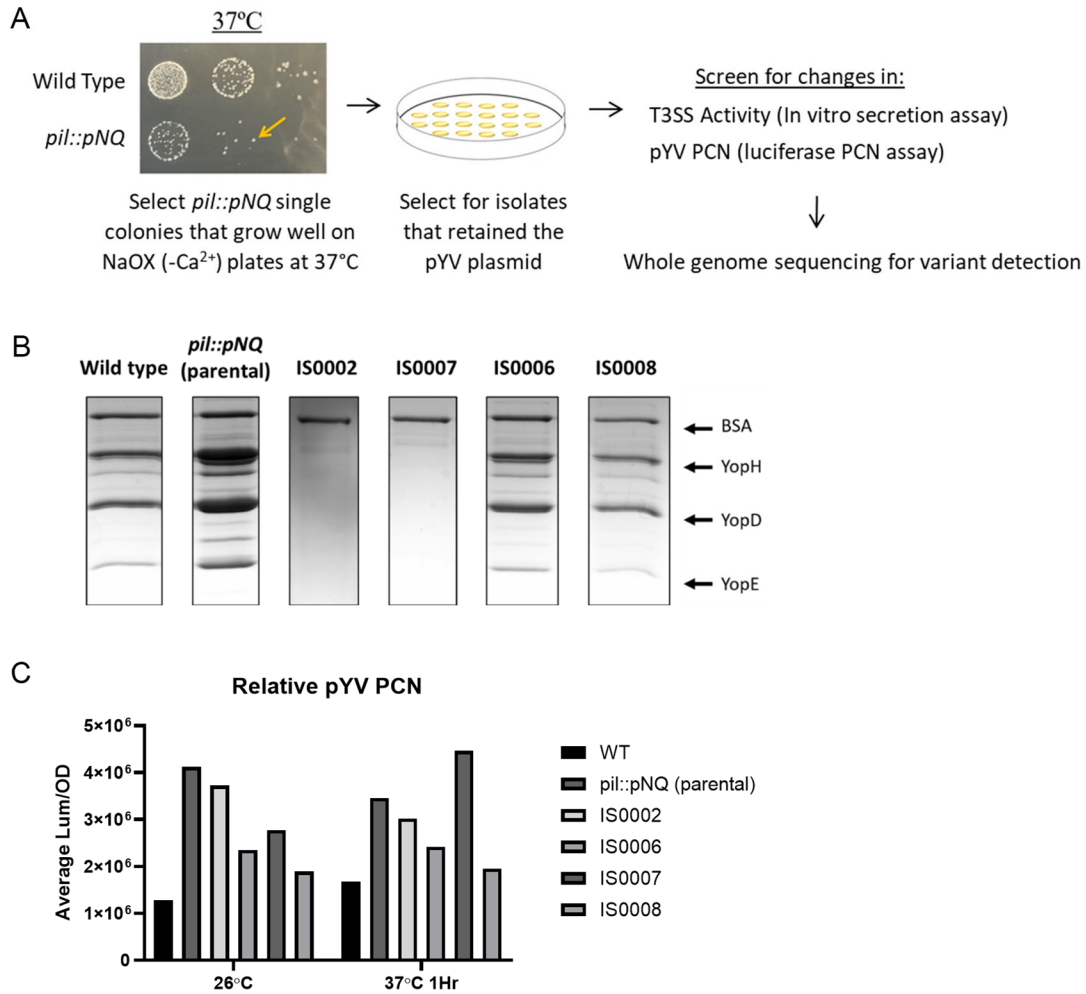


Figure 8. The *pil::pNQ* mutant readily produces suppressor mutations affecting T3SS activity and/or pYV PCN.

(A) Schematic representing the general work flow for induction of suppressor mutation formation and selection for suppressor isolates to generate a suppressor mutant library in the *pil::pNQ* YPIII/pIBX background. A potential suppressor isolate is indicated by an orange arrow. **(B)** YPIII/pIBX strains were grown at 37°C in low calcium media and secreted proteins (Yops) were precipitated and visualized by SDS-Page gel separation and Coomassie blue staining. Bovine serum albumin (BSA) was added as a loading and precipitation control. Data shown is representative data of three biological replicates. **(C)** Relative pYV PCN was estimated for YPIII/pIBX strains using a luciferase PCN assay. Data shown is representative of three biological replicates.

Table 2. Summary of suppressor isolates and identified mutations

Strain:	PCN 26°C ¹	PCN 37°C ¹	T3SS activity ³	Identified Mutation:	Comments:
pil::pNQ (parental)	High ²	High ²	High ²	N/A	
IS0001	Lower	Lower	Lower	N.D. ⁴	
IS0002	No Change	No change	No Activity	<u>T</u> G A → <u>G</u> G A mutation abolishing YscI stop codon	Predicted to decrease or abolish translation of YscI
IS0003	No Change	Lower	Lower	G → C mutation in tRNA-Gly	Unknown how this mutation impacts pYV PCN and/or T3SS activity.
IS0004	No Change	Lower	Lower	G → A mutation in <i>prepA</i> -35 region	Predicted to decrease <i>prepA</i> promoter firing
IS0005	No Change	No Change	Lower	N.D.	
IS0006	Lower	Lower	Lower	L291R mutation in PAP I	Mutation lowers PAP I protein levels at 37°C
IS0007	No Change	No Change	No Activity	K175E mutation abolishing the essential lysine in walker box A of YscN	Mutation predicted to impair YscN ATP binding and hydrolysis
IS0008	Lower	Lower	Lower	L291R mutation in PAP I	Mutation lowers PAP I protein levels at 37°C
IS0009	No Change	No Change	No Activity	N.D.	
IS0016	Lower	Lower	Lower	A272E in RNA Pol alpha factor	Mutation may impact RNA Pol binding to some promoters

¹ Relative pYV PCN was determined using a luciferase PCN assay

² Changes in pYV PCN and T3SS activity relative to the parental strain

³ T3SS activity was determined using an in vitro secretion assay

⁴ N.D. = Not Determined for isolates that did not have a mutation identified

We identified two suppressor mutant isolates (IS0002 and IS0007) that retained the increased pYV PCN phenotype of the *pil::pNQ* parental strain but completely lacked T3SS activity (Fig. 8B-C) (Table 2). One of these isolates, IS0002, was found to harbor a single nucleotide change that conferred a non-stop mutation in the *yscI* gene that encodes the T3SS inner rod protein and is essential for assembly of a functional T3SS. Another isolate, IS0007, was found to harbor a mutation in the *yscN* gene that conferred a lysine to glutamate missense mutation at position 175 (K175E). YscN is the ATPase that powers the T3SS and a mutation affecting YscN activity is expected to significantly impact T3SS function [38, 39]. While the K175 residue of YscN has not been reported as critical for YscN function in the literature as far as we know, it resides within a Walker motif and is predicted to be critical for ATP binding. We therefore hypothesize that the YscN^{K175E} mutation in the *pil::pNQ* background disrupted YscN ATPase activity, leading to the loss of T3SS activity and improved growth on low calcium media at 37°C (Fig. 8B-C). Together, this highlights the power of the *pil::pNQ* mutant as a genetic tool that can be used to identify genes and even specific residues important for T3SS function.

The suppressor screen in the *pil::pNQ* background also produced several isolates harboring mutations that affected pYV PCN. For example, one suppressor isolate (IS0004) displayed a partial rescue in pYV PCN and T3SS at 37°C (i.e. PCN and secretion was lower than the *pil::pNQ* parental but higher than wild type). Whole genome sequencing of IS0004 identified a G to A single nucleotide mutation in the -35 region of *prepA*, the promoter responsible for driving the majority of RepA expression (Table 2). Two additional suppressor mutants (IS0006 and IS0008) displayed reduced pYV PCN and T3SS activity to near wild type levels despite being

in the *pil::pNQ* mutant background. These isolates were found to harbor a mutation in the *pcnB* gene encoding the polyadenylase PAP I, which has been implicated in PCN control in *E. coli* but has never been identified as a PCN regulator in *Yersinia*. Chapter 3 of this dissertation describes the role of *pcnB* in regulating pYV PCN. For the rest of this chapter, we will refer to a mutant with a clean deletion of *pcnB* ($\Delta pcnB$). Briefly, we found that the $\Delta pcnB$ mutant displays low pYV PCN, pYV stability, and T3SS activity (see Chapter 3). These data suggest that suppression of pYV PCN by the *pcnB* suppressor mutation partially alleviated the strong growth arrest observed for the *pil::Tn* mutant at 37°C/-Ca²⁺.

RNA-seq analysis of *pil* insertion and $\Delta pcnB$ *Y. pseudotuberculosis* mutants.

To better understand the impacts of the *pil::Tn* and $\Delta pcnB$ mutations, we carried out RNA-seq analysis to measure global changes in mRNA levels following growth at 26°C or 37°C/-Ca²⁺. We identified 89 and 67 differentially expressed (DE) genes in $\Delta pcnB$ compared to wildtype at 26°C and 37°C, respectively (Supplementary Dataset 1). Both pYV-encoded and chromosomally-encoded genes were DE, supporting a global role for PAP I in gene expression regulation. The *pil::Tn* mutant displayed DE of 115 genes at 26°C and 184 genes at 37°C/-Ca²⁺ compared to wildtype (Supplementary Dataset 1). Many of the DE genes in the *pil::Tn* mutant were pYV-encoded genes and were overexpressed compared to wildtype, consistent with the high pYV PCN observed for this strain.

To visualize global changes in RNA levels, we carried out cluster analysis (Fig. 9A-B). At 26°C cluster 3 contains the majority of pYV-encoded genes. Overall,

we found that expression levels of pYV-encoded T3SS genes closely matched the pYV PCN and stability phenotypes of the various strains. For example, we found that the $\Delta pcnB$ mutant, which has reduced pYV PCN and stability, displays decreased expression of cluster 3 genes compared to wildtype at 26°C. In contrast, the *pil::Tn* mutant overexpressed cluster 3 genes, while the *pil::Tn*/ $\Delta pcnB$ double mutant had intermediate expression levels that were lower than the *pil::Tn* parental strain but higher than wildtype. Interestingly, the *pil::Tn* mutant was found to overexpress several T3SS genes at 26°C that are controlled by the transcription factor LcrF, which normally ensures that these genes are only expressed at 37°C. Indeed, the *pil::Tn* mutant was found to overexpress *lcrF* RNA at 26°C in our RNA-seq analysis and was also found to express a small amount of LcrF protein at 26°C by western blot analysis (Fig. 9E and Supplementary Data set 1). These data suggest that the high pYV PCN in the *pil::Tn* mutant increases pYV-encoded gene dosage and leads to partial derepression of T3SS gene expression at 26°C. This indicates that keeping pYV PCN low at environmental temperatures contributes to repression of the T3SS outside the mammalian host.

At 37°C, the majority of pYV-encoded genes are grouped into cluster 2 (Fig. 9B). At this temperature, we observed a smaller difference in T3SS gene expression between wildtype and $\Delta pcnB$ compared to what was seen at 26°C. Meanwhile, the *pil::Tn* mutant displays significantly higher expression of most pYV-encoded genes in cluster 2 compared to wild type at 37°C/-Ca²⁺. The *pil::Tn*/ $\Delta pcnB$ mutant was again found to have an intermediate expression profile of cluster 2 genes, indicative of an intermediate pYV PCN phenotype at this temperature.

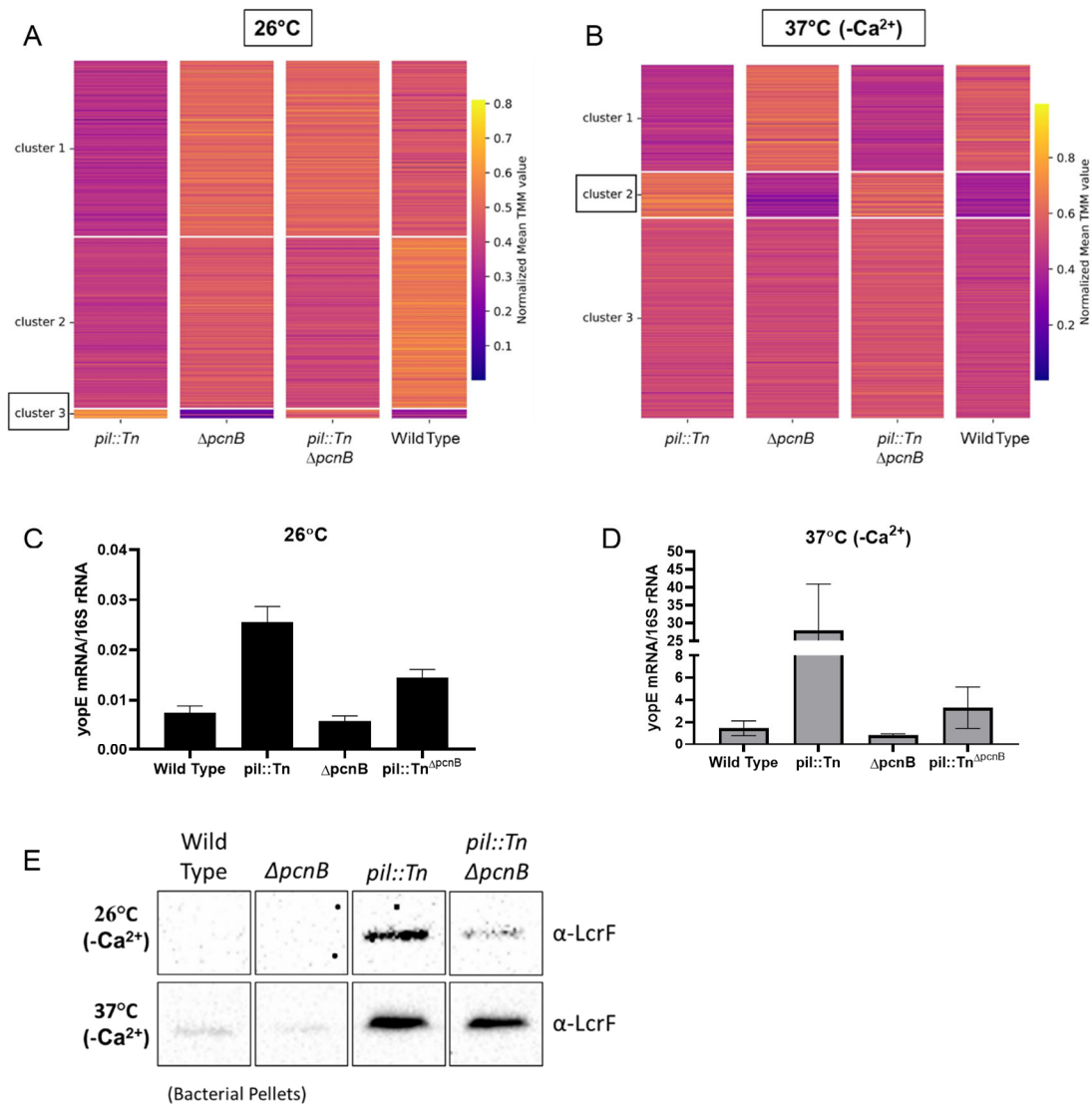


Figure 9. The *pil::Tn* mutant globally over-expresses pYV-encoded genes at 26°C/37°C

(A-B) Strains in the IP2666pIB1 background were grown in LB media at 26°C or LB low calcium media at 37°C before extracting RNA for RNA-seq and RT-qPCR analysis. At 26°C, the majority of pYV-encoded genes group into cluster 3 while at 37°C (low calcium) most pYV-encoded genes are grouped into cluster 2. Data shown is averaged from three biological replicates. **(C-D)** The same RNA samples used for RNA-seq in (A) were used to validate RNA-seq results using RT-qPCR. Depicted is validation of YopE transcript levels between different strains in the IP2666pIB1 background after growth at 26°C or 37°C (low calcium). Data shown is the average of three biological replicates. **(E)** Strains in the IP2666pIB1 background were grown at 26°C or 37°C (low calcium) before preparing samples for western blot and probing for LcrF expression. Images that are in the same row represent bands that were cropped from the same original blot/image. Data shown is representative of three biological replicates.

To validate our RNA-seq data, we carried out RT-qPCR using the same original RNA samples for a subset of genes, including *yopE* (Fig. 9C-D). The relative differences in *yopE* mRNA levels for these strains closely matched the PCN phenotypes previously observed. These data suggest that the *pil::Tn* insertion increases pYV PCN particularly at 26°C, increasing pYV-encoded gene dosage and subsequent transcription levels.

Discussion

In this study, we describe a previously unannotated locus near the highly conserved *cop-rep* locus of the *Y. pseudotuberculosis* pYV plasmid which we have named the plasmid copy number inhibitory locus (*pil*). This locus encodes a putative ORF with homology to PLD endonucleases, although our results suggest that this locus is not highly transcribed *in vitro*. However, insertion into the *pil* locus at amino acid 99 of the predicted *pil* ORF led to an increase in *pil-cop-rep* transcripts as well as higher pYV plasmid copy number (PCN). This correlated with increased expression and activity of the pYV-encoded type III secretion system (T3SS) and a more rapid growth arrest following induction of T3SS activity. Surprisingly, deletion of the *pil* ORF had no effect on pYV PCN or T3SS activity. In contrast, IPTG-inducible expression of transcripts encoding all or part of the *pil* ORF decreased pYV PCN but this did not correlate with *pil* ORF protein levels. Taken together, these data suggest that the *pil* locus is not normally transcribed, but disruption of this region allows abnormal transcription of a factor encoded within *pil* and disruption of pYV PCN homeostasis. The growth defect of the *pil::Tn* mutant provides a mechanism to

isolate suppressor mutations that impact T3SS function or pYV replication or stability.

The *pil* locus is bioinformatically predicted to encode a 122 amino acid protein with a single copy of a highly conserved phospholipase D (PLD) motif that is indicative of bacterial endonucleases such as Nuc from *E. coli* [33, 34]. Analysis using BLAST and multiple sequence alignment identified several Pil orthologues in other species of Gram negative bacteria and at least some of these are plasmid-encoded and located near the plasmid *cop-rep* locus. For example, the pKF3-94 plasmid found in some species of *Klebsiella pneumoniae* contain *repA*, *copB*, and CopA orthologues and have a *pil* locus located just upstream of the *copB* gene similar to the pYV plasmid. The *pil* locus may therefore represent a regulatory element found near the *cop-rep* locus of several naturally occurring bacterial plasmids. However, very little *pil* mRNA was detected in wildtype *Y.*

pseudotuberculosis and no promoters could be detected that were active *in vitro*. In addition, deletion of the entire *pil* ORF had no impact on pYV PCN, T3SS activity, or *Yersinia* virulence. As both Tn5 and pNQ insertions into the same location within the *pil* ORF led to similar phenotypes in two different strain backgrounds, we conclude that the phenotypes of the *pil::Tn* strain are not due to the nature of the Tn5 insertion itself but rather a disruption of this locus. In fact, we were not able to isolate most mutations that we attempted to recombine into the *pil* native locus (data not shown).

Surprisingly, ectopic overexpression of RNA containing the *pil* ORF drove down pYV PCN but only at 37°C where it also drives down activity of the T3SS. This was unexpected given that the *pil* locus is expressed more at 26°C in wildtype *Y. pseudotuberculosis* and the PCN defect of the *pil::Tn* mutant occurs specifically at

26°C. While this suggests that a *pil* RNA product provided in *trans* disrupts pYV PCN homeostasis, the effect is different when *pil* is disrupted in *cis*. The mechanism behind both of these phenotypes remains unclear. However, it is interesting to note that the RNA-seq pattern of *rep-cop-pil* locus in the *pil::Tn* mutant is different from wildtype. It is possible that expression of RepA, CopB, and CopA is perturbed when the *pil* locus is disrupted in *trans*. In fact, preliminary Northern blot analysis showed that *repA* and *copB* mRNA levels were increased specifically at 26°C in the *pil::pNQ* mutant compared to wildtype (data not shown).

By making a series of truncations and mutations, we found that the region of the *pil* locus that encodes the first 99 amino acids of the predicted Pil protein is required to drive down pYV PCN and T3SS activity upon *pil* over-expression. This region is also overexpressed in the *pil::Tn* mutant, but apparently with significant differences in terms of the consequence on pYV PCN. Ectopic overexpression experiments also found that *pil* activity can be uncoupled from the PLD motif and expression of a Pil protein, again supporting that the *pil* locus does not affect pYV PCN via production of a protein. We found that the region of the *pil* ORF predicted to encode the first 13 amino acids of the putative Pil protein is required to drive down pYV PCN upon over-expression. This region of *pil* is not conserved in Pil orthologues from other Gram negative bacteria but is 100% conserved between *Y. pseudotuberculosis* and *Y. pestis*. Since *pil* over-expression drives down pYV PCN independently of protein production, it is possible that this *Yersinia*-specific region represents a DNA sequence that encodes an active RNA molecule or alters DNA topology in *cis*. Interestingly, this is the region of the *pil* locus that is duplicated in the pYV/pCD1 plasmids of *Y. pseudotuberculosis* and *Y. pestis*. It is possible that a

recombination event led to duplication of this region and gave rise to the divergent 5' end of the predicted *pil* region. Indeed, the *pil* locus is flanked by upstream transposase genes and a transposase remnant suggestive of a historical recombination event near the *cop-rep* locus where *pil* is presently located.

One hypothesis for the role of *pil* is that the locus may represent a binding site for a factor that regulates pYV topology and/or expression of the adjacent *cop-rep* locus. There is existing evidence that the pYV plasmid undergoes topological changes upon shifting from 26°C to 37°C [40], and it is possible that these topological changes are important for regulating expression of T3SS and/or *cop-rep* locus genes. Indeed, RNA-seq analysis found that the *pil::Tn* mutant overexpresses nearly the entire pYV plasmid, which might be explained by a global change in pYV plasmid topology, and the fact that a region of the *pil* DNA sequence is duplicated in the pYV plasmid may hold additional implications for its role in regulating pYV topology. Bacteria express several factors that can regulate DNA structure and topology including the histone-like nucleoid structuring protein (H-NS) [41-44]. The pYV plasmid encodes several putative H-NS binding sites, some of which are known to directly regulate transcription of T3SS and other virulence genes [11, 45]. A preliminary experiment where H-NS expression was knocked down using a CRISPRi system found evidence that loss of H-NS increases expression of many loci around the pYV plasmid at 26°C including T3SS genes, several other pYV-encoded genes, and the *pil* locus (data not shown). Future studies to examine the topology of the pYV plasmid are likely to not only increase our understanding of the *pil::Tn* mutant but will also greatly improve our understanding of how the pYV plasmid responds to different temperatures in human pathogenic *Yersinia*.

In addition to exploring the role and mechanism of *pil*, we also describe in this work how the *pil::pNQ* mutant can be used to identify other factors regulating pYV PCN. While working with *pil* insertion strains, we noted that these mutants were particularly susceptible to forming secondary mutations under T3SS-inducing conditions that allowed certain CFU to break growth arrest, suggesting that they had acquired mutations affecting pYV PCN and/or the T3SS. By isolating these suppressor mutants and sequencing their genomes, we identified mutations in several genes impacting regulation of the pYV plasmid and/or function of the T3SS. Mutation of one gene in particular, *pcnB*, decreases pYV PCN in the *pil::pNQ*, *pil::Tn*, and wildtype *Y. pseudotuberculosis* backgrounds. This gene is the focus of Chapter 3. Interestingly, when we subjected wildtype YPIII/plBX *Y. pseudotuberculosis* to the same suppressor selection process used for the *pil::pNQ* mutant, we observed that cells would occasionally lose the pYV plasmid during the selection process but found no evidence of suppressor mutations being formed in the wildtype background (data not shown). In contrast, while the *pil::pNQ* mutant readily formed suppressor mutations, we never isolated clones that lost the pYV plasmid. This may be due to overexpression of the putative *parDE* toxin-antitoxin system in the *pil::pNQ* background, which is expected to stabilize the pYV plasmid by killing plasmidless daughter cells [46]. This makes the *pil::pNQ* background a particularly strong tool for identifying genes related to pYV PCN as it experiences significant selective pressure to escape the high pYV PCN under T3SS inducing conditions but is simultaneously unable to drop the pYV plasmid to escape this selective pressure. Interestingly, we did not identify any mutations in the *pil* locus itself through our suppressor screen, supporting the notion that the *pil::pNQ* mutant phenotypes are not due to expression

a specific factor such as a regulatory RNA or protein whose function could be affected by a secondary mutation.

Overall, the underlying cause of the increased pYV PCN displayed by the *pil::Tn/pNQ* mutant remains unclear despite extensive efforts. However, the *pil::Tn* mutant teaches us several important lessons about pYV PCN regulation in pathogenic *Yersinia*. The growth defect of the *pil::Tn* and *pil::pNQ* strains suggests that the reason *Y. pseudotuberculosis* normally keeps pYV PCN low at 26°C is to optimize growth in the environment and enforce proper regulation of T3SS activity. Furthermore, the finding that the *pil::Tn* mutant, but not wild type, expresses a detectable amount of LcrF protein at 26°C suggests that the RNA thermometer of the *lcrF* transcript is not strong enough to completely block LcrF expression when pYV gene dosage is high. Regulation of T3SS gene dosage is therefore just as critical for *Y. pseudotuberculosis* to keep gene expression low at 26°C as it is for promoting gene expression at 37°C. Finally, the *pil::Tn* and *pil::pNQ* mutants represent valuable novel tools for pYV PCN studies in *Y. pseudotuberculosis* regardless of its underlying molecular mechanism.

Materials & Methods

Bacterial strains, Plasmids, and Growth Conditions: All strains and plasmids used in this study are listed in Tables 3 and 5, respectively. *Y. pseudotuberculosis* strains were grown in LB or 2xYT media (pH=7) at 26°C or 37°C shaking at 250rpm, unless otherwise indicated.

Construction of *Y. pseudotuberculosis* mutant strains: All primers and plasmids used to generate *Y. pseudotuberculosis* strains for this study are listed in Tables 4 and 5, respectively. Inserts were amplified by PCR using primers designed for Gibson Assembly and final recombinant plasmids were all made using the pCVD442 and/or pSR47S suicide vector backbones (λ pir-dependent replicon, *sacB* gene conferring sucrose sensitivity) (Addgene). All ligations were performed using NEBuilder HiFi DNA Assembly kit (New England Biolabs, Inc.). The original 823B and *pil::Tn* *Y. pseudotuberculosis* IP2666pIB1 mutants were previously generated as part of a global screen for genes impacting T3SS activity [32]. The *pil::pNQ* *Y. pseudotuberculosis* YPIII/pIBX mutant was independently generated and gifted to us by Dr. Helen Wang (Uppsala University, Sweden). To make the Δpil and $\Delta pcnB$ mutations in their native genetic loci, homology regions spanning ~500bp upstream and ~500bp downstream of the gene/locus were independently PCR amplified from *Y. pseudotuberculosis* IP2666pIB1 genomic DNA and ligated into pCVD442 [Carb^R] that was previously linearized by digestion with NdeI and BbsI. The inserts to make Δpil were also cloned into pSR47s [Kan^R] previously digested with BamHI and SacI. Recombinant pCVD442/pSR47S plasmids were transformed into *E. coli* S17-1 λ pir competent cells and introduced into *Y. pseudotuberculosis* IP2666pIB1 or YPIII/pIBX via conjugation. Clones that were Carb^R (for pCVD442) or Kan^R (for pSR47s) and irgansan-resistant (*Yersinia* selective antibiotic) were grown in the absence of antibiotics and plated onto media containing 10% sucrose to induce *sacB*-mediate toxicity for pCVD442 loss. Clones that were Carb sensitive (for pCVD442) or Kan sensitive (for pSR47s), sucrose-resistant, and Congo red-positive (pYV+) were screened by PCR and sequence verified by Sangar sequencing.

Construction of overexpression constructs: All primers and plasmids used to generate *Y. pseudotuberculosis* strains for this study are listed in Tables 4 and 5, respectively. To over-express full length Pil, the region of the *pil* locus that encodes the putative ORF was PCR amplified and ligated into the IPTG-inducible over-expression vector pTrc99a [Carb^R] after linearizing with NcoI and XbaI. Constructs for over-expressing truncated *pil* alleles [Pil(M14), Pil(M41), and Pil(1-99)] were made by PCR amplifying the corresponding region of the *pil* locus and inserting into digested pTrc99a. Point mutations to introduce residue changes or early stop codons were introduced using a Q5 site-directed mutagenesis kit (New England Biolabs, Inc.) according to manufacturer's guidelines. To generate over-expression vectors that include a C terminal 6xHis tag, the region of the *pil* locus encoding amino acids 1-122 or 1-99 of the putative Pil protein was PCR amplified from *Y. pseudotuberculosis* genomic DNA. The 6xHis tag was amplified independently from pET28 and the resulting *pil* and 6xHis fragments were cloned into pTrc99a. All ligations were performed using NEBuilder HiFi DNA Assembly kit (New England Biolabs, Inc.) and all recombinant plasmids were propagated in DH5α *E. coli* before introducing to *Y. pseudotuberculosis* IP2666pIB1 and/or YPIII/pIBX via electroporation and selection on antibiotic plates.

Induction of *pil* overexpression: *Y. pseudotuberculosis* strains electroporated with IPTG-inducible promoter fusion constructs in the pTrc99 vector backbone were grown at the indicated temperatures in the presence or absence of IPTG. Strains

electroporated with empty vector constructs were used as controls and over-expression was induced by the addition of 0.2mM IPTG.

Secretion Assay: Visualization of secreted T3SS cargo to assess T3SS activity was carried out as previously described [61]. *Y. pseudotuberculosis* cultures were grown shaking overnight at 26°C before subculturing in LB low calcium media (LB containing 20mM sodium oxalate and 20mM MgCl₂). Cultures were grown shaking at 26°C for 1.5h before shifting to 37°C to grow shaking for an additional 1.5h. Cultures were normalized to the same number of cells using OD₆₀₀ and pelleted at 13,200 rpm for 15min at room temperature. Supernatants were transferred to new tubes and proteins precipitated with 10% trichloroacetic acid (TCA). A BSA protein control was spiked into each sample immediately before the addition of TCA. Samples were incubated in TCA on ice for at least 1h before pelleting at 13,200 rpm for 15 min at 4°C. Pellets were then washed twice with ice-cold 100% acetone and resuspended in final sample buffer (FSB) containing 0.2M dithiothreitol (DTT). Samples were boiled for 10 mins at 95°C before loading and separating on a 12.5% SDS-PAGE gel. Gels were stained with Coomassie blue to visualize protein and imaged using Bio-Rad Image Lab Software Quantity and Analysis tools.

Western Blot Analysis: Cells were grown at the indicated temperature before preparing samples for Western blotting. Samples were normalized to the same number of cells using OD₆₀₀ at the time of sample collection. Bacterial pellets were then resuspended in FSB containing 0.2M DTT and boiled at 95°C for 15 minutes.

Secreted proteins were precipitated using TCA in the same way as described for the in vitro secretion assay above. The same volume of each sample was separated on a 12.5% SDS-PAGE gel and proteins were transferred to a blotting membrane (Immunobilon-P) using a wet mini trans-blot cell (Bio-Rad) at 4°C. Blots were blocked for one hour in Tris-buffered saline containing Tween 20 and 5% milk. Blots were probed with anti-RpoA (gift from Melanie Marketon), goat anti-YopE (Santa Cruz Biotech), mouse anti-His Tag (Santa Cruz Biotech), rabbit anti-LcrF (gift from Gregory Plano), and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech). Bands were visualized using a BioRad ChemiDoc and quantification analysis was performed using Image Lab software (Bio-Rad).

Luciferase plasmid copy number (PCN) assay: Overnight cultures were grown shaking at 26°C before subculturing to $OD_{600}=0.2$ in 2xYT low calcium media (containing 20mM sodium oxalate and 20mM $MgCl_2$). Cultures were then grown shaking for 1.5h at 26°C before shifting to 37°C. Timepoints to measure luminescence and OD_{600} were collected after 1.5h at 26°C just before shifting to 37°C and every hour after shifting to 37°C. Samples were collected at each timepoint in white walled, clear bottom 96 well plates and luminescence (using lum aperture for 96 well plate with measurement time=0.1s) and OD_{600} (using Photometric 600 excitation filter) was measured using a PerkinElmer Multimode Plate Reader EnVision system. Relative plasmid copy number was determined by dividing luminescence by OD_{600} for the same sample. Biological replicates were graphed and statistical analysis was performed using GraphPad Prism 8 (10.2.0).

Droplet digital PCR (ddPCR): ddPCR to determine pYV plasmid copy number was carried out as described by Schneiders et al 2021. Total DNA was extracted from bacterial cells using GeneJet Genomic DNA Purification kit (Thermo Scientific) according to the manufacturer's recommendations for Gram negative bacteria. The final concentration of DNA was determined by nanodropping before setting up samples for ddPCR. Each gDNA sample was split into two reactions and the primer pair F_chrom_ddPCR & R_chrom_ddPCR was used for to amplify chromosomal DNA and F_pYV_ddPCR & R_pYV_ddPCR was used to amplify pYV DNA. Individual ddPCR samples were prepared as follows: 10.5uL of total genomic DNA was combined with 12.5uL of 2xEVA-Green master mix for unlabeled probes (Bio-Rad), 1uL of HindIII-HF (NEB), and 1uL of respective primer pairs (200nM final concentration) in 8-strip PCR tubes. PCR tubes were incubated at room temperature for 10 minutes to allow HindIII to digest genomic DNA. Droplet generation was carried out using an Automated Droplet Generator (Bio-Rad) and droplets were subsequently transferred to a deep-dish qPCR plate (Bio-Rad) for PCR amplification. PCR was carried out with hot-start activation at 95°C for 5 mins following by denaturation at 94°C for 30s, amplification at 58°C for 1 min over 40 cycles, and signal stabilization at 4°C for 10 min and 90°C for 5 min. A ramp rate of 2C/s was used for all PCR steps. Droplets were analyzed using a QX200 droplet reader (Bio-Rad) and data was analyzed using QuantaSoft Analysis Standard Edition (version 1.2) (Bio-Rad).

Growth curves: *Y. pseudotuberculosis* strains were cultured overnight in LB or 2xYT media at 26°C and subcultured to OD₆₀₀ = 0.1 the following morning in 20-25mL of either regular media or low calcium media (containing 20mM sodium oxalate and 20mM MgCl₂). Cultures were then grown shaking at 250rpm at either 26°C or 37°C and optical density at 600nm was measured every hour for 5-8 hours.

Comparative Genomics, Alignments, and Bioinformatic Analysis of *pil*

Identification of *Pil* homologs/orthologs: *Pil* homologs and orthologues were identified using the protein Basic Local Alignment Search Tool (BLAST) available through the National Library of Medicine and alignments were generated using Clustal Omega available through EMBL-EBI [47]. Alignments comparing *Pil* homologs from the three species of human pathogenic *Yersinia* were generated using *Pil* [AZA32661.1] from the pYV plasmid [CP032567] of *Y. pseudotuberculosis* IP2666pIB1, *Pil* [not annotated] from pCD plasmid [CP009835] of *Y. pestis* Kim5, and *Pil* [WP_016266442] from pYVe8081 plasmid of *Y. enterocolitica* 8081. Alignments between orthologues from other species of Gram negative bacteria were generated using *Pil* from *Y. pseudotuberculosis* [AZA32661.1], *Escherichia coli* [WP_016266442], *Salmonella enterica* [ECH9336700], and *Pil* from the *Klebsiella pneumoniae* plasmid pKF3-94 [WP_013609507.1]. Identification of putative *pil* sigma 70 promoters: Potential sigma 70 (σ^{70}) promoters within or near *pil* were predicted using the SoftBerry Prediction of bacterial promoters (BPRM) σ^{70} promoter recognition program.

Spot Tests: Overnight cultures were pelleted at 6000rpm and resuspended in PBS (pH=7.4) before measuring OD₆₀₀. Samples were then diluted to OD₆₀₀ = 0.4 in PBS and used to make a series of 1:10 dilutions in 1mL PBS. Dilutions were spotted onto the following plates: LB only plates, NaOX plates (LB containing 20mM NaOX and 20mM MgCl₂) and/or congo red plates (NaOX plates containing 1% congo red dye). Plates were incubated at their respective temperatures for ~16 hours before imaging.

Mouse Infections: Mouse infections were carried out using the bread feeding model for oral *Yersinia* infection [36]. In brief, LB *Y. pseudotuberculosis* cultures were grown shaking at 250rpm at 26°C overnight before using to inoculate 126S1/SvlmJ mice (Jackson Laboratories) with 2x10⁸ bacterial cells via bread feeding. Mice were sacrificed five days post inoculation and Peyer's patches, mesenteric lymph nodes, spleens, and livers were isolated, homogenized, and plated to determine CFU per gram (CFU/g) tissue.

Beta-Galactosidase Assays: *Y. pseudotuberculosis* strains harboring promoter-lacZ fusion plasmids in the pFU99 vector backbone were grown in 2xYT low calcium media (2xYT plus 20mM NaOX and 20mM MgCl₂) for 3h at 26°C or for 1.5h at 26°C followed by growth at 37°C for 1.5h. Samples were prepared and β-galactosidase activity was measured as described in Miller 1972. In brief, cultures were incubated on ice to stop protein synthesis after incubation, spun down, and resuspended in Z buffer before adding chloroform and 0.1% sodium dodecyl sulfate to permeabilize membranes. Samples were then incubated with 0.8mg/mL ONPG to initiate the

reaction and 1M sodium bicarbonate was subsequently added to terminate β -galactosidase enzymatic activity. B-galactosidase activity is reported as miller units, which were calculated as described in Miller 1972.

In vitro Suppressor Screen: LB cultures for the *pil::Tn* YPIII/pIBX YPIII mutant were grown at 26°C shaking overnight before diluting in fresh media and spotting for single colonies onto low calcium NaOX plates (LB plate containing 20mM sodium oxalate and 20mM MgCl₂). Plates were incubated at 37°C for approximately 16 hours to induce T3SS expression and selective pressure for suppressor mutation formation. CFU that broke growth arrest and thus formed large colonies were selected and glycerol stocks were made to construct a *pil::Tn* suppressor mutant library. High throughput screening for changes in pYV PCN was carried out using the luciferase PCR assay (described below). Suppressor mutants were screened for changes in T3SS activity using an in vitro secretion assay (see below).

Identification of Mutations from Suppressor Screen: A subset of suppressor mutants from the *pil::Tn* YPIII/pIBX suppressor mutant library were selected for whole genome sequencing. In brief, cells were grown shaking at 26°C overnight and total genomic DNA was extracted using the Blood & Tissue DNeasy kit (Qiagen) according to manufacturer recommendations. Final genomic DNA concentration was determined by nanodrop. Qbit quantification, Hiseq single read 150 (Hiseq 4000 SR150) library prep, and illumina sequencing was carried out by the UC Davis DNA Technologies Core. Adapters and low-quality reads were trimmed by trimmomatic (Version 0.39) [48]. The trimmed sequences from each sample were aligned to the

reference genome using BWA [49]. The reference sequence is composed of the YPIII chromosome (CP009792.1) [50] and its plasmid (CP032567.1) [51]. Samtools was used to sort and index the aligned reads [52]. Duplicate reads originated from a single DNA fragment during sample preparation were located and tagged by MarkDuplicates in Picard (<http://broadinstitute.github.io/picard/>). Single nucleotide polymorphisms were detected and joined by GATK (Version 4.1.6).

RNA Isolation and RNAseq Analysis: *Y. pseudotuberculosis* strains were grown in 3mL LB overnight at 26°C and diluted the following morning to an OD₆₀₀ of 0.2 in 5mL fresh LB. Each strain was diluted into two separate cultures (one for each temperature). For 26°C samples, cultures were diluted in plain LB media and grown shaking at 26°C for 3h before extracting RNA. For 37°C samples, cultures were diluted into LB low calcium media (containing 20mM sodium oxalate and 20mM MgCl₂) and grown shaking at 26°C for 1.5h before shifting to 37°C to growing shaking for an additional 1.5 hours. After incubating, 1-2mL of each culture was collected and pelleted for 5 min at 4,000 rpm. Supernatants were removed and cell pellets were resuspended in 1mL RNA Protect (Qiagen) and total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer's protocol. DNA was removed from samples using the TURBO DNA-free kit (Life Technologies/Thermo Fischer) and RNA quality was determined using an Agilent 2000 Bioanalyzer. Illumina library prep and differential expression analysis was performed by Seqcenter, LLC. To generate heat maps, average TMM (Trimmed Mean of the M-Values) were utilized as the raw input data for the clustering pipeline. Subsequently, L2 normalization was applied to the dataset to ensure consistent

scaling prior to the application of the elbow method and K-means clustering. The elbow method was then employed to find the optimal number of clusters required for the subsequent K-means clustering step. Once the clusters were successfully generated, a heatmap of the clusters were visualized using matplotlib and seaborn which are Python visualization packages. Hub tracks from this analysis can be viewed using the following link:

http://lowelab.ucsc.edu/hubs/yersinia_pcnB_pil_expression/hub.txt

Quantitative PCR (qPCR) Analysis: All primers used for qPCR analysis are listed in Table 4. *Y. pseudotuberculosis* strains were grown at the indicated temperatures/conditions before pelleting 1mL of culture at 4000rpm for 5min. Supernatants were removed and cell pellets were resuspended in 1mL of Bacterial RNA Protect (Qiagen) according to manufacturer's recommendations. Total RNA was isolated using an RNeasy minikit (Qiagen) according to manufacturer's protocol. Genomic DNA was removed using the TURBO-DNA-Free kit (Life Technologies/Thermo Fisher) and Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) was used to generate cDNA libraries as previously described [34]. Target gene levels were measured using SYBR green PCR master mix (Thermo Fisher Scientific) and expression levels were calculated using the $\Delta\Delta C_T$ method. Final calculated expression levels for each gene was normalized to 16S rRNA expression levels from the same sample. Graphs and statistical analyses were performed using GraphPad Prism 10 (10.2.0).

In vitro plasmid stability assay: Cells were streaked for single colonies onto LB plates and grown at 26°C for 1.5-2 days. To test plasmid stability after passing at 26°C only, single colonies were then patched onto LB plates for ~16 hours at 26°C before heavily streaking for single colonies onto fresh LB plates at 26°C for 1.5-2 days. To test plasmid stability after exposure to T3SS-inducing conditions, the same colonies were also patched onto NaOX plates to induce T3SS activity for ~16 hours at 37°C before streaking for single colonies onto LB plates and returning to 26°C to grow for 1.5-2 days. Final plasmid stability was determined for both conditions by patching single colonies from the final 26°C streak plate onto NaOX plates containing 1% congo red. The percentage of cells that retained the pYV plasmid was calculated by dividing the number of red (pYV+) CFU by the total number of CFU assessed. Single colony PCR was used on a subset of patches to validate that red CFU retain pYV and while white CFU do not (data not shown).

Table 3. *Y. pseudotuberculosis* strains used in this study

Strain	Background	Mutation(s)	Ref
Wildtype	IP2666pIB1	Naturally lacks full-length YopT	[53]
$\Delta yopHEMOJ$	IP2666pIB1	$\Delta yopHEMOJ$	[54]
$\Delta yscNU$	IP2666pIB1	$\Delta yscNU$	[54]
823B	IP2666pIB1	Tn5 insertion in <i>pil</i> , $\Delta yopEMOJ$ background	[32]
pYV-	IP2666pIB1	Plasmid cured wildtype	[54]
<i>pil::Tn</i>	IP2666pIB1	Tn insertion in <i>pil</i>	[32]
Δpil	IP2666pIB1	Δpil	This work
$\Delta pcnB$	IP2666pIB1	$\Delta pcnB$	This work
<i>pil::Tn</i> $\Delta pcnB$	IP2666pIB1	$\Delta pcnB$ in <i>pil::Tn</i> background	This work
Wildtype	YP111 pIBX	Contains <i>lux</i> reporter in pYV	[14]
YP111:(cIBX) _{n=1}	YP111 pIBX	Single copy of pYV inserted into chrom	[14]
<i>pil::pNQ</i>	YP111 pIBX	pNQ insertion in <i>pil</i>	This work
Δpil	YP111 pIBX	Δpil	This work
IS0002	YP111 pIBX	S.I. [†] in <i>pil::pNQ</i> background (YscI ^{GGA})	This work
IS0006	YP111 pIBX	S.I. [†] in <i>pil::pNQ</i> background (PAP I ^{L291R})	This work
IS0007	YP111 pIBX	S.I. [†] in <i>pil::pNQ</i> background (YscN ^{K175E})	This work
IS0008	YP111 pIBX	S.I. [†] in <i>pil::pNQ</i> background (PAP I ^{L291R})	This work

[†]Suppressor Isolate (S.I.)

Table 4. Primers used in this study

Primer	Sequence (5'-3')	Ref
Primers used to make pSR47s::Δpil		
Fpil_500up_pSR47	agggaacaaaagctggagctcGCTGATGCGGGAATGTGGTC	This work
Rpil_500up_pSR47	tgagatcagtCATTCACCGATATCTTGTACCGC	This work
Fpil_500d_pSR47	tcggtgaatgACTGATCTCACTCTTTTCGCTGAGTCATTAA TGTG	This work
Rpil_500d_pSR47	cgaattcctgcagcccggggatccACCTTACGGCGTGGCCAGCC	This work
Primers used to make pCVD442::Δpil		
Fpil_500up_pCVD	caacataaagtgatcccatatGCTGATGCGGGAATGTGG	This work
Rpil_500up_pCVD	aagagtgagaTCACCGATATCTTGTACCGC	This work
Fpil_500d_pCVD	atatcgggtgaTCTCACTCTTTTCGCTGAGTCATTAATG	This work
Rpil_500d_pCVD	acctggcacggctgggacggaagtcACCTTACGGCGTGGCCAG	This work
Primers used to make pCVD442::ΔpenB		
FpB_500up_pCVD	caacataaagtgatcccatatgAGAAGATTTTATTATCCGTCG	This work
RpB_500up_pCVD	ggtaaaattaAATGGTACACCTCGATAG	This work
FpB_500d_pCVD	gtgtaccattTAATTTTACCATGATCCGGGTC	This work
RpB_500d_pCVD	acctggcacggctgggacggaagtcTGAGCCGGCGATATTACC	This work
Primers used to sequence inserts in pCVD442		
F_pCVD	CTTCCTGCTGAACATCAAAG	This work
R_pCVD	AAGGGAAAGCTGCACGTAAT	This work
Primers used to make over-expression constructs in pTrc99		
Fpil(wt)_pTrc	attcacacaggaaacagaccATGGATACAAAGTTATTACAAC ACACC	This work
Rpil(wt)_pTrc	tgcattgcctgcaggctgactctagaTCAGTACGACGAACGCCAG	This work
Fpil(H61A)_pTrc	CAAAGCGCTTGCCGACAAGGTGATTATC	This work
Rpil(H61A)_pTrc	AAACTGTTGACAGTTCGG	This work
Fpil(57stop)_pTrc	CAACAGTTTctAAGCGCTTCAC	This work

Rpil(57stop)_pTrc	ACAGTTCGGACAGGAATG	This work
Fpil(66stop)_pTrc	GGTGATTATCtgaGATGGCAAGAATACGC	This work
Rpil(66stop)_pTrc	TTGTCGTGAAGCGCTTTG	This work
Fpil(90stop)_pTrc	CTCGGAGAAAtaGCTGATTATATGGGG	This work
Rpil(90stop)_pTrc	CTCGGAGAAAtagCTGATTATATGGGG	This work
Fpil(M14)_pTrc	atttcacacaggaaacagaccATGGTGGATTACCGCCCTG	This work
Rpil(M14)_pTrc	tgcctgcctgcaggtcgactctagaTCAGTACGACGAACGCCAG	This work
Fpil(M41)_pTrc	tgcctgcctgcaggtcgactctagaTCAGTACGACGAACGCCAG	This work
Rpil(M41)_pTrc	atttcacacaggaaacagaccATGGATCTGTTGGTCAATGC	This work
Fpil(1-40)_pTrc	atttcacacaggaaacagaccATGGATACAAAGTTATTACAACAC	This work
Rpil(1-40)_pTrc	tgcctgcctgcaggtcgactctagaTCACCTGTAGTGGATCAC	This work
Fpil(wt)_pET28	ccaccactgaTCTCACTCTTTTCGCTGAG	This work
Rpil(wt)_pET28	acctggcacggctgggacggaagtcGTTGCCATTCAAGCTGCATTAG	This work
Fpil(1-99)_pET28	actttaagaaggagatataccatggATGGATACAAAGTTATTACAA CACA	This work
Rpil(1-99)_pET28	agtgggtggtggtggtgctcgagCGTGAAGTCCCCCATATAATC	This work
Fpil(wt)_His_pTrc	atttcacacaggaaacagaccATGGATACAAAGTTATTACAACA CACC	This work
Rpil(wt)_His_pTrc	tgcctgcctgcaggtcgactctagaTCAGTGGTGGTGGTGGTG	This work
Fpil(99-His)_pTrc	atttcacacaggaaacagaccATGGATACAAAGTTATTACAAC ACACC	This work
Rpil(99-His)_pTrc	tgcctgcctgcaggtcgactctagaTCAGTGGTGGTGGTGGTG	This work

Primers used to sequence inserts in pTrc99

F_pTRC	gccgacatcataacggtct	This work
R_pTRC	cacttctgagttcgcatgg	This work

Primers used to make transcriptional reporter constructs in pFU99

Fpil_p1_pFU	ccttcgtcttcacctcgaggatccTGTGGTCTGGCGAGTCGC	This work
Rpil_p1_pFU	ttcatttttaattcctcctgtcgacTCACCGATATCTTGTACCGCATG	This work
Fpil_p2_pFU	ccttcgtcttcacctcgaggatccAGATATCGGTGAATGGATACAAA	This work
Rpil_p2_pFU	ttcatttttaattcctcctgtcgacTAGGAATGCCGGCATTGAC	This work

Fpil_p3_pFU	ccttcgtcttcacctcgaggatccATGGATCTGTTGGTCAATG	This work
Rpil_p3_pFU	ttcattttaattcctcctgtcgacTATTCTCCGAGTTGGACTG	This work
Fpil_pR1_pFU	ccttcgtcttcacctcgaggatccTTTTTCCACCGCTCTTCG	This work
Rpil_pR1_pFU	ttcattttaattcctcctgtcgacGGGGATGACGATATGGCAC	This work
Fpil_pR2_pFU	ccttcgtcttcacctcgaggatccACGACCCCATCTGCGTATTC	This work
Rpil_pR2_pFU	ttcattttaattcctcctgtcgacACCGCCCTGTGAATACGAAG	This work
Fpil_pR3_pFU	ccttcgtcttcacctcgaggatccGCGACTTAACTTTTGCAG	This work
Rpil_pR3_pFU	ttcattttaattcctcctgtcgacATTATCGTTGATGGCAAG	This work
Fpil_pR4_pFU	ccttcgtcttcacctcgaggatccCTTACGGCGTGGCCAGCC	This work
Rpil_pR4_pFU	ttcattttaattcctcctgtcgacTAAATGTGTTTCATTCAGAAC GATCTCA	This work

Primers used to sequence inserts in pFU99

F_pFU99	ATACGCCCCGGTAGTGATCTT	This work
R_pFU99	TCACGTTGGTGTAGATGGGC	This work

Primers used for ddPCR

F_Chrom_ddPCR	CCTCACCGATACCGAACGAG	[35]
R_Chrom_ddPCR	GTCAGCAGGATAGGGCTACC	[35]
F_pYV_ddPCR	CTCTTTGACCTCGGCTTGAG	[35]
R_pYV_ddPCR	CGCAGCCGTTAGGACAAATG	[35]

Primers used for qPCR

F_pil_qPCR	GGTGGATTACCGCCCTGTGA	This work
R_pil_qPCR	CAGTTCGGACAGGAATGCCG	This work
F_yopE_qPCR	CCATAAACCGGTGGTGAC	[55]
R_yopE_qPCR	CTTGGCATTGAGTGATACTG	[55]
F_16S_qPCR	AGCCAGCGGACCACATAAAG	[56]
R_16S_qPCR	AGTTGCAGACTCCAATCCGG	[56]

Table 5. Plasmids used in this study

Plasmid/vector	Insert	Ref
Plasmids used to make mutations in <i>Y. pseudotuberculosis</i>		
psr47s::empty	Empty [SacB ⁺] [Kan ^R]	[57]
pCVD442::empty	Empty [SacB ⁺] [Carb ^R]	[58]
pSR47s:: Δpil	Δpil	This work
pCVD442:: Δpil	Δpil	This work
pCVD442:: $\Delta pcnB$	$\Delta pcnB$	This work
Plasmids used for over-expression cloning & assays		
pTrc99a::empty	Empty [Carb ^R]	[59]
pTrc99a:: <i>pil</i>	Full length <i>pil</i>	This work
pTrc99a:: <i>pil</i> -H61A	Pil(H61A)	This work
pTrc99a:: <i>pil</i> -57STOP	Pil(57STOP)	This work
pTrc99a:: <i>pil</i> -66STOP	Pil(66STOP)	This work
pTrc99a:: <i>pil</i> -90STOP	Pil(90STOP)	This work
pTrc99a:: <i>pil</i> (14-122)	Pil(M14)	This work
pTrc99a:: <i>pil</i> (41-122)	Pil(M41)	This work
pTrc99a:: <i>pil</i> (1-40)	Pil(1-40)	This work
pTrc99a:: <i>pil</i> (1-122)-6xHis	Full length Pil(1-122)-C-6xHis	This work
pTrc99a:: <i>pil</i> (1-99)-6xHis	Pil(1-99)-c-6xHis	This work
pET28:: <i>pil</i> (1-122)-6xHis	Pil(wt)-C-6xHis	This work
pET28:: <i>pil</i> (1-99)-6xHis	Pil(1-99)-C-6xHis	This work
Plasmids used for β-galactosidase assays		
pFU99a::empty	Empty [Cm ^R]	
pFU99a:: <i>pil</i> -p1	<i>pil</i> -p1-LacZ	This work
pFU99a:: <i>pil</i> -p2	<i>pil</i> -p2-LacZ	This work
pFU99a:: <i>pil</i> -p3	<i>pil</i> -p3-LacZ	This work
pFU99a:: <i>pil</i> -pR1	<i>pil</i> -pR1-LacZ	This work
pFU99a:: <i>pil</i> -pR2	<i>pil</i> -pR2-LacZ	This work
pFU99a:: <i>pil</i> -pR3	<i>pil</i> -pR3-LacZ	This work
pFU99a:: <i>pil</i> -pR4	<i>pil</i> -pR4-LacZ	This work
pFU99a:: <i>yscW</i> -lcrF	<i>yscW</i> -lcrF-LacZ	This work

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Chapter 3.

The polyadenylase PAP I is required for virulence plasmid maintenance in

Y. pseudotuberculosis.

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Abstract

Many species of pathogenic bacteria harbor critical plasmid-encoded virulence factors, and yet the regulation of such plasmids is often poorly understood. Human pathogenic *Yersinia*, including the plague agent *Y. pestis* and its close relative *Y. pseudotuberculosis*, require the type III secretion system (T3SS) virulence factor to cause disease. The *Yersinia* T3SS is encoded on the IncFII plasmid for *Yersinia* virulence (pYV). Expression and activity of the T3SS is dynamically regulated via changes in virulence plasmid copy number (PCN), or the number of pYV plasmids per *Y. pseudotuberculosis* cell. In addition, pYV is highly stable in *Yersinia* despite being dispensable for growth outside the host. In this study, we show that the chromosomal gene *pcnB* encoding the polyadenylase PAP I is required for regulation of pYV PCN, maintenance of pYV in the bacterial population outside the host, robust T3SS activity, and for *Yersinia* virulence in a mouse infection model. We also report that PAP I is broadly required for stabilization of sRNA-regulated plasmids that encode antimicrobial resistance (AMR) genes in *Y. pseudotuberculosis*, and is therefore required for effective antibiotic resistance. These data suggest that *pcnB*/PAP I is involved in the spread and stabilization of virulence and AMR plasmids in bacterial pathogens, and in maintaining the plasmid-encoded gene dosage required to mediate plasmid-encoded traits. Importantly *pcnB*/PAP I has been bioinformatically identified in many species of bacteria despite being studied in only a few species to date. Through this work, we show for the first time that *pcnB*/PAP I regulates a virulence plasmid in its natural pathogenic host with a direct impact on bacterial virulence

Introduction

Many species of pathogenic bacteria harbor plasmids that encode genes important for virulence or antimicrobial resistance (AMR). Virulence plasmids are typically characterized by their relatively large size (>40kb) and low plasmid copy number (PCN), which refers to the average number of plasmid copies per cell or chromosome equivalent [1]. In addition to virulence or AMR genes, these plasmids typically contain additional genes important for plasmid replication, PCN control, and plasmid maintenance. Virulence plasmids are commonly found in members of the Enterobacteriaceae family, which includes several species of Gram negative pathogens. For example, *Y. pseudotuberculosis* and the related plague agent *Y. pestis* naturally harbor a ~70 kb low copy number virulence plasmid known as the plasmid for *Yersinia* virulence (pYV), also called pCD1 in *Y. pestis* [2-4]. Given that *Y. pestis* has killed >200 million people since antiquity [5], pYV has played a grim but important role in human history.

The pYV plasmid encodes several factors important for *Yersinia* virulence, including a major virulence factor known as the type III secretion system (T3SS) [3, 6, 7]. The T3SS is used to inject a series of effector proteins known as *Yersinia* outer proteins (Yops) directly into the cytosol of target host cells during infection. Yops dampen the host immune response and promote *Yersinia* pathogenesis, making pYV essential for *Yersinia* virulence [3, 8-12]. Expression and activity of the T3SS exerts a metabolic burden on the bacteria. Therefore, *Yersinia* repress expression of the T3SS at low environmental temperatures when the T3SS is dispensable, but induce T3SS expression at mammalian body temperature, 37°C [13-18]. Several

mechanisms contribute to this temperature-dependency. For example, the master regulator of the T3SS is only translated at 37°C due to control by an RNA thermometer [17, 19]. More recently, it was reported that temperature-dependent changes in T3SS gene expression is also regulated at the level of gene dosage via changes in pYV PCN [20]. At environmental temperatures such as 26°C, pYV PCN is kept low to about 1-2 copies per cell. Upon exposure to mammalian temperatures of 37°C, pYV PCN is rapidly upregulated to 3+ copies per cell. Importantly, this ability to upregulate pYV PCN and T3SS gene dosage is essential for normal T3SS activity and *Y. pseudotuberculosis* virulence [20, 21]. Dynamic changes in pYV PCN and retention of the pYV plasmid during these rapid changes is therefore required for the *Y. pseudotuberculosis* pathogenic lifestyle.

The pYV plasmid encodes several systems important for plasmid regulation and maintenance, including a *cop-rep* locus that regulates plasmid replication, a ParAB partitioning system that ensures each daughter cell inherits the plasmid, and a ParDE toxin-antitoxin (TA) system that promotes plasmid maintenance in the bacterial population [1, 22]. Together, these systems are predicted to synergistically enforce plasmid stability, or the percentage of cells in the population that maintain the plasmid. The highly conserved *cop-rep* locus places pYV in the IncFII group of plasmids [1, 23, 24]. The *cop-rep* locus encodes the essential replication initiation factor RepA and two negative regulators of RepA expression: the CopA small RNA (sRNA) and the CopB protein. The functions of RepA, CopA, and CopB have been elucidated using plasmid R1, an IncFII plasmid originally isolated from a clinical strain of *S. enterica* that has been extensively studied in *E. coli* model systems [24-

26]. These studies support that CopA and CopB regulate plasmid replication rate to minimize fluctuations above or below the average PCN set-point [24, 27].

Using *Y. pseudotuberculosis* as a model, we identified the chromosomally-encoded *pcnB* gene as a previously unknown regulator of pYV replication and stability. The *pcnB* gene was named for plasmid copy number B since loss of *pcnB* was found to decrease PCN for several model plasmids in *E. coli* [28, 29]. The *pcnB* gene encodes the poly A polymerase (PAP I), the major bacterial polyadenylase ubiquitous in β - and γ -Proteobacteria [28-35]. Polyadenylation, or the addition of adenosine residues to the 3' OH of an RNA transcript, is poorly understood in bacteria compared to eukaryotes. PAP I-mediated polyadenylation promotes RNA degradation as part of a housekeeping mechanism [36], but is also implicated in certain gene regulatory networks [37-42]. PAP I-dependent polyadenylation has been shown to destabilize RNAs that negatively regulate replication of certain plasmid types in *E. coli*, including the CopA from plasmid R1 and RNAI from ColE1 plasmids [28, 43-46]. However, PAP I has not been studied in the context of a naturally occurring virulence or AMR plasmid within its native host, and has never been linked to bacterial virulence.

Here, we report that *Y. pseudotuberculosis* *pcnB* mutants display reduced PCN and stability of pYV. This defect is associated with reduced T3SS activity in bacterial culture and impaired virulence in a mouse infection model. Finally, we show that *pcnB* is required for *Y. pseudotuberculosis* to maintain model AMR plasmids belonging to several different plasmid families.

Results

***Y. pseudotuberculosis* toggles pYV plasmid copy number in response to temperature without plasmid loss**

To determine average plasmid copy number (PCN) at the bacterial population level, we used droplet digital (dd)PCR to measure pYV PCN relative to chromosome equivalents in wildtype *Y. pseudotuberculosis* IP2666pIB1 [21]. Cells were either grown at 26°C to represent growth in environmental conditions outside of the host or at 37°C in low calcium media to represent host cell contact during mammalian infection. The pYV-encoded T3SS is expressed at 37°C and becomes active for secretion following host cell membrane contact or calcium chelation [47-50].

Consistent with previous studies, we observed an increase in pYV PCN from an average of ~1.5 pYV copies per chromosome equivalents at 26°C to ~3 copies at 37°C/low calcium [20, 21] (Fig 1A). In addition, using a YPIII/pIBX strain of *Y. pseudotuberculosis* harboring a pYV-encoded luciferase reporter, we found that pYV PCN relative to cell density increases one hour after shifting the cultures to 37°C/low calcium conditions (Fig 1B).

To examine pYV PCN at the single-cell level, we employed a microscopy-based approach. To visualize and quantify pYV puncta in living *Y. pseudotuberculosis* cells, we generated a YPIII/pIBX *Y. pseudotuberculosis* strain that harbors a ParB-GFP translational fusion in the native *parB* pYV locus. This approach has been used to visualize plasmids in other bacterial species [51] but has never been used to study pYV in *Y. pseudotuberculosis*. At 26°C, cells were found to have an average of ~4 pYV puncta per cell (Fig 1D). While this is greater than the

pYV PCN determined by our population-based approaches that normalize to cell density or per chromosome, observing a higher PCN by microscopy is consistent with previous findings. However, we did observe a temperature-dependent increase in pYV PCN, with an average of ~7 puncta per cell by 1.5 hours after the shift to 37°C (Fig 1D). Therefore, while the absolute values of pYV PCN differ depending on the method used, *Yersinia* pYV PCN consistently increases by ~1.5-fold upon shifting bacterial cultures from 26°C to 37°C. Despite this dynamic change in pYV PCN, we found that the pYV plasmid is highly stable in *Y. pseudotuberculosis*, with the vast majority of clones retaining pYV (Fig 1D, Table 1).

Disruption of the *pcnB* gene encoding the polyadenylase PAP I decreases pYV PCN, T3SS expression and activity

Through a forward genetic screen to identify genes important for T3SS activity [52], we identified a *Y. pseudotuberculosis* IP2666pIB1 transposon (Tn) insertion mutant (Fig S1A). This mutant was found to harbor a Tn insertion in a previously unannotated locus adjacent to the highly conserved pYV *cop-rep* locus. We will refer to this locus as the plasmid copy number inhibitory locus (*pil*) (Fig S1B). The *pil::Tn* *Y. pseudotuberculosis* mutant displayed elevated T3SS function and increased expression of T3SS genes compared to the parental strain (Fig S1C&E). Given the location of the *pil* locus relative to *cop-rep*, we hypothesized that the *pil::Tn* mutation may affect pYV PCN. We introduced the *pil::Tn* insertion mutation into a clean wildtype *Y. pseudotuberculosis* IP2666pIB1 strain background. In addition, we constructed a mutant in the YPIII/pIBX background with a plasmid insertion into the

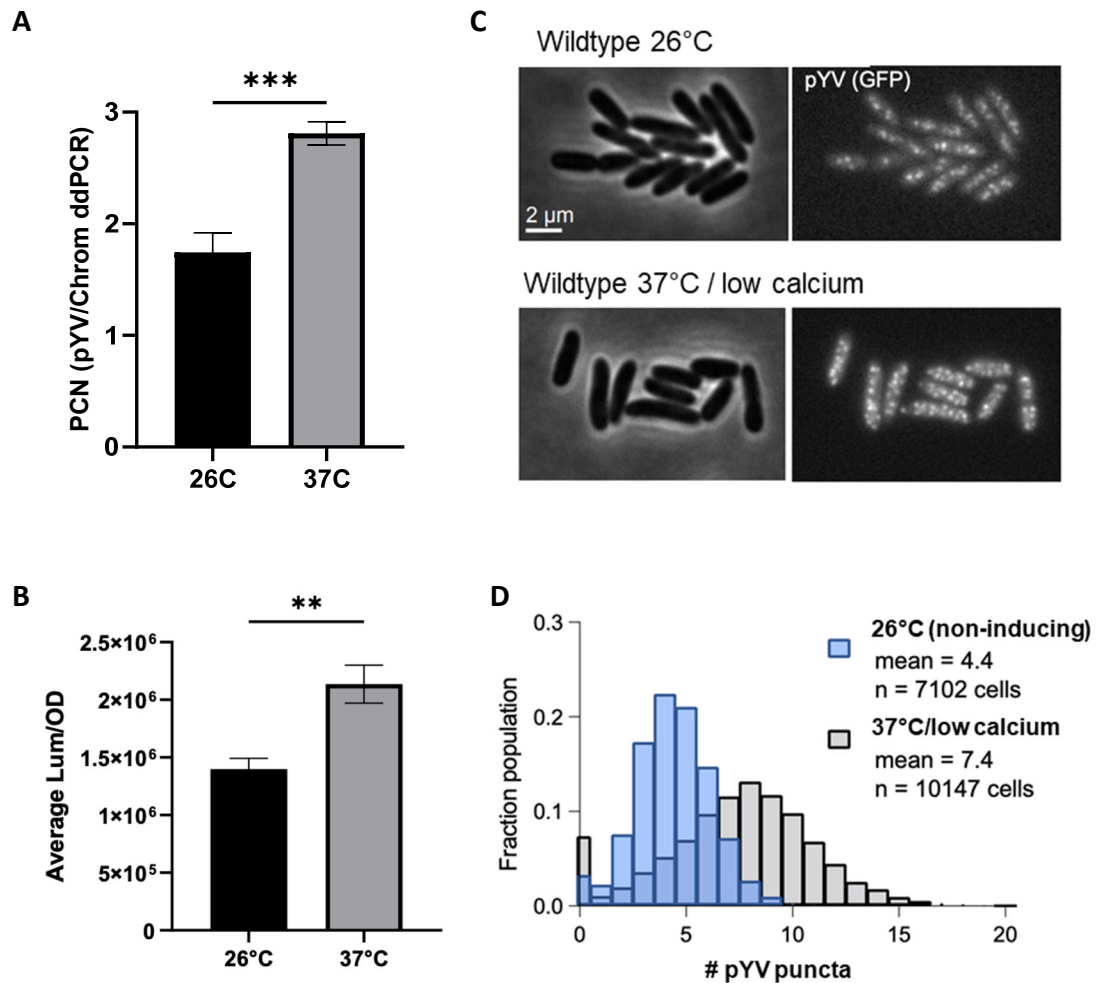


Figure 1. *Y. pseudotuberculosis* pYV plasmid copy number (PCN) dynamically changes in response to temperature. (A) Absolute pYV plasmid copy number was determined for wildtype *Y. pseudotuberculosis* IP2666pIB1 following growth at 26°C or 37°C/low calcium using droplet digital PCR (dd)PCR [21]. (B) Relative pYV plasmid copy number for wildtype *Y. pseudotuberculosis* YPIII/pIBX was determined following growth at 26°C or 37°C/low calcium using a luciferase reporter stably incorporated in the pIBX plasmid [20]. (A-B) Averages of three independent experiments are shown $\pm$ standard deviation (Student t-test, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). (C) The pYV plasmid was visualized in live *Y. pseudotuberculosis* YPIII/pIBX cells using a ParB-GFP fluorescent marker at different temperatures. (D) Histogram depicting the number of pYV puncta quantified from data represented in part C.

same locus in *pil* (*pil::pNQ*). The *pil::Tn* and *pil::pNQ* strains had elevated pYV PCN at 26°C and early after shifting to 37°C relative to the parental strain (Fig S1D), which is likely the underlying cause of the increased T3SS activity and expression in this strain (Fig S1C-E and data not shown).

The *pil::Tn* and *pil::pNQ* mutants displayed poor growth compared to the parental strain under T3SS-inducing conditions, and suppressor mutants could readily be isolated (Fig S1F and data not shown). We hypothesized that constitutively high pYV PCN contributed to this growth defect, resulting in selective pressure favoring suppressor mutations that would alleviate high pYV PCN. Therefore, we used the *pil* insertion mutation as a tool to identify genes important for pYV homeostasis (see Chapter 2). We carried out whole genome sequencing of a subset of *pil::pNQ* suppressor mutants and identified several categories of mutations affecting growth, pYV PCN, and the T3SS (Table S1). Two clones harbored a single point mutation that was associated with reduced pYV PCN and T3SS activity relative to the *pil::pNQ* parental strain. This mutation was mapped to the *pcnB* gene encoding the polyadenylase PAP I, and was predicted to confer a leucine to arginine change at position 291 (L291R) in the PAP I neck domain linked to RNA binding (Fig 2A). The L291 residue is conserved in PAP I from *E. coli*, suggesting that the residue might be important for PAP I structure and/or function (Fig 2A).

To confirm that the *pcnB* gene encoding PAP I affects *Y. pseudotuberculosis* pYV PCN, we generated a full in-frame deletion of the *pcnB* gene ($\Delta pcnB$) in the wildtype and *pil::Tn* IP2666pIB1 strain backgrounds. We used ddPCR to measure relative pYV PCN and found that compared to wildtype, the $\Delta pcnB$ mutant had lower pYV PCN following growth at 26°C and 37°C/low calcium (Fig 2B). In keeping with

Table S1. Phenotypic categories and mutations identified in suppressor screen

Strain:	PCN 26°C ¹	PCN 37°C ¹	T3SS activity ³	Identified Mutation:	Comments:
<i>pil::pNQ</i> (parental)	High ²	High ²	High ²	N/A	
IS0002	No Change	No change	No Activity	<u>T</u> GA → <u>G</u> GA mutation abolishing YscI stop codon	Predicted to decrease or abolish translation of YscI
IS0003	No Change	Lower	Lower	G → C mutation in tRNA-Gly	Unknown how this mutation impacts pYV PCN and/or T3SS activity.
IS0004	No Change	Lower	Lower	G → A mutation in <i>prepA</i> -35 region	Predicted to decrease <i>prepA</i> promoter firing
IS0006	Lower	Lower	Lower	L291R mutation in PAP I	Mutation lowers PAP I protein levels at 37°C
IS0007	No Change	No Change	No Activity	K175E mutation abolishing the essential lysine in walker box A of YscN	Mutation predicted to impair YscN ATP binding and hydrolysis
IS0008	Lower	Lower	Lower	L291R mutation in PAP I	Mutation lowers PAP I protein levels at 37°C
IS0016	Lower	Lower	Lower	A272E in RNA Pol alpha factor	Mutation may impact RNA Pol binding to some promoters

¹ Relative pYV PCN was determined using a luciferase PCN assay with luminescence normalized to OD₆₀₀.

² Changes in pYV PCN and T3SS activity relative to the parental strain. The parental strain for *pil::pNQ* is wildtype *Y. pseudotuberculosis* YPIII/pIBX and the parental strain for all suppressor isolates shown is *pil::pNQ* YPIII/pIBX.

³ T3SS activity was determined by a secretion assay on cells grown at 37°C in low calcium media.

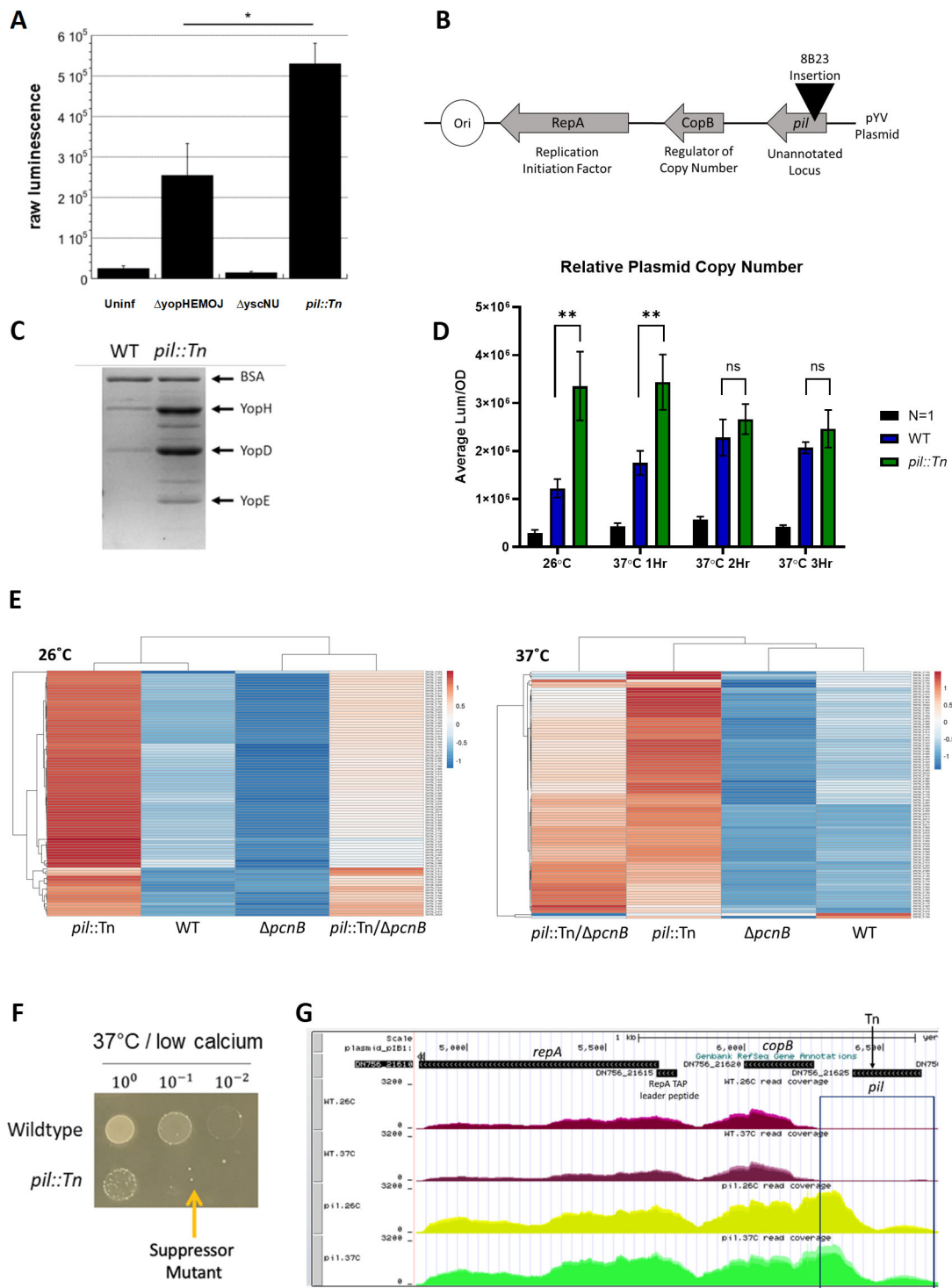


Figure S1. A transposon insertion into *pil* leads to a constitutively high pYV PCN, elevated T3SS expression, and a severe growth defect under T3SS-inducing conditions. (A) HEK293T cells expressing an NF κ B luciferase reporter were left uninfected

(uninf) or were infected with an effectorless $\Delta yop6$ strain ($\Delta yopHEMOJ$), a $\Delta yscNU$ T3SS-deficient strain, or *pil::Tn* in the *Y. pseudotuberculosis* IP2666pIB1 background. Averages of three independent replicates $\pm$ standard error the mean are shown (Student t-test, $p < 0.04$). **(B)** Schematic representation of the location of *pil*. The *pil* locus is located ~123bp away from the *copB* gene. **(C)** Wildtype and *pil::Tn* IP2666pIB1 were grown at 37°C/low calcium and secreted proteins (Yops) were precipitated and visualized by coomassie blue staining. Bovine serum albumin (BSA) serves as a loading and precipitation control. Data shown is representative of three independent replicates. **(D)** Relative pYV PCN was estimated for strains in the pIBX/YPIII background of *Y. pseudotuberculosis* using a luciferase plasmid copy number assay. For each timepoint, luminescence was measured and normalized to optical density. Data shown is the average of three independent replicates and statistical significance was determined using a student t-test (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). **(E)** Heat maps displaying relative expression of pYV-encoded genes from RNA-seq analysis of *Y. pseudotuberculosis* IP2666pIB1 strains grown at 26°C or 37°C/low calcium. **(F)** Serial dilutions for wildtype and *pil::Tn* *Y. pseudotuberculosis* IP2666pIB1 were spotted onto low calcium agar plates for ~16 hours at 37°C before imaging. The yellow arrow indicates an example of a large colony in the *pil::Tn* mutant background that represents a candidate suppressor mutant. **(G)** Reads from RNA-seq analysis mapped to the *cop-rep* locus and adjacent *pil* locus from wildtype and *pil::Tn* (*pil*) *Y. pseudotuberculosis* IP2666pIB1 grown at 26°C or 37°C/low calcium. The full length putative *pil* locus is indicated by a black box and the approximate location of the Tn insertion is indicated with an arrow.

our previous findings, the *pil::Tn* mutant displayed significantly higher pYV PCN at 26°C compared to wildtype. Furthermore, loss of PAP I in the *pil::Tn*/ $\Delta pcnB$ double mutant significantly reduced pYV PCN at 26°C compared to the *pil::Tn* parental strain. These results show that loss of PAP I leads to decreased pYV PCN. Despite this, loss of *pcnB* did not prevent *Yersinia* from increasing pYV PCN upon shift from 26°C to 37°C, although not to the same high PCN as in wildtype. Consistent with this reduction in T3SS gene dosage, the $\Delta pcnB$ mutant displayed two-fold less expression and secretion of the T3SS cargo protein YopE compared to wildtype *Y. pseudotuberculosis* (Fig 2C). Similarly, *pcnB* deletion reduced YopE expression and secretion by 1.5-fold in the *pil::Tn* strain background (Fig 2C). Taken together, these data suggest that *pcnB* is essential for maintaining pYV PCN, and that this is important for enabling robust T3SS expression under T3SS-inducing conditions.

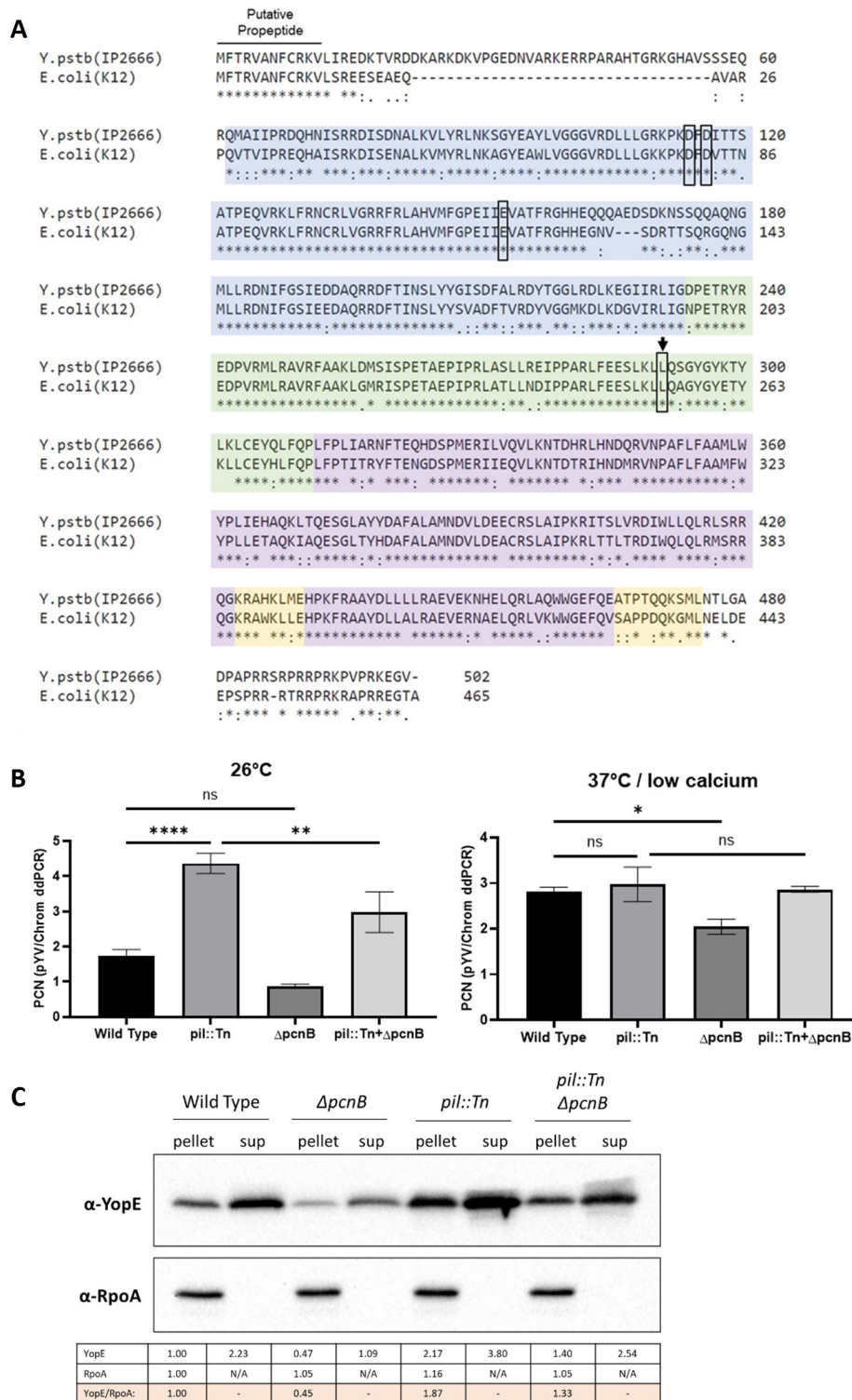


Figure 2. Disruption of the *pcnB* gene encoding poly(A) polymerase PAP I decreases pYV PCN as well as T3SS expression and activity. (A) PAP I from *Y. pseudotuberculosis*

IP2666pIB1 and *E. coli* K12 are highly conserved. The first 10-13 amino acids represent a putative propeptide that affects PAPI localization in *E. coli* [53]. Other domains include the PAP I head domain (blue), the neck domain (green), the body domain (purple), and the leg domain (yellow). Residues in plain black boxes represent known catalytic residues from EcPAP and the residue in a black box indicated with an arrow represents the residue that was mutated in the L291R *pcnB* suppressor mutants. **(B)** Absolute pYV PCN was determined for *Y. pseudotuberculosis* IP2666pIB1 strains following growth at 26°C or 37°C/low calcium using ddPCR. Data shown represents the average of three independent experiments and $\pm$ standard error of the mean. Statistical significance was calculated using a one-way ANOVA for multiple comparisons (**** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$). **(C)** *Y. pseudotuberculosis* IP2666pIB1 strains were grown in low calcium media at 37°C before preparing samples for western blotting. Bacterial pellets were collected and secreted proteins (Yops) were precipitated from the supernatant (sup). Images shown represent a single blot that was independently probed for YopE and an RpoA loading control.

To determine how the *pcnB* gene and the *pil::Tn* disruption broadly impact expression of pYV-encoded genes, we carried out RNA-seq analysis on wildtype, $\Delta pcnB$, *pil::Tn*, and *pil::Tn/\Delta pcnB* *Y. pseudotuberculosis* IP2666pIB1 grown at 26°C or 37°C/low calcium (Supplementary Dataset 1). Most pYV-encoded gene transcripts were present at higher steady-state level in the *pil::Tn* mutant compared to wildtype *Y. pseudotuberculosis* at both temperatures, consistent with an increase in pYV gene dosage (Fig S1E). Interestingly, the *pil::Tn* insertion appeared to alter the RNA-Seq read pattern of the region containing *pil*, *copB*, and *repA* under both conditions (Fig S1G). In previous studies, we observed no difference in pYV PCN or T3SS expression when we generated a full in-frame deletion of the predicted ORF found in the *pil* locus (data not shown, see Chapter 2). Therefore, we propose that Tn or pNQ insertion into *pil* leads to derepressed transcription of the *cop-rep* locus leading to elevated pYV replication through an as-yet unknown mechanism. In contrast, deletion of *pcnB* resulted in fewer transcripts of most pYV-encoded genes compared to wildtype *Yersinia* at both temperatures, consistent with a decrease in pYV gene

dosage (Fig S1F). In addition, *pcnB* deletion in the *pil::Tn* background also led to a decrease in the steady-state levels of most T3SS genes compared to the *pil::Tn* parental strain. T3SS genes are not translated at 26°C due to the action of several RNA thermometers in wildtype *Yersinia* [47], and this regulation is retained in the $\Delta pcnB$ and *pil::Tn* mutant backgrounds (data not shown). We conclude that loss of *pcnB* leads to a decrease in pYV PCN in both the wildtype and *pil::Tn* backgrounds, leading to decreased transcription of pYV-encoded genes and mitigating T3SS activity-induced growth arrest.

PAP I is required for pYV plasmid stability

When working with the $\Delta pcnB$ strain, we noted that cells occasionally lost the pYV plasmid at an unusually high rate compared to wildtype *Y. pseudotuberculosis*. To determine if PAP I affects pYV plasmid stability in addition to pYV PCN, we generated a $\Delta pcnB$ strain in the ParB-GFP YPIII/pIBX *Y. pseudotuberculosis* background and measured the number of ParB-GFP puncta to determine pYV molecules per cell (Fig 3A). The $\Delta pcnB$ mutant showed the expected increase in the average number of ParB-GFP puncta per cell from 26°C to 37°C/low calcium, but had ~1.5-fold fewer ParB-GFP puncta per cell compared to the wildtype strain at both temperatures, consistent with our previous results (Fig 3B-D). In addition, we found that over 25% of cells lacked pYV in the $\Delta pcnB$ mutant compared to less than 5% for wildtype at 26°C (Fig. 3C). Loss of PAP I also resulted in more pYV plasmid loss compared to wildtype under 37°C/low calcium conditions (Fig. 3D).

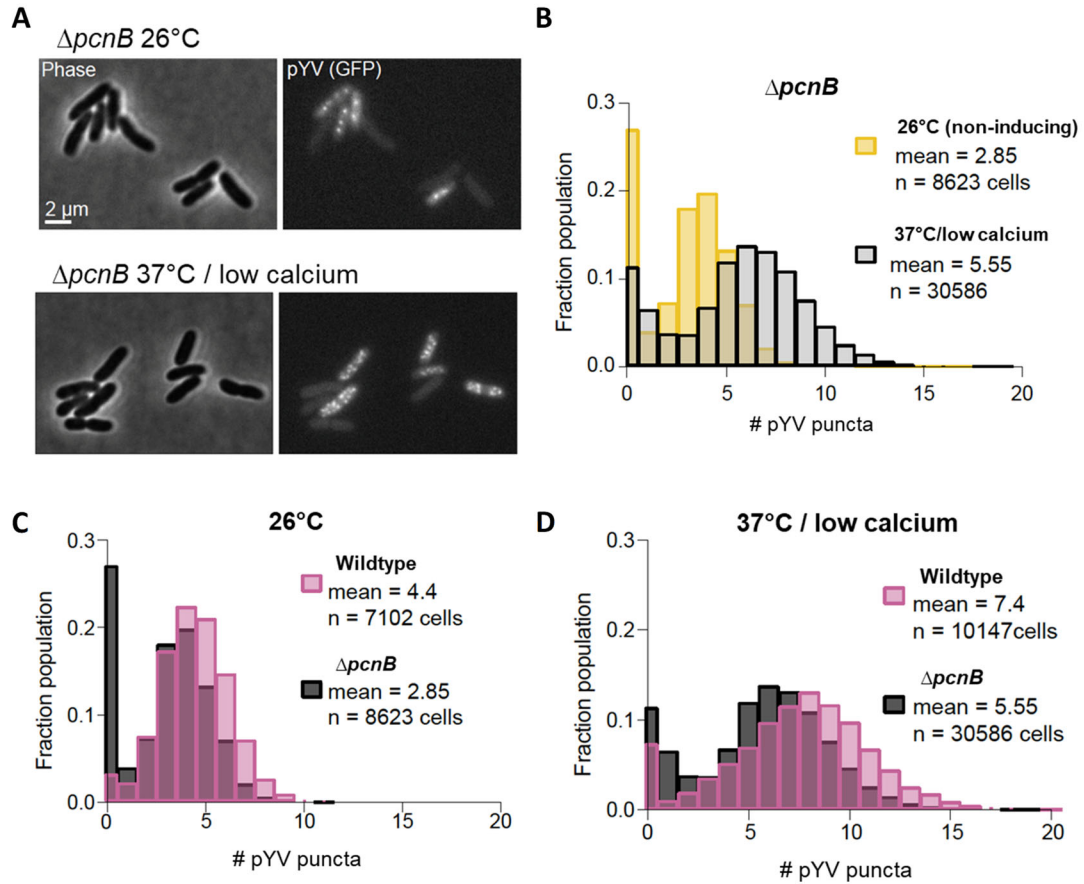


Figure 3. The *pcnB* gene is required for maintenance of the pYV plasmid. (A) *Y. pseudotuberculosis* YPIII/pIBX $\Delta pcnB$ expressing ParB-msfGFP was grown at 26°C or at 37°C/low calcium and live cells were imaged using phase contrast microscopy (left) and fluorescence microscopy (right). **(B)** The number of cells and pYV puncta were determined and plotted for the data represented in A. **(C)** The number of cells and pYV puncta for *Y. pseudotuberculosis* YPIII/pIBX wildtype or $\Delta pcnB$ strains expressing ParB-msfGFP was quantified and overlayed following growth at 26°C (non T3SS-inducing conditions) or **(D)** 37°C/low calcium (T3SS-inducing conditions).

To further understand the extent of the pYV plasmid stability defect in the $\Delta pcnB$ mutant, we passaged bacteria on solid media for several days at 26°C and assessed pYV retention for each clone using the T3SS indicator dye congo red. Wildtype retained the pYV plasmid at 100% while the $\Delta pcnB$ mutant only retained pYV in 82% of cells (Table 1). When cells were exposed to T3SS-inducing conditions at 37°C/low calcium and returned to 26°C to mimic the *Y. pseudotuberculosis* facultative lifecycle, we found that only 58% of $\Delta pcnB$ cells retained pYV compared to 99% for wildtype. This observed increase in pYV loss likely stems from the growth arrest of pYV⁺ clones under T3SS-inducing conditions [14, 48, 49] leading to the cultures becoming dominated by $\Delta pcnB$ clones that have lost pYV. The standard deviation for pYV stability was much higher in the $\Delta pcnB$ mutant compared to other strains, indicative of perturbed plasmid maintenance mechanisms. Altogether, these data support a role for PAP I in regulation of pYV PCN and plasmid stability during the *Y. pseudotuberculosis* facultative lifecycle.

Table 1. PAP I is required for pYV maintenance in *Y. pseudotuberculosis*

Strain:	Percent Retention [†] of pYV after growth at 26°C:	Percent Retention [†] of pYV after exposure to 37°C:
Wildtype	100.0 ±0	99.1 ±1.2
$\Delta pcnB$	81.6 ±17.3	57.7 ±10.7
<i>pil::Tn</i>	100.0 ±0	99.1 ±1.3
<i>pil::Tn</i> $\Delta pcnB$	98.1 ±1.8	75.9 ±13.1

[†]Percent retention of the pYV plasmid was averaged from three independent experiments where ± represents the standard deviation between experiments.

The PAP I^{L291R} mutation affects PAP I protein levels.

The original suppressor screen that identified *pcnB* as a potential regulator of pYV PCN and *Yersinia* type III secretion contained a single point mutation that changed leucine in position 291 to an arginine (L291R) (Fig 2A). Since the corresponding Leu291 residue is conserved in *E. coli* but has never been identified as a critical PAP I residue in any studies, we investigated how this mutation affects PAP I expression and function. We introduced the L291R missense mutation into the native *pcnB* locus of both *Y. pseudotuberculosis* IP2666pIB1 (with or without a PAP I C terminal FLAG tag) and YPIII/pIBX to generate PAP I^{L291R} strains. Since the L291R mutation represents a nonconservative change from an uncharged nonpolar amino acid to a positively charged amino acid, we hypothesized that the L291R mutation might impact PAP I protein stability. To test this hypothesis, we also constructed a more conservative leucine to alanine mutation at position 291 (L291A) to generate PAP I^{L291A} strains. While the PAP I^{L291A} protein was expressed at both 26°C and 37°C/low calcium, the PAP I^{L291R} protein appeared to be expressed at 26°C but expressed poorly at 37°C/low calcium (Fig. 4A). These data suggest that the PAP I^{L291R} allele may be unstable, leading to low PAP I expression at least at 37°C/low calcium. Consistent with these data, the PAP I^{L291A} allele, but not PAP I^{L291R}, supported wildtype levels of pYV PCN (Fig 4C). In addition, the PAP I^{L291R} strain showed enhanced loss of pYV compared to the wildtype and PAP I^{L291A} mutant, as indicated by the emergence of white, fast-growing colonies following growth under 37°C/low calcium conditions on congo red agar, which is indicative of pYV loss (Fig 4B). Interestingly, the PAP I^{L291R} strain displayed a greater loss of pYV following long passage at 37°C/low calcium compared to when bacteria were passaged at 26°C

(Table 2), reflective of the relative stability of the PAP I^{L291R} protein under these different conditions. Lastly, the PAP I^{L291A} allele, but not the PAP I^{L291R} allele, was able to support wildtype levels of T3SS activity (Fig 4D). Taken together, these data suggest that the PAP I^{L291R} mutation disrupts PAP I expression, explaining why we identified this mutation in our suppressor screen.

Mutation of residues predicted to affect polyadenylase activity decreases pYV PCN, plasmid maintenance, and T3SS activity

Asp80 and Asp82 have been shown to be essential for PAP I polyadenylase catalytic activity in *E. coli* [54, 55] and are conserved in PAP I from *Y. pseudotuberculosis* (Fig. 2A). To determine if PAP I polyadenylase catalytic activity is responsible for the phenotypes of the *Y. pseudotuberculosis* $\Delta pcnB$ mutant, we mutated the two corresponding aspartic acid residues (Asp114 and Asp116) to alanine residues to produce a PAP I^{D2A} catalytic mutant. These mutations did not affect PAP I protein levels (Fig 4A). We found that the PAP I^{D2A} mutant displayed reduced pYV PCN at 26°C and 37°C/low calcium compared to wildtype *Y. pseudotuberculosis*, and this reduction in relative pYV PCN was similar to the $\Delta pcnB$ strain which lacks PAP I completely (Fig. 4C). Spot test analysis found that PAP I^{D2A} grows similarly to wildtype and $\Delta pcnB$ at 26°C. At 37°C/low calcium, PAP I^{D2A} and $\Delta pcnB$ display 10-fold more growth compared to wildtype, consistent with a reduction in growth arrest due to less robust T3SS activity (Fig. 4B). PAP I^{D2A} was also observed to produce plasmid-less, rapidly-growing colonies similar to the $\Delta pcnB$ mutant (Fig. 4B) and reduced pYV stability after long passage (Table 2). Lastly, the PAP I^{D2A} allele was

unable to support normal T3SS activity (Fig 4D). Taken together, these data suggest that *Yersinia* PAP I is likely to function as a polyadenylase, and that this activity is critical to maintain pYV PCN, plasmid stability, and normal T3SS gene dosage and activity.

Table 2. Important PAP I residues are required for stability of the pYV plasmid

Strain:	Percent Retention [†] of pYV after growth at 26°C:	Percent Retention [†] of pYV after exposure to 37°C:
Wildtype	100.0 ±0	97.3 ±3.8
$\Delta pcnB$	80.2 ±7.8	47.7 ±14.0
PAP I ^{D2A}	94.0 ±1.7	21.0 ±7.0
PAP I ^{L291R}	98.6 ±2.5	68.6 ±7.9
PAP I ^{L291A}	100.0 ±0	100 ±0

[†]Percent retention of the pYV plasmid was averaged from three independent experiments where ± represents the standard deviation between experiments.

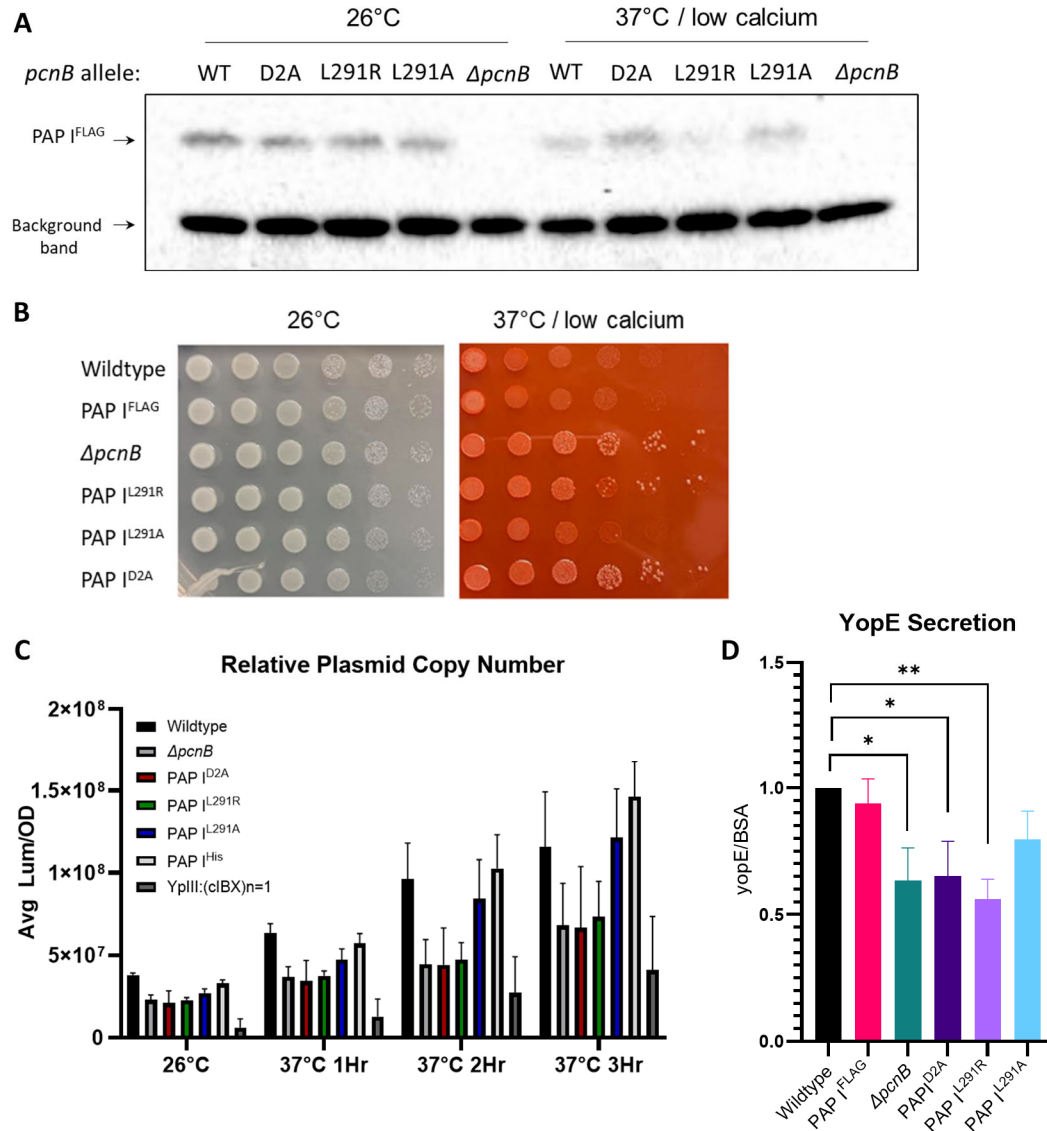


Figure 4. PAP I point mutations lead to a decrease in pYV PCN, plasmid stability, and T3SS activity. (A) *Y. pseudotuberculosis* IP2666pIB1 strains harboring various PAP I^{FLAG} alleles were grown at 26°C or 37°C/low calcium before pelleting and preparing for western blot analysis. Images shown represent a single blot that was cut after transferring and independently probed for expression of PAP I^{FLAG} (top) and an RpoA loading control (bottom). Data shown is representative of three biological replicates. (B) *Y. pseudotuberculosis* IP2666pIB1 strains were grown at 26°C before diluting and spotting onto either LB or low calcium plates containing congo red dye and incubated at 26°C or 37°C, respectively, prior to imaging. (C) Relative pYV PCN was estimated for strains in the *Y. pseudotuberculosis* pIBX/YPIII background. For each timepoint, luminescence was measured and normalized to optical density. Data shown is the average of three biological replicates $\pm$ standard error of the mean. (D) *Y. pseudotuberculosis* IP2666pIB1 strains were grown at 37°C/low calcium

and secreted proteins (Yops) were precipitated and visualized by Coomassie blue staining. Bovine serum albumin (BSA) was used as a loading and protein precipitation control. The YopE band from four independent experiments was visualized, quantified, and normalized to BSA from the same samples. Bars represent the mean fold change in YopE/BSA relative to wildtype and error bars represent the standard error of the mean between different experiments. Statistical significance was determined using an ordinary one-way ANOVA for multiple comparisons (**** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$).

***Yersinia* PAP I is required for plasmid stability and plasmid-mediated antibiotic resistance from a broad range of plasmid families**

To determine if *Yersinia* PAP I contributes to regulation of PCN and retention of other plasmid types, we introduced a ColE1-like pGFPuv plasmid into wildtype and $\Delta pcnB$ *Y. pseudotuberculosis*. This plasmid lacks partitioning systems but can be maintained by antibiotic resistance selection. We observed about a ten-fold decreased in fluorescence intensity in $\Delta pcnB$ colonies compared to wildtype upon growth on carbenicillin, suggesting that pGFPuv PCN is lower in the absence of PAP I in *Y. pseudotuberculosis* leading to less robust antibiotic resistance (Fig. 5A). To determine if the decreased PCN of the pGFPuv plasmid in the $\Delta pcnB$ strain promotes eventual plasmid loss, we measured kinetics of plasmid retention over 40 hours after removing antibiotic selection. We found that over 70% of $\Delta pcnB$ cells lose the pGFPuv plasmid within 10 hours of removing carbenicillin and the plasmid is completely lost from the population by ~36 hours. In contrast, 100% of wildtype cells retained pGFPuv over the same 36 hour time course (Fig. 5B).

To ensure that the growth defect seen for $\Delta pcnB$ harboring pGFPuv was due to loss of PAP I specifically, we next constructed a PAP I over-expression vector in the pTrc99 vector backbone. Importantly, pTrc99 is also a ColE1-like plasmid and lacks partitioning systems. We found that $\Delta pcnB$ harboring pTrc99::empty displays a

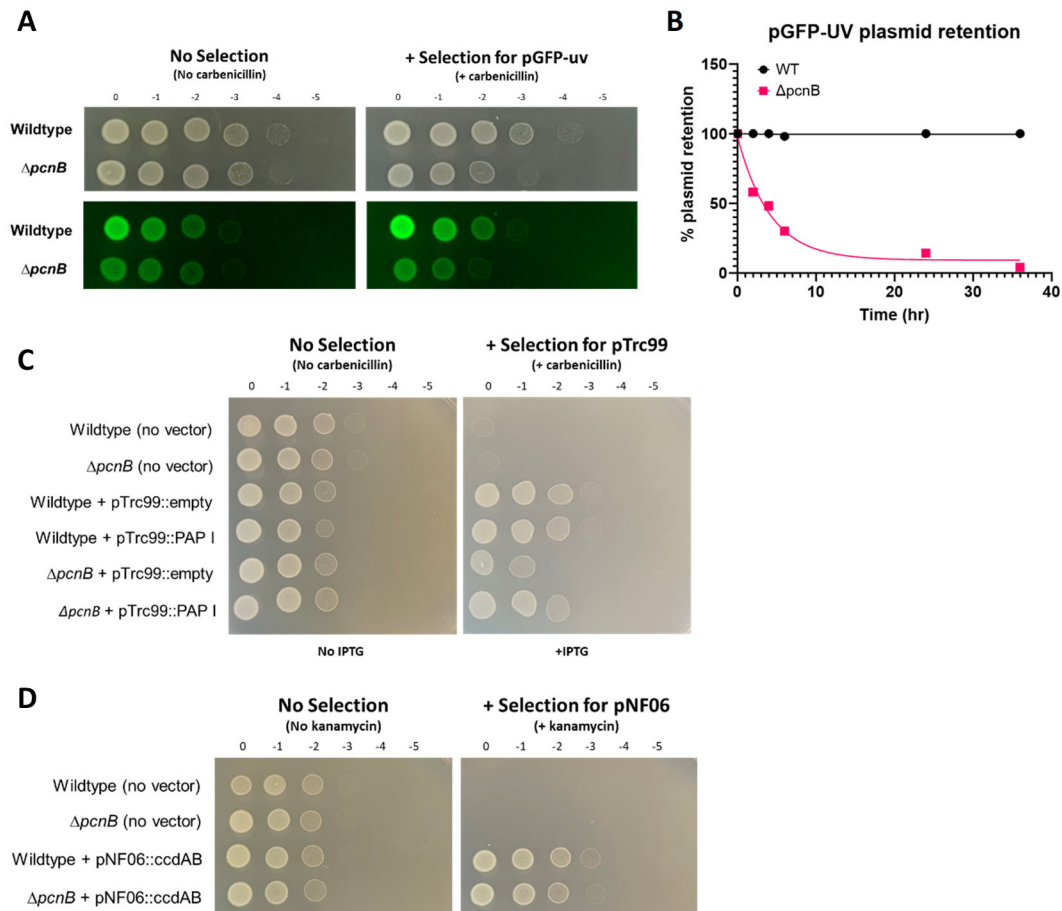


Figure 5. The *pcnB* gene is required for plasmid-mediated antibiotic resistance in *Y. pseudotuberculosis*. (A) Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 harboring pGFP-uv were spotted simultaneously onto either plain LB plates or LB plates containing carbenicillin to select for retention of pGFP-uv. Plates were incubated at 26°C for ~16 hours before imaging spots under a GFP filter. (B) Retention of the pGFP-uv plasmid by wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 was assessed at 26°C over 40 hours following the removal of carbenicillin. (C) Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 harboring either no vector, a pTrc99::empty vector control, or a pTrc99::PAP I recombinant plasmid were spotted onto either plain LB plates or LB plates containing carbenicillin to select for pTrc99 and 0.2mM IPTG to induce PAP I expression. Plates were incubated at 26°C for ~16 hours prior to imaging. (D) Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 harboring either no vector or a pNF06 mini-F plasmid with a ccdAB TA system (pNF06::ccdAB) were spotted onto either plain LB plates or LB plates containing kanamycin to select for pNF06. Plates were incubated at 26°C for ~16 hours prior to imaging.

10-fold growth defect when spotted onto LB-carbenicillin but not LB-only plates, consistent with what was previously observed with the pGFPuv plasmid (Fig. 5C). Importantly, this growth defect was nearly completely abolished by expression of PAP I from the pTrc99 plasmid (Fig. 5C). In addition to ColE1 plasmids, we assessed the ability of the $\Delta pcnB$ mutant to maintain a series of commonly-used cloning and expression vectors from different plasmid families. We found that PAP I is required for normal plasmid-mediated antibiotic resistance for plasmids that have ColE1-like and p15A-like origins of replication in general (Fig 5A-C and data not shown). Interestingly, the replication locus of all of these plasmid types is regulated by a plasmid-specific sRNA [56-58], suggesting a general role for PAP I in regulating plasmids with replicons regulated by a sRNA. To test this, we introduced a mini-F plasmid that contains the CcdAB toxin-antitoxin system (miniF::ccdAB) from *E. coli* into wildtype and $\Delta pcnB$ *Y. pseudotuberculosis*. Importantly, F plasmid replication is not known to be regulated by a sRNA [29, 43, 59]. The $\Delta pcnB$ strain maintained miniF::ccdAB to wildtype levels with or without antibiotic selection (Fig. 5D). These data suggest that PAP I is important for *Yersinia* to maintain normal PCN and stability of plasmids whose replication is regulated by a sRNA.

PAP I is required for *Y. pseudotuberculosis* virulence in mice

It is well established that the pYV plasmid and T3SS are critical for *Y. pseudotuberculosis* virulence in mouse infection models [60-65]. To determine if loss of PAP I contributes to *Yersinia* virulence, we infected mice via the intraperitoneal (IP) route and monitored colonization of the spleen and liver. Four days post-inoculation, significantly fewer $\Delta pcnB$ -infected mice had robust colonization of the

spleen and liver compared to wildtype-infected mice (Fig 6A and B). Interestingly, $\Delta pcnB$ -infected mice formed two distinct populations: one population where the deeper tissues were colonized to the same levels as wildtype-infected mice, and a second population that had completely cleared the infection (Fig. 6A and B). We isolated several $\Delta pcnB$ clones from the livers of colonized mice and found that their pYV stability and T3SS activity phenotypes were similar to the inoculum strain (data not shown). In addition, we sequenced the genomes of these isolates and could not identify any significant mutations (data not shown), suggesting that host tissue colonization by the $\Delta pcnB$ mutant in a subset of mice does not stem from suppressor mutations that stabilize pYV. Taken together, these data suggest that PAP I increases the likelihood of *Yersinia* colonization of host tissues.

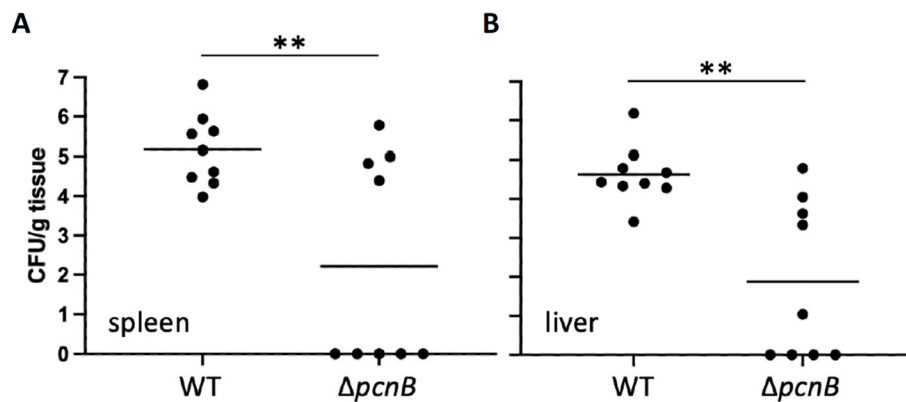


Figure 6. The *pcnB* gene is required for normal *Y. pseudotuberculosis* virulence in a mouse IP infection model. Six- to eight-week-old C57Bl/6 mice were infected with $1-3 \times 10^3$ *Y. pseudotuberculosis* IP2666pIB1 via intraperitoneal (IP) injection. Tissues were collected, homogenized, and plated to determine CFU per gram (CFU/g) tissue 4 days post-inoculation. The CFU/g tissue from the spleens (A) and livers (B) of infected mice are shown.

PAP I does not impact *Y. pseudotuberculosis* stress response to temperature, acid shock, or osmotic shock

While analyzing our RNA-seq data, we identified several differentially expressed chromosomal transcripts in the $\Delta pcnB$ mutant that are involved in stress responses (Supplemental Dataset 1). EcPAP from *E. coli* has been previously reported to play a role in regulating mRNA transcripts important for responding to stress, where loss of PAP I leads to stabilization of stress response transcripts [42, 66]. This stabilization makes $\Delta pcnB$ *E. coli* cells more tolerant of several stresses, including osmotic stress, acid stress, heat shock, and cold shock [66]. Given that many of the major domains and residues are conserved between PAP I and EcPAP (Fig 2A), we next determined if loss of PAP I influences *Y. pseudotuberculosis* growth following stress exposure. Interestingly, we found no significant difference in growth between wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 following heat shock at 42°C, growth at 4°C, acid shock (pH 3.0), osmotic shock (+800mM NaCl), or after treatment with 5% bile salts (Fig. 7A-E). These data suggest that PAP I has species-specific effects on gene expression and stress responses, but maintains PCN and stability of sRNA-regulated plasmids in both *E. coli* and *Yersinia*.

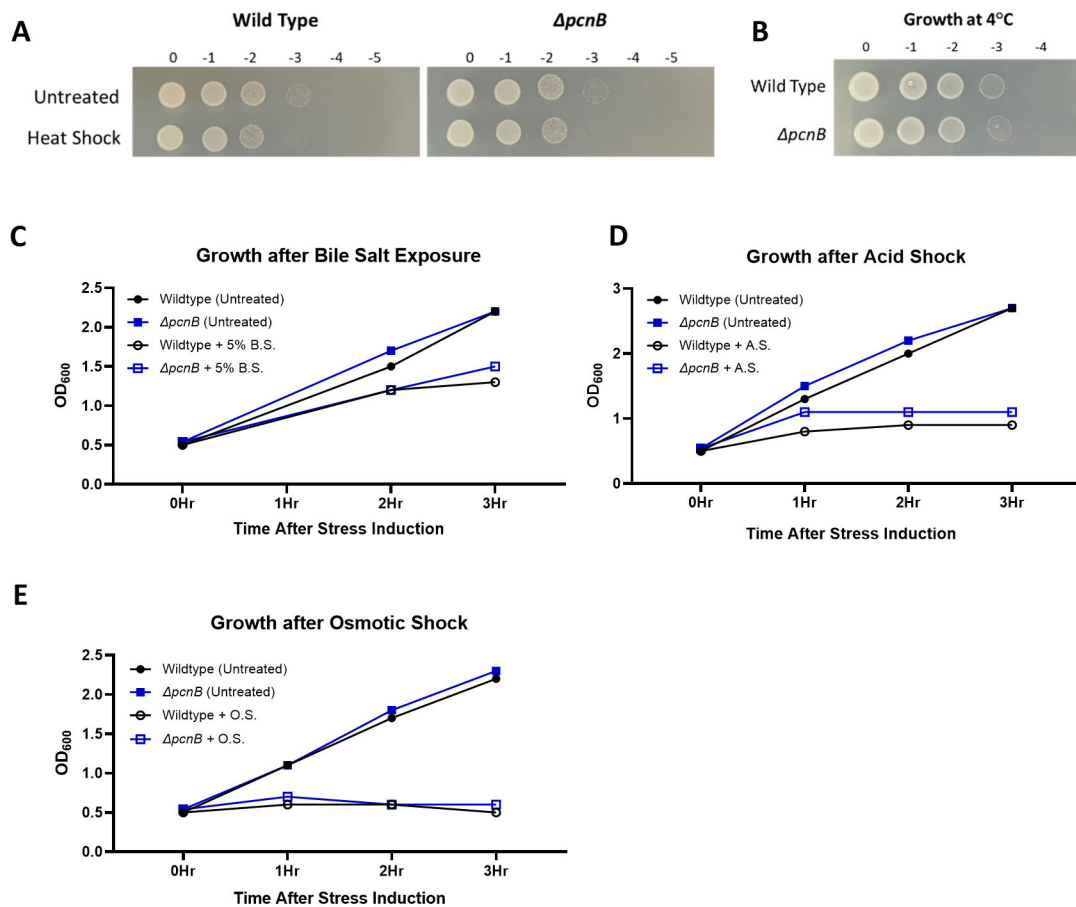


Figure 7. Loss of PAP I does not affect *Y. pseudotuberculosis* growth following stress exposure. (A) Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 cultures were grown to midlog phase at 26°C before shifting to 42°C for 1 hour to induce heat shock. Spot dilutions were plated onto LB plates and incubated at 26°C for ~16 hours before imaging. (B) Strains in the IP2666pIB1 background were grown at 26°C overnight (stationary phase) before diluting and spotting into LB plates. Plates were incubated at 26°C for 4 hours before transferring to 4°C and allowing growth for ~2 days prior to imaging. (C-E) Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 were grown at 37°C (low calcium) to midlog phase before inducing stress. Growth was assayed by plating spot dilutions following stress exposure. Stress was induced by (C) incubation in the presence of 5% bile salts (B.S.), (D) adjusting media to pH=3.0 to induce acid shock (A.S.), or (E) addition of 800Mm NaCl₂ to induce osmotic shock (O.S.).

Discussion

Y. pseudotuberculosis modulates the plasmid copy number (PCN) of its pYV virulence plasmid to control the dosage of pYV-encoded genes in response to temperature [20, 21]. This ability of *Y. pseudotuberculosis* to upregulate pYV PCN at mammalian body temperature is critical for its ability to cause disease. Here, we show that in addition to dynamic regulation of pYV PCN, *Y. pseudotuberculosis* requires the chromosomal gene *pcnB*, encoding the PAP I polyadenylase, to maintain temperature-specific pYV PCN homeostatic while preventing pYV loss from the bacterial population. Although *pcnB*/PAP I has been shown to regulate plasmid replication of model plasmids in *E. coli*, this is the first study to link this gene to bacterial pathogenesis. In addition, the requirement of *Yersinia pcnB*/PAP I for robust plasmid-encoded antibiotic resistance suggests that PAP I represents a potential new drug target.

Putative PAP I orthologs have been bioinformatically identified in many bacterial species hailing from diverse phyla, and yet studies on *pcnB*/PAP I have historically been confined to small number of non-pathogenic bacterial species [35]. *E. coli* PAP I (EcPAP) has been shown to promote RNA degradation of target transcripts. Degradation of RNA is generally initiated by the endonuclease RNase E and, in the absence of 3' secondary structures, the RNA fragments produced by RNase E are then digested by the 3'-5' exonucleases PNPase and RNase II [67]. However, RNase II- and PNPase-mediated RNA degradation is significantly impaired for transcripts that have terminal stem-loop structures, such as mRNAs containing intrinsic terminators at their 3' ends and structured sRNAs [67]. PAP I has been shown to add poly(A) tails to the 3' ends of transcripts containing terminal stem-loops

structures to promote their decay [68-70] and it is thought that PAP I-dependent polyadenylation of these transcripts provides a “toe-hold” for PNPase and RNase II to bind and digest the RNA [70-73]. Components of the RNA degradeosome such as PNPase are known to be important for T3SS function in *Y. pseudotuberculosis* [74-76] and PAP I has been shown to copurify with PNPase in *E. coli* [77], suggesting that PAP I may represent a novel component of the *Yersinia* RNA degradeosome. Importantly, many of the major domains and catalytic residues are conserved between PAP I and EcPAP. EcPAP polyadenylase activity is abolished when the two catalytic residues Asp80 and Asp82 are mutated to alanine residues in *E. coli* [54, 55]. To test if the phenotypes of the $\Delta pcnB$ strain was due to loss of PAP I polyadenylase activity, we mutated the corresponding catalytic residues (Asp114 and Asp116) in PAP I from *Y. pseudotuberculosis* to generate a PAP I^{D2A} strain with both catalytic residues replaced by alanine residues. The PAP I^{D2A} mutant recapitulated the phenotypes observed for the $\Delta pcnB$ strain, including reduced pYV PCN, plasmid stability, and T3SS activity. This supports that PAP I acts as a polyadenylase to regulate plasmid maintenance and T3SS activity in *Y. pseudotuberculosis*.

EcPAP has been shown to polyadenylate both chromosomally-derived and plasmid-derived transcripts and was first identified due to its regulation of a sRNA that regulates plasmid replication. EcPAP has been shown to regulate IncFII family plasmid R1 PCN by polyadenylating and targeting CopA regulatory sRNA for degradation, leading to de-repression of R1 plasmid replication and increasing R1 PCN [46]. This is consistent with our finding that PAP I is required for normal PCN and maintenance of the IncFII family pYV plasmid and suggests that PAP I might also target CopA sRNA from the pYV *cop-rep* locus for polyadenylation in *Y.*

pseudotuberculosis. Bacteria are thought to maintain large extrachromosomal plasmids such as IncF virulence plasmids within a tight range near a specific “set point” that is optimal for that species lifestyle [24]. This is particularly complicated in the case of pYV since this plasmid seems to have a different optimal “set point” depending on temperature. Given that the $\Delta pcnB$ mutant displays a PCN defect at both 26°C and 37°C, this suggests that pYV plasmid replication rates are unusually low in the absence of PAP I in general, possibly due to the buildup of CopA sRNA. Reduced plasmid replication rates can also lead to plasmid stability defects when the plasmid replication rate drops low enough that cells can no longer reliably pass a copy of the plasmid to daughter cells during cell division [78]. Both reduced PCN and reduced plasmid stability can theoretically drive compounding disturbances in plasmid maintenance systems due to reduced gene dosage of stability systems such as partitioning and toxin-antitoxin (TA) systems. In keeping with this idea, our RNA-seq analysis supports that the decrease in pYV-encoded gene dosage in the absence of PAP I leads to decreased expression of the ParDE TA system in $\Delta pcnB$ cells that may be responsible for killing plasmidless cells [22]. Altogether, this supports that PAP I is required for maintenance of the pYV PCN “set point” at both 26°C and 37°C, possibly through modulation of CopA sRNA levels.

CopA is a structured sRNA that can repress expression of the essential plasmid replication factor RepA [24, 46]. Interestingly, a study looking at the *Yersinia* RNA structurome in response to temperature found evidence that CopA sRNA secondary structure is maintained at 26°C and becomes less structured at mammalian temperatures of 37°C [79]. Given that CopA sRNA stem-loop structure is important to its ability to regulate replication of plasmid R1 [46], this suggests that

CopA sRNA might be a more potent repressor of pYV replication at 26°C compared to 37°C and changes in CopA structure may therefore contribute to the temperature-dependent shift in pYV PCN. Shortly after the shift to T3SS-inducing conditions, *Y. pseudotuberculosis* undergoes growth arrest, which may also contribute to increased pYV PCN specifically at 37°C. While there is an overall reduction in pYV PCN in the absence of *pcnB*/PAP I, $\Delta pcnB$ cells that retain the pYV plasmid still increase pYV PCN upon shifting from 26°C to 37°C. In addition, we have found that the *pcnB* gene is transcribed at 26°C and 37°C (data not shown) and the PAP I protein is also produced at both temperatures. Taken together, this suggests that PAP I is not required for the temperature-dependent shift in pYV PCN but rather for maintenance of the pYV plasmid in general as it undergoes dynamic shifts in PCN during the *Yersinia* facultative lifestyle. We hypothesize that PAP I polyadenylates and promotes degradation of pYV-encoded CopA sRNA to regulate cellular CopA levels at both 26°C and 37°C. This would help maintain CopA levels within a range that promotes maintenance of pYV PCN near its optimal “set point” and ensuring that pYV-encoded gene dosage remains high enough to support replication and maintenance of the plasmid under conditions where pYV PCN is normally kept low. In the absence of PAP I, we hypothesize that pYV plasmid replication rates decrease due to abnormally high CopA sRNA levels, leading to reduced pYV PCN, plasmid stability, and T3SS activity. Additional studies are needed to determine how changes in CopA sRNA structure contribute temperature-dependent shifts in pYV PCN as well as to determine exactly how PAP I from *Y. pseudotuberculosis* impacts the half-life of pYV-encoded CopA sRNA.

We found that PAP I is required for normal *Y. pseudotuberculosis* virulence in a mouse intraperitoneal infection model, consistent with its role in promoting high pYV-encoded gene dosage and pYV plasmid stability, and therefore T3SS activity. Interestingly, $\Delta pcnB$ -infected mice fell into two groups: one in which the spleens and livers were colonized with *Yersinia* to similar levels as wildtype-infected mice, and another in no $\Delta pcnB$ cells could be recovered from spleens and livers four days post-inoculation. Overall, this led to a significant decrease in spleen and liver colonization by $\Delta pcnB$ compared to wildtype *Y. pseudotuberculosis*. Consistent with our finding that deletion of *pcnB* leads to enhanced pYV loss from the bacterial population *in vitro*, wildtype cultures used for mouse inoculation had 100% retention of pYV whereas the $\Delta pcnB$ inoculum had an average retention of 81%. This 19% decrease in pYV+ $\Delta pcnB$ cells in the inoculum are not likely to explain the difference in virulence, as wildtype inoculums of 10^3 to 3×10^3 yielded similar spleen and liver colonization levels. These data suggest that *pcnB*/PAP I may aid *Yersinia* in survival of a colonization bottleneck during infection. To our knowledge, this is the first time that PAP I has been directly linked to virulence of a bacterial pathogen and highlights the importance of understanding how PAP I and virulence plasmid gene dosage contribute to bacterial pathogenesis.

All $\Delta pcnB$ cells recovered from the spleen and livers of infected mice retained at least one copy of the pYV plasmid despite the plasmid stability defect observed for this strain *in vitro* (data not shown). These data suggest a selective pressure to retain pYV during growth in mouse tissues, consistent with the importance of the T3SS to *Yersinia* virulence. This is in contrast to what we observe *in vitro* when pYV and T3SS expression is a metabolic burden and not a benefit. These data suggest that

pcnB/PAP I is required for maintenance of the pYV plasmid under all conditions, even when pYV is dispensable to survival. This is necessary to ensure that enough of the *Y. pseudotuberculosis* population is pYV⁺ to be able to colonize a new host and survive in host tissues.

In addition to pYV, we found that *pcnB* is important for robust replication of sRNA-regulated plasmids encoding antimicrobial resistance (AMR) genes in *Yersinia*. When *Y. pseudotuberculosis* $\Delta pcnB$ cells are grown in the presence of the plasmid-selective antibiotic, we observed a ten-fold decrease in growth, consistent with previous studies in *E. coli* [28]. Similarly, we found a ten-fold decrease in GFP fluorescence in the $\Delta pcnB$ strain carrying the ColE1 pGFPuv plasmid compared to wildtype, suggesting a ten-fold decrease in plasmid-encoded gene expression in the absence of *pcnB*. In addition, time course experiments found that the majority of the $\Delta pcnB$ population loses the pGFPuv plasmid within just 10 hours of removing the selective antibiotic. These data support that ColE1 PCN and plasmid stability is reduced in the absence of PAP I, rendering cells more antibiotic sensitive. Interestingly, the rate at which pYV is lost from *Y. pseudotuberculosis* *in vitro* under non-T3SS inducing conditions was far slower than for pGFPuv. We passaged wildtype and $\Delta pcnB$ strains in LB media at 26°C and while 100% of wildtype retained pYV, only ~60% of $\Delta pcnB$ cells retained pYV by 13 days with a still increasing rate of loss (data not shown). As pGFPuv has no active plasmid partitioning or maintenance systems but pYV encodes ParAB and ParDE, we hypothesize that the rate of plasmid loss in cells lacking PAP I activity will depend on plasmid-encoded mechanisms that promote plasmid stability.

Through our RNA-seq analysis we identified several differentially regulated transcripts in the $\Delta pcnB$ mutant that are involved in responding to several types of stress, including the universal stress protein UspB, the *Yersinia* urease operon, and several cold-shock proteins (Supplemental Dataset 1). Importantly, these differentially regulated genes are encoded on the *Y. pseudotuberculosis* chromosome and so should not be significantly affected by the pYV PCN defect in the $\Delta pcnB$ mutant. In addition, EcPAP from *E. coli* has been previously reported to play a critical role in regulating mRNA transcripts important for responding to stress where loss of PAP I leads to stabilization of stress response transcripts [42]. Deletion of *pcnB* in *E. coli* results in increased tolerance to several stresses, including osmotic stress, acid stress, heat shock, and cold shock [66]. Interestingly, and we found that loss of PAP I does not affect *Y. pseudotuberculosis* viability following heat shock, acid shock, osmotic shock, or exposure to bile salts. Loss of PAP I also did not affect *Y. pseudotuberculosis* growth at 4°C. In addition, EcPAP activity is regulated at least in part by phosphorylation of Y202 where this phosphorylation is associated with reduced EcPAP activity during stress, leading to stabilization of transcripts important for stress response in wildtype *E. coli* [66]. However, mutation of the corresponding tyrosine residue (Y239) to an alanine (Y239A) or a glutamate (Y239E) in PAP I from *Y. pseudotuberculosis* had no detectable phenotypes (data not shown). Our RNA-seq analysis also found that some stress response genes such as CspE are downregulated in $\Delta pcnB$ cells, suggesting that PAP I normally promotes stability of at least some stress-related transcripts in *Y. pseudotuberculosis*. These data suggest that while PAP I regulates plasmid-derived sRNA in multiple bacterial species, its effect on stress responses is species-specific.

While *E. coli* EcPAP and PAP I from *Y. pseudotuberculosis* are highly conserved in general, some regions of the proteins are less conserved and might account for their different impacts on stress tolerance. Interestingly, while the N terminal region following the initial propeptide sequence is predicted to be disordered for both PAP I and EcPAP, this is also the region that displays the least amount of conservation between the two proteins. In contrast, this N terminal region is 100% conserved between *Y. pseudotuberculosis* and *Y. pestis* (data not shown). This disordered region is also significantly expanded in length for PAP I from *Y. pseudotuberculosis* and *Y. pestis* compared to EcPAP. Given that disordered regions of RNA-binding proteins have been proposed to mediate interactions with target RNAs and/or other proteins [80], it is possible that PAP I has evolved to target different RNA transcripts and/or interacts with one or more unique protein partners compared to EcPAP. This suggests that bacterial PAPs like PAP I have conserved roles in polyadenylase function and regulating plasmid-derived sRNAs but may be adapted to regulate other mRNA transcripts in a more-species specific way.

In this study, we show for the first time that *pcnB*/PAP I is required for maintenance of a large IncFII virulence plasmid (pYV) that undergoes dynamic yet specific changes in PCN to regulate virulence gene dosage in *Y. pseudotuberculosis*. PAP I was also found to be required for normal *Y. pseudotuberculosis* virulence, providing the first direct evidence that a PAP I regulates virulence of a Gram negative pathogen. PAP I is required for maintenance of plasmids from many families in *Y. pseudotuberculosis*, including families that commonly include virulence and antimicrobial resistance plasmids. PAP I homologs can be found in many species of Gram negative pathogenic bacteria, many of which harbor extrachromosomal

plasmids important for their pathogenic lifestyles, and genomic studies show that the *pcnB* gene is highly subject to horizontal gene transfer [35]. Our data supports that bacterial PAP I has a conserved role in promoting the stability of many sRNA-regulated plasmids and may be critical for the establishment of new plasmids in a bacterial population following horizontal transfer by promoting plasmid replication. However, given the different effects of PAP I loss to stress resistance in *E. coli* and *Yersinia*, PAP I targeting of transcripts that are chromosomally-encoded likely varies between species. These data suggest that bacterial polyadenylation may regulate different pathways in different bacterial species. Future studies will explore the possibility of targeting PAP I activity as a novel antibiotic and/or plasmid-curing strategy as well as the contribution of PAPI-mediated polyadenylation to regulation of gene expression in different pathogenic bacteria.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

All strains used in this study are listed in Table 3. *Y. pseudotuberculosis* strains were grown in LB or 2xYT media (pH=7) at 26°C or 37°C shaking at 250rpm, unless otherwise indicated.

Construction of *Y. pseudotuberculosis* mutant strains

All primers and plasmids used to generate *Y. pseudotuberculosis* strains for this study are listed in Tables 4 and 5, respectively. Inserts were amplified by PCR using

primers designed for Gibson Assembly and final recombinant plasmids were all made using the pCVD442 suicide vector backbone (λ pir-dependent replicon, carbenicillin resistant [Carb^R], *sacB* gene conferring sucrose sensitivity) (Addgene) following digestion with NdeI and BbsI. All ligations were performed using NEBuilder HiFi DNA Assembly kit (New England Biolabs, Inc.). Recombinant pCVD442 plasmids were transformed into *E. coli* S17-1 λ pir competent cells and introduced into *Y. pseudotuberculosis* IP2666pIB1 or YPIII/pIBX via conjugation. Clones that were Carb^R and irgansan-resistant (*Yersinia* selective antibiotic) were grown in the absence of antibiotics and plated onto media containing 10% sucrose to induce *sacB*-mediate toxicity for pCVD442 loss. Clones that were Carb sensitive, sucrose-resistant, and Congo red-positive (pYV+) were screened by PCR and sequence verified by Sangar sequencing. Construction of *pil* insertion strains: The *pil::Tn* *Y. pseudotuberculosis* IP2666pIB1 strain was generated previously [52]. The *pil::pNQ* *Y. pseudotuberculosis* YPIII/pIBX mutant was independently generated and gifted to us by Dr. Helen Wang. Construction of Δ *pcnB*: ~500bp upstream of *pcnB* and ~500bp downstream of *pcnB* was PCR amplified using genomic DNA (gDNA) from *Y. pseudotuberculosis* IP2666pIB1. Amplified fragments were cloned into pCVD442 to produce pCVD442:: Δ *pcnB*. Construction of ParB-GFP: To make ParB-GFP YPIII/pIBX strains, three fragments were amplified for insertion into pCVD442: (1) the last ~500bp of the *parB* gene excluding the stop codon was amplified from *Y. pseudotuberculosis* gDNA, (2) msfGFP was amplified from pDK13 and (3) 500bp downstream of the *parB* gene was amplified from *Y. pseudotuberculosis* gDNA. The resulting fragments were cloned into pCVD442 to generate a *parB*-msfGFP construct that includes 500bp of downstream homology after msfGFP. Construction of PAPI-C-

6xHis and PAPI-C-3xFLAG: To make PAPI-6xHis strains, *pcnB* was amplified from *Y. pseudotuberculosis* gDNA and cloned into pET28 that was previously digested with XhoI and NcoI to produce PAPI-C-6xHis. PAPI-C-6xHis was then amplified from pET28 and 500bp downstream of *pcnB* was amplified from gDNA for insertion into pCVD442 to make pCVD442::PAPI-c-6xHis containing 500bp of homology flanking the 6xHis tag. We subsequently found that the 6xHis tag was not sufficient to visualize the PAPI protein, and so alternative PAPI-C-3xFLAG strains were made in order to carry out Western blot analyses. To make PAPI-FLAG, three fragments were amplified for insertion into pCVD442: (1) the *pcnB* gene was amplified from *Y. pseudotuberculosis* gDNA (2) 3xFLAG was amplified from pET28 and (3) 300bp downstream of *pcnB* was amplified from *Y. pseudotuberculosis* gDNA. The fragments were then cloned into pCVD442 to make pCVD442::PAPI-c-3xFLAG containing 300bp of downstream homology following the 3xFLAG tag. Construction of PAPI(D2A)/(L291R)/(L291A): To make PAPI(D2A)/(L291R)/(L291A) mutations, we used the Q5 site-directed mutagenesis kit (New England Biolabs, Inc.) to introduce base pair changes at the corresponding residues using the pCVD442::PAPI-c-6xHis or pCVD442::PAPI-c-3xFLAG recombinant plasmids as templates.

In vitro suppressor screen

LB cultures for the *pil::pNQ* YPIII/pIBX mutant were grown at 26°C shaking overnight before diluting in fresh media and spotting for single colonies onto low calcium NaOX plates (LB plate containing 20mM sodium oxalate and 20mM MgCl₂). Plates were incubated at 37°C for approximately 16 hours to induce T3SS expression and selective pressure for suppressor mutation formation. CFU that broke growth arrest

and thus formed large colonies were selected and glycerol stocks were made to construct a *pil::Tn* suppressor mutant library. High throughput screening for changes in pYV PCN was carried out using the luciferase PCR assay (see below). Suppressor mutants were screened for changes in T3SS activity using an in vitro secretion assay (see below).

Identification of mutations arising from suppressor screen

A subset of suppressor mutants from the *pil::pNQ* YPIII/pIBX suppressor mutant library were selected for whole genome sequencing. In brief, cells were grown shaking at 26°C overnight and total genomic DNA was extracted using the Blood & Tissue DNeasy kit (Qiagen) according to manufacturer recommendations. Final genomic DNA concentration was determined by nanodrop. Qbit quantification, Hiseq single read 150 (Hiseq 4000 SR150) library prep, and illumina sequencing was carried out by the UC Davis DNA Technologies Core. Adapters and low-quality reads were trimmed by trimmomatic (Version 0.39) [81]. The trimmed sequences from each sample were aligned to the reference genome using BWA [82]. The reference sequence is composed of the YPIII chromosome (CP009792.1) [83] and its plasmid (CP032567.1) [84]. Samtools [85] was used to sort and index the aligned reads. Duplicate reads originated from a single DNA fragment during sample preparation were located and tagged by MarkDuplicates in Picard (<http://broadinstitute.github.io/picard/>). Single nucleotide polymorphisms were detected and joined by GATK (Version 4.1.6) [86].

Droplet digital PCR (ddPCR)

ddPCR to determine pYV plasmid copy number was carried out as previously described [21]. Total DNA was extracted from bacterial cells using GeneJet Genomic DNA Purification kit (Thermo Scientific) according to the manufacturer's recommendations for Gram negative bacteria. The final concentration of DNA was determined by nanodropping before setting up samples for ddPCR. Each gDNA sample was split into two reactions and the primer pair F_chrom_ddPCR/R_chrom_ddPCR was used for to amplify chromosomal DNA and F_pYV_ddPCR/R_pYV_ddPCR was used to amplify pYV DNA. Individual ddPCR samples were prepared as follows: 10.5uL of total genomic DNA was combined with 12.5uL of 2xEVA-Green master mix for unlabeled probes (Bio-Rad), 1uL of HindIII-HF (NEB), and 1uL of respective primer pairs (200nM final concentration) in 8-strip PCR tubes. PCR tubes were incubated at room temperature for 10 minutes to allow HindIII to digest genomic DNA. Droplet generation was carried out using an Automated Droplet Generator (Bio-Rad) and droplets were subsequently transferred to a deep-dish qPCR plate (Bio-Rad) for PCR amplification. PCR was carried out with hot-start activation at 95°C for 5 mins following by denaturation at 94°C for 30s, amplification at 58°C for 1 min over 40 cycles, and signal stabilization at 4°C for 10 min and 90°C for 5 min. A ramp rate of 2C/s was used for all PCR steps. Droplets were analyzed using a QX200 droplet reader (Bio-Rad) and data was analyzed using QuantaSoft Analysis Standard Edition (version 1.2) (Bio-Rad). Statistical significance was calculated using an ordinary one-way ANOVA for multiple comparisons (**** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05) and graphs were generated using GraphPad Prism (10.2.0).

Luciferase plasmid copy number assay

Overnight cultures for strains in the YPIII/pIBX background were grown shaking at 26°C before subculturing to OD₆₀₀=0.2 in 2xYT low calcium media (containing 20mM sodium oxalate and 20mM MgCl₂). Cultures were then grown shaking for 1.5h at 26°C before shifting to 37°C. Timepoints to measure luminescence and OD₆₀₀ were collected after 1.5h at 26°C just before shifting to 37°C and every hour after shifting to 37°C. Samples were collected at each timepoint in white walled, clear bottom 96 well plates and luminescence (using lum aperture for 96 well plate with measurement time=0.1s) and OD₆₀₀ (using Photometric 600 excitation filter) was measured using a PerkinElmer Multimode Plate Reader EnVision system. Relative plasmid copy number was determined by dividing luminescence by OD₆₀₀ for the same sample. Statistical analysis was determined using a 2way ANOVA for multiple comparisons (**** P<0.0001, *** P<0.001, ** P<0.01, * P<0.05) where applicable. All graphs were made using GraphPad Prism (10.2.0).

ParB-msfGFP microscopy and analysis

Wildtype and $\Delta pcnB$ YPIII/pIBX cells expressing ParB-GFP were grown shaking at 200rpm overnight at 26°C in EZ Rich Defined Medium (EZRDM) (Teknova) to reduce media autofluorescence. A secretion analysis was performed to confirm that *Y. pseudotuberculosis* displays normal T3SS activity when grown in EZRDM under inducing conditions (37°C/low calcium) (data not shown). For non-inducing conditions, overnight cultures were diluted 1:10 in fresh EZRDM and grown shaking for 3 hours at 26°C. Cells were then spotted onto a 1% agarose-EZRDM pad and

covered with a no. 1.5 coverslip for imaging. For inducing conditions, overnight cultures were diluted 1:10 in low calcium EZRDM (containing 20mM sodium oxalate and 20mM MgCl₂) and grown shaking for 1.5 hours at 26°C before shifting to 37°C for an additional 1.5 hours. Cells were prepared for imaging by spotting onto a 1% agarose-EZRDM low calcium pad and covering with a no. 1.5 coverslip. Imaging was carried out using fluorescence microscopy (exposure time and conditions = 50ms GFP 50% power and 100ms Phase). For image analysis, cells were segmented and cell outlines were generated using Oufi with the same parameters developed for visualizing *E. coli* cells [87]. Cell meshes were further curated by SVM_curate.m to identify and remove misdected cells [88]. Detection of pYV spots was performed using the ImageJ plug-in package ThunderSTORM [89] and detected spots were correlated with cell meshes using the code ypstb_spotdetection.m. Three biological replicates were performed per strain/condition and data was plotted into histograms using GraphPad Prism (10.2.0).

In vitro pYV plasmid stability assay

Cells were struck for single colonies onto LB plates and grown at 26°C for 1.5-2 days. To test plasmid stability after passing at 26°C only, single colonies were then patched onto LB plates for ~16 hours at 26°C before heavily streaking for single colonies onto fresh LB plates at 26°C for 1.5-2 days. To test plasmid stability after exposure to T3SS-inducing conditions, the same colonies were also patched onto NaOX plates to induce T3SS activity for ~16 hours at 37°C before streaking for single colonies onto LB plates and returning to 26°C to grow for 1.5-2 days. Final plasmid stability was determined for both conditions by patching single colonies from

the final 26°C streak plate onto NaOX plates containing 1% congo red. The percentage of cells that retained the pYV plasmid was calculated by dividing the number of red (pYV+) CFU by the total number of CFU assessed. Single colony PCR was used on a subset of patches to validate that red CFU retain pYV and while white CFU do not (data not shown).

In vitro Type III secretion assay

Visualization of secreted T3SS cargo to assess T3SS activity was carried out as previously described [90]. *Y. pseudotuberculosis* cultures were grown shaking overnight at 26°C before subculturing in LB low calcium media (LB containing 20mM sodium oxalate and 20mM MgCl₂). Cultures were grown shaking at 26°C for 1.5 hours before shifting to 37°C to grow shaking for an additional 1.5 hours. Cultures were normalized to the same number of cells using OD₆₀₀ and pelleted at 13,200 rpm for 15min at room temperature. Supernatants were transferred to new tubes and proteins precipitated with 10% trichloroacetic acid (TCA). A bovine serum albumin (BSA) protein control was spiked into each sample immediately before the addition of TCA. Samples were incubated in TCA on ice for at least 1 hour before pelleting at 13,200 rpm for 15 min at 4°C. Pellets were then washed twice with ice-cold 100% acetone and resuspended in final sample buffer (FSB) containing 0.2M dithiothreitol (DTT). Samples were boiled for 10 mins at 95°C before loading and separating on a 12.5% SDS-PAGE gel. Gels were stained with Coomassie blue to visualize protein and imaged using Bio-Rad Image Lab Software Quantity and Analysis tools.

RNA isolation and RNAseq analysis

Y. pseudotuberculosis IP2666pIB1 strains were grown in 3mL LB overnight at 26°C and diluted the following morning to an OD₆₀₀ of 0.2 in 5mL fresh LB. Each strain was diluted into two separate cultures (one for each temperature). For 26°C samples, cultures were diluted in plain LB media and grown shaking at 26°C for 3 hours before extracting RNA. For 37°C samples, cultures were diluted into LB low calcium media (containing 20mM sodium oxalate and 20mM MgCl₂) and grown shaking at 26°C for 1.5 hours before shifting to 37°C to growing shaking for an additional 1.5 hours. After incubating, 1-2mL of each culture was collected and pelleted for 5 min at 4,000 rpm. Supernatants were removed and cell pellets were resuspended in 1mL RNA Protect (Qiagen) and total RNA was isolated using the RNeasy minikit (Qiagen) according to the manufacturer's protocol. DNA was removed from samples using the TURBO DNA-free kit (Life Technologies/Thermo Fischer) and RNA quality was determined using an Agilent 2000 Bioanalyzer. Illumina library prep and differential expression analysis was performed by Seqcenter, LLC. To generate heat maps, average TMM (Trimmed Mean of the M-Values) were utilized as the raw input data for the clustering pipeline. Subsequently, L2 normalization was applied to the dataset to ensure consistent scaling prior to the application of the elbow method and K-means clustering. The elbow method was then employed to find the optimal number of clusters required for the subsequent K-means clustering step. Once the clusters were successfully generated, a heatmap of the clusters were visualized using matplotlib and seaborn which are Python visualization packages. Hub tracks from this analysis can be viewed using the

following link:

http://lowelab.ucsc.edu/hubs/yersinia_pcnB_pil_expression/hub.txt

Western blot analysis

Cells were grown at the indicated temperature before preparing samples for Western blotting. Samples were normalized to the same number of cells using OD₆₀₀ at the time of sample collection. Bacterial pellets were then resuspended in FSB containing 0.2M DTT and boiled at 95°C for 15 minutes. Secreted proteins were precipitated using TCA in the same way as described for the in vitro secretion assay above. The same volume of each sample was separated on a 12.5% SDS-PAGE gel and proteins were transferred to a blotting membrane (Immunobilon-P) using a wet mini trans-blot cell (Bio-Rad) at 4°C. Blots were blocked for one hour in Tris-buffered saline containing Tween 20 and 5% milk. Blots were probed with anti-RpoA (gift from Melanie Marketon), goat anti-YopE (Santa Cruz Biotech), mouse M2 anti-FLAG (Sigma), and horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotech). Bands were visualized using a BioRad ChemiDoc and quantification analysis was performed using Image Lab software (Bio-Rad).

Mouse Infections

C57Bl/6 mice were purchased from Jackson Laboratory. Six- to eight-week-old C57Bl/6 mice were infected with $1-3 \times 10^3$ *Y. pseudotuberculosis* via intraperitoneal (IP) injection as previously described [91]. In brief, LB overnight cultures of *Y. pseudotuberculosis* were grown shaking at 26°C before spinning down at 5000rpm and resuspending in sterile PBS (pH=7.4). Samples were then diluted in PBS such

that 100uL contained $1-3 \times 10^3$ cells. Mice were injected with 100uL of inoculum and sacrificed four days post-inoculation and spleens and livers were harvested, homogenized, and serial dilutions of the homogenate were plated to determine CFU per gram (CFU/g) of tissue. Original inoculums used for the infection were plated for single colonies to confirm the inoculation dose was in the $1-3 \times 10^3$ range and to estimate the percentage of cells that were pYV+. CFU used for the inoculum cultures were confirmed to contain pYV prior to infection using single colony PCR with primers specific to the pYV plasmid (data not shown). 100uL of the inoculums used for the infection were plated for single colonies to confirm the inoculation dose was in the $1-3 \times 10^3$ range and to estimate the percentage of cells that were pYV+ by patching onto NaOX plates containing 1% congo red dye. The plasmid retention of the starting inoculums was found to be 100% for wild type and 81% for $\Delta pcnB$ (after averaging the inoculums for all infections).

Construction of PAPI expression vector:

The *pcnB* gene was PCR amplified from *Y. pseudotuberculosis* gDNA using the primer pair FpcnB_pTrc/RpcnB_pTrc and ligated into pTrc99a previously digested with NcoI and XbaI using NEBuilder HiFi DNA Assembly kit (New England Biolabs, Inc.). Constructs were transformed into DH5 α *E. coli* for propagation and sequence verified via Sanger sequencing. The resulting pTrc99a::PAPI recombinant expression vector was introduced to wild type and $\Delta pcnB$ *Y. pseudotuberculosis* by electroporation and selection on LB plates containing carbenicillin. Expression of PAPI was induced by plating onto LB plates containing 0.2mM IPTG.

Spot tests to analyze retention of pGFPuv, pTrc99, and pNF06

Wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 were electroporated with 1uL of pGFPuv [Carb^R] (Gifted by Dr. Manel Camps), pTrc99a, or pNF06::ccdAB [Kan^R] (Gifted by Dr. Laurence Van Melderren) and single colonies were selected by plating on LB plates containing carbenicillin (pGFPuv, pTrc99a) or kanamycin (pNF06). For spot tests, overnight cultures were grown at 26°C before diluting in PBS (pH=7.4) and making a series of 1:10 dilutions. Serial dilutions were spotted onto LB plates containing no antibiotic or LB plates containing the selective antibiotic and plates were visualized after 1-1.5 days of growth at 26°C.

Time course to analyze retention of pGFPuv

Wildtype and $\Delta pcnB$ IP2666pIB1 lacking pYV (pYV-) and harboring pGFPuv were grown shaking at 26°C in LB media overnight in the presence of 100ug/mL carbenicillin to select for pGFPuv retention. The following day, cultures were subcultured 1:1000 in 50mL of fresh LB media (without carbenicillin) and grown shaking at 26°C for the duration of the time course. Cultures were subcultured 1:1000 in plain LB media after 6 and 36 hours to return cells to exponential growth. Retention of the pYV plasmid was determined at the 0 hour (starting population) timepoint immediately following the removal of carbenicillin. Additional timepoints were collected 2, 4, 6, 24, and 36 hours following the transfer to carbenicillin-free media. For each time point, 100uL of culture was collected, diluted, and plated for single colonies onto LB plates (containing no carbenicillin). Plates were incubated at

26°C for 1.5-2 days to allow for colony growth before patching single colonies onto LB plates containing carbenicillin. The percent retention of pGFPuv was determined by dividing the number of colonies that grew on carbenicillin plates by the total number of colonies patched and 50 CFU were analyzed per strain per timepoint.

Stress Assay

To induce heat shock, mid-log ($OD_{600}=0.5-0.7$) cultures of wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* IP2666pIB1 were shifted from 26°C to 42°C for 1h or left at 26°C for the untreated control. After inducing heat shock, cells were diluted 1:100 in PBS (pH=7.4) and a series of spot dilutions were made on LB plates at 26°C. To test growth at 4°C, cultures were grown at 26°C overnight (stationary phase) before diluting 1:10 in PBS (pH=7.4) and spotting serial dilutions onto an LB plate. LB plates were incubated at 26°C for 4 hours before shifting to 4°C and allowing growth for ~2 days before imaging. To induce other types of stress, wildtype and $\Delta pcnB$ cultures were grown to mid-log ($OD_{600}=0.5-0.7$) in 2xYT low calcium media before either adding 800mM NaCl2 (osmotic shock), adjusting to pH=3.0 with HCl (acid shock), or adding 5% bile salts (dehydrated purified Ox bile, Fluka Biochemika). The corresponding volume of sterile water was added to untreated controls for each condition. Cultures were then incubated at 37°C and OD_{600} was measured every hour for 3 hours. At the 1 hour timepoint, samples were collected and diluted 1:10 in PBS (pH=7.4) to make a series of spot dilutions on LB plates at 26°C. Spot plates confirmed that there was no difference in growth between wildtype and $\Delta pcnB$ *Y. pseudotuberculosis* when cells are given days to recover post-stress induction (data not shown).

Table 3. *Y. pseudotuberculosis* strains used in this study

Strain	Background	Mutation(s)	Ref
Wildtype (WT)	IP2666pIB1	Naturally lacks full-length YopT	[92]
<i>pil::Tn</i>	IP2666pIB1	Tn5 insertion in <i>pil</i> , wildtype background	[52]
$\Delta pcnB$	IP2666pIB1	$\Delta pcnB$	This work
<i>pil::Tn$\Delta pcnB$</i>	IP2666pIB1	$\Delta pcnB$, <i>pil::Tn</i> background	This work
PAP I ^{His}	IP2666pIB1	PAP I with C-6xHis tag	This work
PAP I ^{D2A}	IP2666pIB1	D114A+D116A mutations in PAP I (C-6xHis)	This work
PAP I ^{L291R}	IP2666pIB1	L291R mutation in PAP I (C-6xHis)	This work
PAP I ^{L291A}	IP2666pIB1	L291A mutation in PAP I (C-6xHis)	This work
PAP I ^{FLAG}	IP2666pIB1	PAP I with C-3xFLAG tag	This work
PAP I ^{D2A-FLAG}	IP2666pIB1	D114A+D116A mutations in PAPI(C-3xFLAG)	This work
PAP I ^{L291R-FLAG}	IP2666pIB1	L291R mutation in PAP I (C-3xFLAG)	This work
PAP I ^{L291A-FLAG}	IP2666pIB1	L291A mutation in PAP I (C-3xFLAG)	This work
$\Delta yopHEMOJ$	IP2666pIB1	$\Delta yopHEMOJ$	[91]
$\Delta yscNU$	IP2666pIB1	$\Delta yscNU$	[93]
<i>pil::Tn</i>	IP2666pIB1	Tn5 insertion in <i>pil</i> , $\Delta yopEMOJ$ background	[52]
Wildtype (WT)	YPIII pIBX	Naturally lacks full-length YopT	[20]
YpIII:(cIBX) _{n=1}	YPIII pIBX	Single copy of pYV inserted into chromosome	[20]
<i>pil::pNQ</i>	YPIII pIBX	pNQ insertion in <i>pil</i>	This work
$\Delta pcnB$	YPIII pIBX	$\Delta pcnB$	This work
ParB-GFP	YPIII pIBX	ParB-C-msfGFP	This work
$\Delta pcnB^{ParB-GFP}$	YPIII pIBX	$\Delta pcnB$ ParB-C-msfGFP	This work
PAP I ^{His}	YPIII pIBX	PAP I with C-6xHis tag	This work
PAP I ^{D2A}	YPIII pIBX	D114A+D116A mutations in PAP I (C-6xHis)	This work
PAP I ^{L291R}	YPIII pIBX	L291R mutation in PAP I (C-6xHis)	This work
PAP I ^{L291A}	YPIII pIBX	L291A mutation in PAP I (C-6xHis)	This work

Table 4. Primers used in this study

Name	Primer Sequence	Ref
Primers used to make pCVD442::<i>ΔpcnB</i>		
FpcnB_500up_pCVD	caacataaaggatgaatcccatatgAGAAGATTTTATTATCCGTCG	TW [†]
RpcnB_500up_pCVD	ggtaaaattaAATGGTACACCTCGATAG	TW
FpcnB_500d_pCVD	gtgtaccattTAATTTTACCATGATCCGGGTC	TW
RpcnB_500d_pCVD	acctggcacggctgggacggaagtcTGAGCCGGCGATATTACC	TW
Primers used to make pCVD442::<i>PAP</i>^{His}		
FpcnB_pET28	agtgggtgggtgggtgctcagTACCCCTTCTTTACGGGG	TW
RpcnB_pET28	actttaagaaggagatataccatggATTTTACCCGAGTAGCC	TW
FpcnB_500up_His	gaggtgtaccATTTTACCCGAGTAGCCAATTTC	TW
RpcnB_500up_His	catggtaaaaTCAGTGGTGGTGGTGGTG	TW
FpET_pB_His_300d	caacataaaggatgaatcccatatgAGGGGAATAAGCTCTCCAAG	TW
RpET_pB_His_300d	gggtaaaaatGGTACACCTCGATAGTGG	TW
FpcnB_300d_pCVD	ccaccactgaTTTTACCATGATCCGGGTC	TW
RpcnB_300d_pCVD	tgacagtctccggaagacggTGAGCCGGCGATATTACC	TW
Primers used to make pCVD442::<i>PAP</i>^{FLAG}		

FpcnB_FLAG	caacataaaggtgaatcccaTGAAAAATACCGACCACC	TW
RpcnB_FLAG	ctttgtagtcTACCCCTTCTTTACGGGG	TW
FpET_FLAG_pcnB	agaaggggtaGACTACAAAGACCATGACG	TW
RpET_FLAG_pcnB	ggtaaaattaCATATGGTACCAGCTGCAG	TW
FpcnB_500d_pCVD	gtaccatgTAATTTTACCATGATCCGGGTC	TW
RpcnB_500d_pCVD	tgacagtctccggaagacggTGAGCCGGCGATATTACC	TW

Primers used to make pCVD442::ParB-msfGFP

FparB_GFP	caacataaaggtgaatcccatgTTGCTAATGAGTATCGTC	TW
RparB_GFP	cgcctttgaCAAAGAATGTTTCCTTTGC	TW
FpKD_GFP_parB	acattctttgTCAAAAGGCGAAGAACTTTTAC	TW
RpKD_GFP_parB	tattcaggcaTTATTTATACAATTCATCCATTCCATGAGTGAT TCCTGCCGCAGTGACAAATTC	TW
FparB_500d_pCVD	gtataaataaTGCCTGAATAAGATCAGAAC	TW
RparB_500d_pCVD	tgacagtctccggaagacggAATCTCCCTAAAGCTATCAC	TW

Q5 mutagenesis primers used to introduce L291R mutation

FpB_L291R_pCVD	ACTCAAGCTGaggCAATCCGGCTAC	TW
RpB_L291R_pCVD	GACTCCTCAAACAGGCGG	TW

Q5 mutagenesis primers used to introduce L291A mutation

FpB_L291A_pCVD ACTCAAGCTGgcgCAATCCGGCTAC TW

RpB_L291A_pCVD GACTCCTCAAACAGGCGG TW

Q5 mutagenesis primers used to introduce D114A + D116A (D2A) mutations

FpB_D2A_pCVD tcgctATCACCACCAGCGC TW

RpB_D2A_pCVD aagcTTTGGGTTTTCTGCCC TW

Primers used to make pTrc99::PAP I

FpcnB_pTrc atttcacacaggaaacagaccatggATTTTTACCCGAGTAGCCAATTTC TW

RpcnB_pTrc tgcctgcctgcaggctgactctagaCAGCGCGATATAGACCCG TW

Primers used for ddPCR

F_Chrom_ddPCR CCTCACCGATACCGAACGAG [21]

R_Chrom_ddPCR GTCAGCAGGATAGGGCTACC [21]

F_pYV_ddPCR CTCTTTGACCTCGGCTTGAG [21]

R_pYV_ddPCR CGCAGCCGTTAGGACAAATG [21]

[†]TW indicates primers made for This Work

Table 5. Plasmids used in this study

Vector/Plasmid	Insert	Ref
pCVD442::empty	None [SacB ⁺]	[94]
pET28::empty	-C-3xFLAG and -C-6xHis	[95]
pKD13-msfGFP	-C-msfGFP	Gifted ¹
pCVD442:: <i>ΔpcnB</i>	<i>ΔpcnB</i>	This work
pCVD442::PAP I ^{His}	PAP I-C-6xHis	This work
pCVD442::PAP I ^{L291R}	PAP I ^{L291R} -C-6xHis	This work
pCVD442::PAP I ^{L291A}	PAP I ^{L291A} -C-6xHis	This work
pCVD442::PAP I ^{D2A}	PAP I ^{D2A} -C-6xHis	This work
pCVD442::PAP I ^{FLAG}	PAP I-C-3xFLAG	This work
pCVD442::PAP I ^{L291R-FLAG}	PAP I ^{L291R} -C-3xFLAG	This work
pCVD442::PAP I ^{L291A-FLAG}	PAP I ^{L291A} -C-3xFLAG	This work
pCVD442::PAP I ^{D2A-FLAG}	PAP I ^{D2A} -C-3xFLAG	This work
pCVD442::ParB-msfGFP	ParB-C-msfGFP	This work
pTrc99A::empty	None [IPTG-inducible T7]	[96]
pTrc99::PAP I	PAP I	This work
pGFP-uv	GFPuv	[97, 98] Gifted ²
pNF06::ccdAB	ccdAB	[99]

¹Gifted by Dr. Jacobs-Wanger (Stanford University)

²Gifted by Dr. Camps (U.C. Santa Cruz)

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