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4 **Coiled-coils in the YopD translocator family: a predicted structure**
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8 **unique to the YopD N-terminus contributes to full virulence of**
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11 *Yersinia pseudotuberculosis*
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34 **Running head:** Coiled-coil domain required for full YopD function
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52 **Abbreviations:** T3S(S), type III secretion (system); Yops, *Yersinia* outer proteins; Ysc, *Yersinia*
53 secretion; CD, calcium dependent; TS, temperature dependent; BHI, Brain Heart Infusion; LB, Luria-
54 Bertani; TMH, Modified Higuchi's Medium; BLAST, Basic Local Alignment Search Tool; CFU, colony
55 forming unit
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1 **ABSTRACT**

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3 Pathogenic *Yersinia* all harbor a virulence plasmid-encoded Ysc-Yop T3SS. In this system, translocator
4 function is performed by the hydrophobic proteins YopB and YopD. With the goal to better understand
5 how YopD orchestrates *yop*-regulatory control, translocon pore formation and Yop effector translocation,
6 we performed an *in silico* prediction of coiled-coil motifs in YopD and YopD-like sequences from other
7 bacteria. Of interest was a predicted N-terminal coiled-coil that occurred solely in *Yersinia* YopD
8 sequences. To investigate if this unique feature was biologically relevant, two *in cis* point mutations were
9 generated with a view to disrupting this putative structure. Both mutants maintained full T3SS function *in*
10 *vitro* in terms of environmental control of Yops synthesis and secretion, effector toxin translocation and
11 evasion of phagocytosis and killing by cultured immune cells. However, these same mutants were
12 attenuated for virulence in a murine oral-infection model. The cause of this tardy disease progression is
13 unclear. However, these data indicate that any structural flaw in this element unique to the N-terminus
14 will subtly compromise an aspect of YopD biology. Sub-optimal T3SSs are then formed that are unable to
15 fortify *Yersinia* against attack by the host innate and adaptive immune response.

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17 **Keywords:** Ysc-Yop, Type III secretion system, YopD, YopB, hydrophobic, translocon pore

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6 2 **1. Introduction**
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10 4 The coiled-coil is a tertiary motif frequently present in a broad variety of proteins. It consists of a
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12 5 repeated number of amphipathic α -helices (usually 2 to 5) that interlace around each other creating a
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14 6 super-coiled structure characterized by hydrophobic residue periodicity, called the heptad-repeat (Cohen
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16 7 and Parry, 1990; Lupas et al., 1991). This motif is a popular scaffold for diverse protein-protein
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18 8 interaction interfaces because of its inherent flexibility and heterogeneous architecture (Burkhard et al.,
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20 9 2001; Grigoryan and Keating, 2008). Their biological significance is further highlighted by recent in
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22 10 silico-based observations that suggested as much as 5% of the eukaryote proteome and 10% of the
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24 11 prokaryotic and archaeobacteria proteomes contain predicted coiled-coil motifs (Liu and Rost, 2001; Rose
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26 12 et al., 2005). Interestingly, some of those prokaryotic proteins were predicted to be substrates of the type
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28 13 III secretion system (T3SS), a prominent virulence determinant of many Gram-negative bacteria (Delahay
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30 14 and Frankel, 2002; Pallen et al., 1997). Since many of these T3SS substrates are prone to remodel
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32 15 biological functions inside the eukaryotic cell, it is no surprise that some have incorporated this motif
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34 16 during their evolutionary course as a means to cross-talk with host cell proteins.
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40 17 Like many Gram-negative bacteria, pathogenic *Yersinia* sp. utilizes a T3SS to translocate virulence
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42 18 factors directly into the interior of immune cells to subvert host innate immunity that promotes
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44 19 extracellular bacterial colonization in lymphoid tissue (Cornelis, 2006; Galan and Wolf-Watz, 2006).
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46 20 These are evolutionarily related to the flagellar biogenesis system and are thought to have evolved by
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48 21 horizontal gene transfer among the Gram-negative proteobacteria (Gophna et al., 2003; Troisfontaines
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50 22 and Cornelis, 2005). The *Yersinia* system – denoted Ysc-Yop for *Yersinia* secretion and *Yersinia* outer
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52 23 protein – is encoded on a large virulence plasmid common among all pathogenic strains (Cornelis et al.,
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54 24 1998). While T3SSs are widespread, variations exist among them. In fact, the Ysc-Yop T3SS forms a
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56 25 distinct phylogenetic clade that among others, includes T3SSs encoded by *Pseudomonas aeruginosa*,
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58 26 *Photorhabdus luminescens*, pathogenic *Aeromonas* sp., pathogenic *Vibrio* sp. and *Photobacterium*
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4 1 *damselae* (Troisfontaines and Cornelis, 2005). It is possible that the Ysc-Yop clade has evolved
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6 2 specifically for bacterial evasion of host phagocytosis so that they can remain as an extracellular
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8 3 pathogen, although no doubt each individual pathogen has adapted their cognate systems for use in
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10 4 different hosts and/or host-specific niches.

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13 5 Although most of the structural components of all T3SSs share similar structural and functional
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15 6 characteristics, the cognate substrates secreted by each individual system display considerable genetic and
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17 7 functional diversity (Erhardt et al., 2010; Tampakaki et al., 2004). Despite this variation, several among
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19 8 them are predicted to incorporate α -helical coiled-coil structural elements, indicating that such domains
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21 9 might play important roles in their function (Delahay and Frankel, 2002; Pallen et al., 1997). However, a
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23 10 structure-function analysis has only been experimentally verified in some cases [for example: (Daniell et
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25 11 al., 2001; Delahay et al., 1999; Gazi et al., 2008; Hamad and Nilles, 2007; Knodler et al., 2011; Lawton et
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27 12 al., 2002; Schubot et al., 2005)].

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31 13 The *Yersinia* secreted translocator YopD is a multifunctional protein that is involved in governing
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33 14 multiple T3SS related activities such as translocon pore assembly in the eukaryotic plasma membrane,
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35 15 effector translocation to the host-cell interior and maintenance of *ysc-yop* regulatory control in the
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37 16 bacterial cytoplasm. Given this functional repertoire, it is not surprising that YopD possesses distinct
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39 17 functional domains and engages numerous interacting partners (e.g. itself, YopB, YopK, YopE, LcrV,
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41 18 TyeA and SycD/LcrH) (Amer et al., 2011; Costa et al., 2010; Edqvist et al., 2006; Francis et al., 2000;
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43 19 Hartland and Robins-Browne, 1998; Iriarte et al., 1998; Neyt and Cornelis, 1999a; Olsson et al., 2004;
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45 20 Sarker et al., 1998; Thorslund et al., 2011). In several cases, these interactions have been attributed to a C-
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47 21 terminal amphipathic α -helix domain that spans residues 278 to 292 (Costa et al., 2010; Olsson et al.,
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49 22 2004; Tengel et al., 2002).

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53 23 As revealed in this and other studies (Bröms et al., 2003; Pallen et al., 1997), a putative C-terminal
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55 24 coiled-coil lies immediately upstream of the amphipathic α -helix. We also noted via the COILS
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57 25 webserver (<http://pbil.univ-lyon1.fr/>) a second putative coiled-coil motif located near to the YopD N-
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59 26 terminus, although this was with a predictive probability of <70% and a small window size <21.

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1 Nevertheless, analysis of the numerous YopD-like sequences harbored by other bacterial members of the
2 Ysc-Yop T3SS phylogenic clade consistently failed to predict this putative N-terminal structure. We
3 therefore wondered whether the N-terminal coiled-coil predicted only in YopD of pathogenic *Yersinia* sp.
4 is important for Ysc-Yop T3SS activity. To address this, we used site directed mutagenesis of residue
5 Ile₃₂ in an attempt to modestly (I32K substitution) or totally (I32P substitution) disrupt the chances of
6 forming this predicted N-terminal coiled-coil. Interestingly, *Y. pseudotuberculosis* producing YopD_{I32K} or
7 YopD_{I32P} maintained a functional T3SS *in vitro*. Within the *in vivo* context of a mouse infection model
8 however, the mutants displayed signs of virulence attenuation. These results suggest that this putative N-
9 terminal YopD coiled-coil domain, unique to the *Yersinia* genus, is required for full function and
10 virulence.

2. Materials and methods

2.1. YopD sequence comparisons

YopD protein sequence from *Y. pseudotuberculosis* YPIII was retrieved from NCBI sequence database
(accession number AAA72322) and used as bait for *BLASTP* (2.2.26+) analysis against all *Yersinia*
genomes (taxonomy identity: 629) in the NCBI databases. Only matched proteins with minimal Expect
(E)-values of 0 were reported. In order to identify YopD-like protein sequence in other bacteria, an
unrestricted *BLASTP* analysis was performed against the entire NCBI genome database. In this case, only
significantly matched proteins with minimal E-values of < E-15 were considered. Selected protein
sequences with YopD homology were retrieved from the NCBI sequence database and alignments
generated using *ClustalW* (2.1).

2.2. Bacterial strains, plasmids and growth conditions

1 Bacterial strains and plasmid list used in this study can be found as Table 1. We used *Y.*
2 *pseudotuberculosis* YPIII/pIB102 (serotype 0:3) as the parental strain which harbors an Ysc-Yop T3SS
3 encoded on the pIB102 virulence plasmid that carries a kanamycin resistance cassette in *yadA* (Bölin and
4 Wolf-Watz, 1984; Gemski et al., 1980) in addition to a chromosomally-encoded inactive PhoP response
5 regulator (Grabenstein et al., 2004). Unless otherwise stated, *Y. pseudotuberculosis* and *E. coli* strains
6 were cultivated at 26°C and 37°C respectively in Luria-Bertani (LB) broth with aeration. When required,
7 broth was supplemented with kanamycin (50µg/ml) or chloramphenicol (25µg/ml). Growth phenotypes
8 produced by the bacterial strains used in this study were achieved by growing the bacterial strains in
9 liquid Modified Higuchi's medium (TMH) supplemented with or without 2.5mM of CaCl₂ at 37°C
10 (Straley and Bowmer, 1986). When normal growth occurs in the presence of 2.5mM of CaCl₂ this
11 phenotype was termed calcium dependent (CD). In contrast, when bacteria are unable to grow irrespective
12 of Ca²⁺ levels, this is termed a temperature sensitive (TS) phenotype (Costa et al., 2010; Francis et al.,
13 2001; Olsson et al., 2004).

14 15 2.3. Mutant construction

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17 Overlap PCR (Higuchi, 1989) was used to generate amplified DNA fragments that incorporate point
18 mutations in *yopD* and flanked with XhoI and XbaI restriction sites using the primer pair combinations
19 listed in Supplementary Table S1. The amplified fragments were first cloned into the pCR®4-TOPO TA
20 cloning vector (Life Technologies Ltd, UK) and sequenced by Eurofins MWG Operon (Ebersberg,
21 Germany). The confirmed fragments were then sub-cloned into the pDM4 suicide vector that contained
22 the *sacB* gene encoding for levansucrase, an enzyme toxic to bacteria when grown in the presence of
23 sucrose. Mutagenesis constructs were transformed into *E. coli* S17-1λpir, which preceded conjugal
24 mating with *Y. pseudotuberculosis*. Primary allelic exchange events were encouraged by growth on
25 *Yersinia* selective agar containing kanamycin (50µg/ml) and chloramphenicol (25µg/ml). The positive
26 selection for secondary allelic exchange events occurred on LB agar plates supplemented with kanamycin

1 and 5% sucrose as detailed previously (Francis and Wolf-Watz, 1998; Milton et al., 1996). All mutants
2 were verified by a diagnostic PCR that amplified DNA flanking both up and down-stream of the mutation
3 and by subsequent sequencing of this amplified product.

4 5 *2.4. Limited chymotrypsin digestion*

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7 Chymotrypsin digestion was essential performed according to established protocol (Olsson et al., 2004).
8 In short, 0.1 volumes of overnight culture grown in LB broth depleted of Ca²⁺ ions was subcultured into
9 10ml of the same fresh media and grown for 1h at 26°C before being shifted to 37°C for 3h. Bacterial-
10 free supernatants were recovered by a 15min centrifugation at 2500xg and passage through a 0.45µm
11 filter. To all samples, CaCl₂ was added to a final concentration of 20mM followed by incubation at 0°C
12 for 20min. Each sample was divided into two 5ml-portions; to one of the vials chymotrypsin was added to
13 a final concentration of 10µg/ml. The duplicate sample remained untreated to control for spontaneous
14 proteolysis. Following incubation for 30min at 0°C, proteinase activity was quenched by the addition of
15 0.1 volume of 100% (v/v) trichloroacetic acid and incubation at 0°C for 1h. Protein was pelleted by
16 centrifugation for 10 min at 2500xg and each precipitate dissolved in 50µl of loading buffer (50mM Tris-
17 HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue). Samples were
18 fractionated in an 18% acrylamide SDS-PAGE gel and cleaved YopD peptides identified by immunoblot
19 on polyvinyl difluoride (PVDF) membrane with rabbit anti-YopD polyclonal antisera.

20 21 *2.5. Chemical cross-linking*

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23 Chemical cross-linking of YopD was adapted from our previous study (Costa et al., 2010). Briefly, 0.1
24 volume of overnight culture grown in BHI broth depleted of Ca²⁺ ions was sub-cultured in 2ml of the
25 same fresh media and grown for 1h at 26°C followed by 3h at 37°C. Cell density of the individual

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4 1 cultures were standardized by spectrophoretic measurement at an optical density of 600nm. Bacterial free
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6 2 supernatants were recovered by centrifugation and the EGS cross-linker added to a final concentration of
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8 3 0.5mM. Following incubation for 2h at 0°C, the cross-linking reaction was terminated during 15min
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10 4 incubation at ambient temperature by the addition of Tris-HCl (pH 7.5) buffer to a final concentration of
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12 5 50mM. Samples were then prepared for fractionation on a 12% acrylamide SDS-PAGE gel by the
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14 6 addition of 4x loading buffer (200mM Tris-HCl, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol,
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16 7 0.4% bromophenol blue) into 150ul of sample reaction. The oligomeric state of YopD was identified by
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18 8 immunoblot on PVDF membrane with rabbit anti-YopD polyclonal antisera.
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25 10 *2.6. Protein stability*

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29 12 Resistance of the generated YopD derivatives to endogenous proteases was tested following growth of
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31 13 the bacteria in BHI (Brain Heart Infusion) broth supplemented with 2.5mM of CaCl₂ (Feldman et al.,
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33 14 2002). Protein fractions collected at various time points subsequent to blocking *de novo* protein synthesis
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35 15 by the addition of 50μg/ml chloramphenicol were analyzed by SDS-PAGE and transferred onto a
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37 16 nitrocellulose membrane using a semi-dry transfer system. The immobilized YopD was identified using a
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39 17 rabbit polyclonal YopD antiserum. An anti-rabbit antibody conjugated with horseradish peroxidase (GE
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41 18 healthcare) and a homemade luminol detection solution was used for Western blot detection.
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47 20 *2.7. In vitro analysis of protein synthesis and secretion*

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51 22 Assessment of synthesis and secreted of T3S substrates was performed according to established
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53 23 protocols (Aili et al., 2008; Francis et al., 2000; Francis et al., 2001; Francis and Wolf-Watz, 1998;
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55 24 Olsson et al., 2004). Briefly, overnight cultures were back diluted 10 times and the production of T3S
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57 25 substrates was induced by a temperature shift from 26°C to 37°C for 3h in BHI broth depleted of Ca²⁺.
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4 1 Bacterial cultures were divided into pellets (synthesis) and supernatant (secretion) fractions and separated
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6 2 by SDS-PAGE followed by immunoblotting. Specific T3S substrates were indentified with rabbit
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8 3 polyclonal antiserum raised against YopD, YopB, YopE and LcrV. After treatment with horseradish
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10 4 peroxidase conjugated anti-rabbit antibody the detection was completed with homemade luminol detection
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12 5 solution.
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17 7 *2.8. Contact-dependent erythrocyte lysis and carbohydrate osmoprotection*

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22 9 For the hemolysis assay using horse blood, overnight bacterial cultures were grown in LB broth depleted
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24 10 of Ca²⁺ ions and supplemented with 75mM of NaCl. Into fresh 2ml medium, 0.1 volumes were
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26 11 subcultured and then incubated for 1h at 26°C. A further 1ml of 42°C prewarmed medium was added
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28 12 before incubating at 37°C for 1h. The horse blood was prepared by washing three times with fresh LB
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30 13 broth and was then resuspended in half of the volume in LB preheated to 37°C. Bacteria cultures were
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32 14 standardized to the same optical density by spectrophoretic measurement at 600nm. The bacteria were
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34 15 then harvested by centrifugation and the pellet thoroughly resuspended in 1ml of blood (equivalent to 2 x
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36 16 10¹¹ erythrocytes). Samples were centrifuged at 3500rpm for 5min and then incubated for 1h. To measure
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38 17 hemoglobin release, samples were mixed and 100µl aliquots transferred to wells of a microtiter plate
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40 18 containing an equal volume of PBS prior to optical density measurement at 545nm. For the
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42 19 osmoprotection assay using the carbohydrates raffinose, dextrin 15 and dextran 4, we followed the
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44 20 method of Holmström and colleagues (Holmström et al., 1997).
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51 22 *2.9. HeLa cells infection assay*

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56 24 For cultivation and infection of human HeLa epithelial cell line for the cytotoxicity assay, we followed
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58 25 well-established protocols (Francis and Wolf-Watz, 1998; Rosqvist et al., 1991). The ability of *Yersinia*
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4 1 expressing various *yopD* derivatives to intoxicate eukaryotic cells with the YopE cytotoxin was accessed
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6 2 by HeLa cell monolayer cytotoxicity assay. The cell morphology change from oblong to round was
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8 3 followed by light microscopy for a period of 2h. The change induced by parental *Yersinia* (YPIII/pIB102)
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10 4 defines the upper limit while an oblong morphology maintained by the $\Delta yopD$ mutant (YPIII/pIB621)
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12 5 defines the lower limit.
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15 6 For the proteinase K digestion and digitonin solubility assay, 10cm diameter tissue culture petri dishes
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17 7 were seeded with 2×10^6 HeLa cells in Eagle Minimal Essential Medium (MEM) with Earle's salts (MP
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19 8 Biomedicals) supplemented with 10% FCS, 1% PeSt, L-glutamine and sodium pyruvate and grown
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21 9 overnight in 5% CO₂ at 37°C. Overnight bacterial cultures were grown at 26°C in 2ml of LB broth with
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23 10 appropriate antibiotic selection. The next day, 0.01 volumes of these were sub-cultured into 12ml of
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25 11 MEM without antibiotic and grown for 30 min at 26°C followed by 1h at 37°C. Prior to infection, HeLa
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27 12 cell monolayers were washed twice with PBS and then overlaid with 5ml MEM without antibiotic.
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29 13 Twenty minutes pre-infection, a final concentration of 0.5µg/ml of cytochalasin D was added and
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31 14 maintained throughout to block bacterial uptake. The cell-free media was then carefully aspirated and
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33 15 monolayers infected in a volume of 5ml in duplicate with a multiplicity of infection (MOI) of ~10.
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35 16 Infections were allowed to proceed for 3h in 5% CO₂ at 37°C, after which time non-adherent bacteria
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37 17 carefully removed by washing thrice with PBS. Monolayers were then treated for 30sec with 500µg of
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39 18 proteinase K solubilized in 1ml of PBS. Following removal of the proteinase K solution, monolayers were
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41 19 further incubated at ambient temperature for 20min. proteinase K activity was then blocked by the
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43 20 addition of 500µl of freshly prepared 4mM phenylmethylsulfonyl fluoride (in PBS). To one of the
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45 21 duplicate monolayers, 400µl of 1% digitonin in PBS was added, while 400µl PBS was added to the other.
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47 22 Material was harvested in collection tubes and incubated for 20min at ambient temperature. Soluble
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49 23 fractions were clarified by centrifugation and the supernatants subsequently analyzed by Western blot.
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51 24 Translocated YopE and YopH effectors were identified with specific rabbit anti-YopE and goat anti-
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1 YopH antisera. Samples were also probed with mouse anti-ERK1 antibody (BD Pharmingen) specific for
2 the C-terminal region of ERK1 to confirm the loading of an equal quantity of protein in each lane.

3 4 *2.10. Resistance to phagocytic uptake and killing*

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6 For measurements of bacterial uptake and killing by murine J774A.1 macrophage-like cell monolayers
7 (ATCC, Virginia, USA) we used the method of Bartra and colleagues (Bartra et al., 2001). Into individual
8 wells of a 24-well tissue culture plate, 1×10^5 J774A.1 macrophages-like cells were seeded with
9 Dulbecco's Modified Eagle Medium (DMEM) High Glucose, GlutaMAX™ (Life Technologies Ltd,
10 Paisley, UK) supplemented with 10% FCS and 1% PeSt and then grown for 24h in 5% CO₂ at 37°C.
11 Additionally, bacterial cultures were grown with aeration overnight at 26°C in BHI broth minus Ca²⁺ ions.
12 Next day, 20µl of overnight culture was transferred into 3ml of antibiotic free DMEM and incubated at
13 26°C for 30min prior to a shift to 37°C for 60min. In preparation for infection with a MOI of ~1, J774A.1
14 monolayers were washed twice with 1ml PBS and then each monolayer overlaid with 1ml antibiotic free
15 DMEM. Bacterial infections were then initiated and synchronized with centrifugation at 1500rpm for
16 5min. After 30min incubation in 5% CO₂ at 37°C, supernatants were carefully aspirated and monolayers
17 then gently overlaid with 250µl of antibiotic free DMEM medium and incubated further. At time intervals
18 2h and 6h, 250µl 1% (v/v) Triton X-100 was added, mixed and then incubated for 10min at ambient
19 temperature. Serial dilutions of cell lysate were performed in PBS to obtain colony forming units. These
20 values are expressed as means ± standard errors (SEM) of the results of six independent experiments. The
21 non-parametric two-tailed Mann-Whitney *U*-test performed using GraphPad Prism version 5.00 for
22 Windows, GraphPad Software, San Diego California USA, www.graphpad.com was used to analyze the
23 differences in data sets. Differences with a probability value of $P < 0.05$ were considered significant.

24 25 *2.11. Murine infection model*

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6 2 Female eight-week-old BALB/c mice (Taconic, Denmark) were given food and water *ad libitum*. For
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8 3 infection, groups of six mice were deprived of food and water 16 h prior to oral infection. For infection,
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10 4 bacteria grown overnight in LB broth at 26°C were pelleted and serially diluted to 10⁹, 10⁸ and 10⁷
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12 5 CFUs/ml in sterile tap water supplemented with 150 mM NaCl. Cultures were serially diluted and plated
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14 6 to establish viable bacterial cell counts for verification of the infection dose. Mice were individually
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16 7 monitored for 14 days post infection for weight, ruffled fur, diarrhea, crumpled back, and listlessness. The
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18 8 ID₅₀ (50% infectious dose) was determined using the Reed–Muench method (Reed and Muench, 1938).
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20 9 Infected mice showing symptoms of a terminal infection were immediately euthanized. The experiments
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22 10 were conducted in accordance with the guidelines of the Animal Ethics Committee of Umeå University
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24 11 (permit number A90-11).
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31 13 **3. Results**

32 33 14 34 35 15 *3.1. Heterogeneity of YopD sequences*

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39 17 It was apparent from the first report of YopD primary sequence that it represented a novel class of
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41 18 protein (Håkansson et al., 1993). Using this original YopD protein sequence with accession number
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43 19 AAA72322 as a consensus – designated YPIII_YopD after the source strain *Y. pseudotuberculosis* YPIII
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45 20 serotype 0:3 – we first performed a *BLASTP* analysis against all other *Yersinia* sequences available in the
46
47 21 NCBI database. This comprised of >60 entries and corresponded to >50 *Y. pestis*, 2 *Y. pseudotuberculosis*
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49 22 and >8 *Y. enterocolitica* isolates (Table 2). This approach revealed 5 different homology groups of YopD
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51 23 sequences, designated A to E (Fig. 1 and Table 2). Consistent with the close evolutionary relationship
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53 24 between *Y. pseudotuberculosis* and *Y. pestis* (Achtman et al., 1999; Skurnik et al., 2000; Wren, 2003),
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55 25 group A sequences exhibited a single amino acid change (G283S) across the entire 306 residues, and are
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57 26 derived entirely from other *Y. pseudotuberculosis* and *Y. pestis* isolates. Moreover, we observed a greater
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1 heterogeneity with YopD sequences derived from *Y. enterocolitica*, which is a reflection of the much
2 earlier divergence of this species from *Y. pseudotuberculosis* (Achtman et al., 1999). *Y. enterocolitica*
3 group B sequences boast six genetic alterations (K27G, I32V, T113S, E172D, G183S and I185A), group
4 C has seven (Q17R, K27G, I32V, T113S, E172D, G183S and I185A), group D with eight (L18I, K27G,
5 I32V, T111A, E172D, G183S, I185T and G186S) and group E with nine (L18I, K27G, I32V, T111A,
6 E172D, M179L, G183S, I185T and G186S). Based on these comparisons, the G183S substitution is
7 recognized as common to all other YopD sequences, whereas the additional K27G, I32V and E172D
8 divergences are also common to all *Y. enterocolitica* isolates (Fig. 1).

9 Outside of the *Yersinia* genus, YopD-like proteins exist in the genera *Aeromonas*, *Photothabdus* and
10 *Vibrio* as well as *Pseudomonas aeruginosa* and *Photobacterium damsela* (Table 2). Of these, AopD
11 from *A. veronii* exhibits 49.3% amino acid identity to YPIII_YopD over much of the protein length. This
12 reduces to a modest 27.8% identity for the open reading frame annotated as VDA_000190 from *Pb.*
13 *damsela*. Only twenty one residues are identical among all YopD-like sequences (Fig. 1). However,
14 these gather in two groups; one located in a segment bordering the internal YopD putative membrane
15 spanning domain and the other in a C-terminal segment overlapping with a putative coiled-coil. These
16 two discrete foci correspond to domains that in YopD of pathogenic *Yersinia* are critical for Ysc-Yop
17 T3SS function (Olsson et al., 2004) (Costa et al., unpublished data). We therefore posit that these are core
18 functional domains of all YopD-like proteins.

3.2. A predicted coiled-coil structure unique to the YopD sequences of *Yersinia* sp.

22 With the aim to benefit our understanding of YopD function, we further scrutinized YopD sequences
23 for the presence of coiled-coils using the unweighted method of Lupas and colleagues (Lupas et al.,
24 1991). We focused on this structural motif because it is a critical functional domain in many diverse
25 proteins (Liu and Rost, 2001; Rose et al., 2005). Corroborating earlier reports (Bröms et al., 2003; Pallen
26 et al., 1997), a coiled-coil domain in the C-terminus was predicted with high probability in YopD

1 sequences derived from *Yersinia* (Supplementary Fig. S1 and Table 2). Significantly, mutational analysis
2 of this region in YPIII_YopD indicates a critical role in T3SS function and virulence (Olsson et al., 2004)
3 (Costa et al., unpublished data). A similarly predicted C-terminal coiled-coil segment was also observed
4 in many of the YopD-like sequences sourced from other bacterial genera, reinforcing the idea that this
5 represents a core functional domain. The two exceptions to this were LopD from *Phototrhabdus* sp. and
6 PopD from *P. aeruginosa* (Supplementary Fig. S1 and Table 2). Hence, unique features can evolve
7 among individual YopD-like homologues that is presumably necessary to support function within their
8 cognate T3SS (Bröms et al., 2003).

9 Albeit with much lower probability, *in silico* analysis predicted a second modestly sized coiled-coil
10 structure to lie at the N-terminus of YopD sequences from *Yersinia* (Supplementary Fig. S1 and Table 2).
11 Interestingly, this prediction was unique to these sequences alone. Analysis of no other YopD-like
12 sequence sourced from the other genera was suggestive of an N-terminal coiled-coil. Hence, this N-
13 terminal segment might exclusively contribute to YopD function during *Yersinia* pathogenesis.

14 3.3 Disruption of the N-terminal coiled-coil of YopD

15 Short of solving the YopD structure, it is difficult to demonstrate that the N-terminal domain forms a
16 coiled coil. However, if the coiled-coil is disrupted, this may lead to differences in YopD structure and
17 function. As a first step, we therefore generated by site directed mutagenesis and allelic exchange two
18 variant *yopD* alleles *in cis* on the virulence plasmid and coding for YopD_{I32K} and YopD_{I32P} that were
19 anticipated to reduced coiled-coil prediction probability from 80% to 60% or 0%, respectively
20 (Supplementary Fig. S1). In addition, we also took advantage of a previously constructed *yopD* mutation
21 that codes for a poorly secreted YopD_{Δ23-47} variant that essentially lacks the N-terminal putative coiled-
22 coil (Olsson et al., 2004).

23 To probe for structural differences between the wild-type and mutant proteins, we performed a limited
24 chymotrypsin digestion of secreted YopD. Chymotrypsin was chosen because PeptideCutter
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1 (<http://us.expasy.org/tools/peptidecutter>) predictions suggested it should recognize only five sites evenly
2 distributed in YopD with a cleavage probability of $\leq 80\%$ (data not shown). All secreted YopD variants
3 essentially exhibited a similar peptide fragmentation pattern, although modest alterations in the mobility
4 of smaller digested fragments were identified for YopD_{I32P} (Fig. 2). As an alternative, we also examined
5 for changes in YopD oligomeric state by EGS crosslinking of *Yersinia* secreted fractions. Crosslinking
6 was able to capture higher order structures for all three secreted YopD variants that quite possibly
7 represented dimers, trimers and tetramers (Fig. 3). However, not only was the extent of YopD_{I32P}
8 crosslinking and/or stable oligomer formation quite obviously reduced, but their banding patterns also
9 varied from native YopD and YopD_{I32K} (Fig. 3).

10 Although this combined analysis does not conclusively show that the N-terminus forms a coiled coil,
11 data from chymotrypsin digestion and probing of oligomeric state collectively favors a subtle structural
12 alteration that specifically resulted from introduction of a proline helix-breaker in the YopD_{I32P} variant. In
13 contrast, the seemingly equivalent dramatic change of swapping the hydrophobic isoleucine for a charged
14 lysine in mutant YopD_{I32K} did not induce any measurable structural change.

16 *3.4 Stable YopD N-terminal variants that maintain yop post-transcriptional control*

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18 To facilitate our analysis of YopD function, we first of all used the method of Feldman and colleagues
19 (Feldman et al., 2002) to demonstrate that YopD_{wild type}, YopD _{$\Delta 23-47$} , YopD_{I32K} and YopD_{I32P} all had
20 comparable stability in the presence of endogenous proteases (Fig. 4A). Next, we grew these bacteria in
21 BHI broth restrictive (plus Ca²⁺) and permissive (minus Ca²⁺) for Yops production and secretion. As
22 reported previously (Olsson et al., 2004), bacteria were slightly affected in their ability to produce
23 YopD _{$\Delta 23-47$} as well as other T3SS substrates including YopB, LcrV and YopE (Fig. 4B). This naturally
24 corresponded to lower levels of their secretion into the culture supernatant; although obviously the severe
25 reduction of YopD _{$\Delta 23-47$} secretion cannot be attributed to reduced synthesis alone (Fig. 4C). Interestingly,
26 point mutations in this same region (YopD_{I32K} and YopD_{I32P}) did not cause any obvious deviation in the

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4 1 synthesis and secretion profiles of YopD or other substrates, since they were all comparable to parental
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6 2 bacteria (Fig. 4B and 4C). At least during laboratory culturing, it seems unlikely that this domain *per se* is
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8 3 involved in translation control and/or as a secretion signal for recognition by the Ysc-Yop T3S apparatus
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10 4 of *Yersinia* (Fig. 4C).

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13 5 YopD is a well known component of *yop* post-transcriptional control (Chen and Anderson, 2011). This
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15 6 is illustrated by the phenotype of *Yersinia* lacking YopD (Francis and Wolf-Watz, 1998; Williams and
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17 7 Straley, 1998), which constitutively produces Yops even in the non-permissive growth media (plus Ca²⁺)
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19 8 (Fig. 4B). Critically, this strain is also temperature sensitive, being unable to grow at elevated temperature
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21 9 regardless of Ca²⁺ levels (data not shown). Importantly, parent bacteria and those bacteria producing
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23 10 YopD_{Δ23-47}, YopD_{I32K} and YopD_{I32P} respectively, all maintained normal regulatory control, severely
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25 11 restricting Yops synthesis in high-Ca²⁺ conditions (Fig. 4B). All these bacteria also displayed normal
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27 12 calcium dependent growth phenotypes when grown at 37°C in TMH medium (data not shown). Taken
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29 13 together, targeted disruption of the putative N-terminal coiled-coil region of YopD does not perturb its
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31 14 stability or capacity to maintain post-transcriptional control of Yops synthesis when bacteria are grown *in*
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33 15 *vitro* in laboratory media.
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40 17 *3.5 Maintenance of contact-dependent erythrocyte cell lysis and formation of the translocon pore*

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44 19 All three mutants that produce a YopD variant with a defect in its N-terminus still maintain a typical
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46 20 Ca²⁺-dependent Yop synthesis and secretion profile and normal growth – even though YopD_{Δ23-47} is
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48 21 poorly secreted. Hence, we embarked on assessing the impact of this mutants on T3SS activity. Together
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50 22 with two additional translocators – the highly hydrophobic YopB and the needle tip hydrophilic protein
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52 23 LcrV, secreted YopD contributes to contact-mediated cellular lysis via the formation of a T3S translocon
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54 24 pore in target cell membrane. This membrane-spanning pore maybe the conduit through which anti-host
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56 25 effectors gain access to the cell interior. We infected red blood cells with *Yersinia* and then determined
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58 26 the extent of cell lysis by measuring spectrophoretically the degree of hemoglobin released into the
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1 extracellular milieu. Apart from the *yopD* null mutant or bacteria producing YopD $_{\Delta 23-47}$, other *Yersinia*
2 bacteria were able to induce appreciable hemoglobin release (Fig. 5A). To confirm that hemoglobin
3 release was initiated by the formation of T3S translocon pores in the erythrocyte membrane, we
4 performed an osmoprotection assay in which carbohydrates of various dimensions were used to block the
5 pores formed in erythrocyte membranes to prevent osmolysis. Critically, the smaller sized sugar raffinose,
6 with a diameter (ϕ) of 1.2–1.4nm only blocked ~10% of hemoglobin release caused by *Yersinia* infection
7 (Fig 5B). On the other hand, the larger sized dextrin 15 (2.2nm ϕ) reduced hemolysis to ~50% achieved
8 in the absence of sugars, while dextran 4 (3–3.5nm ϕ) further limited hemolysis in the range of ~70%
9 (Fig 5B). Importantly, all sugars blocked pores formed by parent bacteria or bacteria producing YopD $_{I32K}$
10 or YopD $_{I32P}$ to equivalent degrees. These three bacteria must therefore form similarly sized translocon
11 pores in target cell membranes.

13 3.6 T3SS function in vitro is not compromised

15 Next we endeavored to determine whether these *Yersinia* variants have preserved T3SS function that
16 permits the bacteria to intoxicate eukaryotic cells with anti-host effectors for escaping immune cell
17 phagocytosis and killing. As a gauge of Yop effector translocation into eukaryotic cells, we performed a
18 HeLa cell cytotoxicity assay. This assay is based upon the localization of the GTPase-activating protein
19 YopE in the infected host cell cytosol. Because YopE activity destabilizes host cell actin, intoxicated cells
20 will display a changed morphology from oblong to well-rounded. As expected, in as little as 30 min post-
21 infection with parental *Y. pseudotuberculosis*, a rapid morphological change in all of the infected cells
22 was observed. This was true also of bacteria producing YopD $_{I32K}$ or YopD $_{I32P}$ that is indicative of a
23 functional T3SS *in vitro* (Fig. 6). In contrast, cell monolayer morphology was unperturbed when infected
24 with bacteria completely lacking YopD or producing the very poorly secreted YopD $_{\Delta 23-47}$ variant, even
25 following prolonged incubation (>120 min) (Fig. 6). As has been reported previously (Francis and Wolf-

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4 1 Watz, 1998; Olsson et al., 2004), the inability of these two mutants to intoxicate HeLa cell monolayers is
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6 2 consistent with them producing a dysfunctional T3SS.

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8 3 To verify Yop effector translocation into epithelial cell monolayers by *Yersinia* producing either the
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10 4 YopD_{I32K} or YopD_{I32P} variant, we performed a proteinase K-digtonin detergent solubility assay. With
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12 5 anti-YopE and anti-YopH antiserum, we could detect equivalent levels of proteinase K protected and
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14 6 detergent soluble YopE and YopH in the cytosolic fractions of HeLa cells regardless of them being
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16 7 infected with parental bacteria or bacteria producing either YopD_{I32K} or YopD_{I32P} (Fig. 7A). In contrast,
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18 8 much less YopE and, to some extent YopH, were detected in cytosolic fractions of HeLa cell monolayers
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20 9 infected with *yopD* null mutant or bacteria producing YopD_{Δ23-47} (Fig. 7A). Hence, Yop effector
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22 10 tranlocation into tissue culture cells is intact in bacteria producing either YopD_{I32K} or YopD_{I32P}.

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26 11 Pathogenic *Yersinia* sp. escape host cell phagocytosis through the combined action of translocated
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28 12 anti-host effector toxins (Viboud and Bliska, 2005). Therefore, we measured the capacity of our YopD
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30 13 mutants to resist phagocytosis and killing by J774A.1 macrophage-like immune cells. In this assay,
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32 14 bacteria with a dysfunctional T3SS will be engulfed by the professional phagocytic cell and subsequently
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34 15 killed by anti-microbial effectors present in the host cell cytosol. However, bacteria harboring a fully
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36 16 functional T3SS will resist phagocytosis to permit their proliferation in the extracellular environment
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38 17 (Bartra et al., 2001). Bacterial infections were monitored up to 6h post-infection. At 2h and 6h post-
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40 18 infection, bacteria in close association with host cell were quantified for viability by measuring colony
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42 19 forming units. As a control, we used the translocation defective and growth restricted $\Delta yopD$ null mutant.
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44 20 As observed previously (Amer et al., 2011), this variant cannot resist immune cell phagocytosis and is
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46 21 efficiently killed, which significantly lowers the recovery of viable bacteria at 2h ($P=0.015$) and 6h
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48 22 ($P=0.005$) post-infection compared to the parental strain (Fig. 7B). Bacteria producing YopD_{Δ23-47} also
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50 23 exhibits an inferior ability to defend against phagocytosis given the lower recovery of viable bacteria after
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52 24 6h ($P<0.0001$) (Fig. 7B). This can be explained by an impairment of YopD secretion *in vitro* (see Fig.
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54 25 2C). On the other hand, bacteria producing YopD_{I32K} or YopD_{I32P} could efficiently resist immune cell
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56 26 engulfment as measured by extensive extracellular replication at levels equivalent to parental bacteria
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1 (Fig. 7B). These *in vitro*-based assays suggest that both YopD_{I32K} and YopD_{I32P} support a fully functional
2 T3SS, despite incorporating disruptive structural mutations at their N-terminus. In contrast, a full deletion
3 of this segment leads to poor secretion of YopD_{Δ23-47}, which is a serious impediment to T3SS function at
4 the zone of *Yersinia*-host cell contact.

3.7 YopD N-terminus contributes to *Yersinia* virulence

8 Obtaining YopD variants that maintain *ysc-yop* regulatory control and normal growth gave us the
9 opportunity to assess their virulence in an *in vivo* murine infection model. Oral infections of female
10 BALB/c mice were performed and virulence attenuation on the basis of ID₅₀ was determined by the
11 appearance of symptoms such as diarrhea, ruffled fur, apathy and weight lost. Mice infected with *Y.*
12 *pseudotuberculosis* parental strain was terminally infected at days 5, 7 or 9 with oral infection doses in the
13 range of 10⁹, 10⁸ and 10⁷ respectively. On this basis, an ID₅₀ lower than 3.6 x 10⁷ CFU/ml could be
14 calculated for the parental stain (Fig. 8A). Supporting *in vitro* experimental observations, no signs of
15 infection were observed with mice infected with the YopD_{Δ23-47}-producing mutant, suggesting an ID₅₀ for
16 this strain that exceeds 2.0 x 10⁹ CFU/ml and corresponds to at least a ~55 fold attenuating effect (Fig.
17 8A). Interestingly, mice infected with bacteria producing YopD_{I32K} and YopD_{I32P} clearly displayed milder
18 signs of infection at the lower infection dose (10⁷) resulting in an ID₅₀ of 4.7 x 10⁷ CFU/ml and 2.9 x 10⁷
19 CFU/ml respectively. Moreover, at this lower infection dose more than 50% of the mice infected with
20 these variants survived the entire 14 day experiment (Fig. 8A). Additionally, these same infected mice
21 lost less body weight (an indicator of general well-being) than did mice infected with the parental strain
22 (Fig. 8B). Clearly therefore, the infection did not progress as rapidly with bacteria producing YopD_{I32K}
23 and YopD_{I32P}. These results support the idea that an intact putative YopD N-terminal coiled-coil might be
24 critical for full virulence of *Y. pseudotuberculosis*.

4. Discussion

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The YopD protein is a critical component of *Yersinia* Ysc-Yop T3SS function. As a multi-functional protein, it is essential for post-transcriptional control of *yop* expression (Chen and Anderson, 2011) and also assembles into a translocon pore within the host cell plasma membrane through which effectors might gain access to the cell interior (Costa et al., 2010; Edqvist et al., 2007; Goure et al., 2005; Montagner et al., 2011; Neyt and Cornelis, 1999a; Olsson et al., 2004). It may even possess an effector activity inside the target cell as a portion of YopD is also translocated (Francis and Wolf-Watz, 1998). Reflecting these critical functions, YopD sequences are broadly conserved throughout the genomes of human pathogenic *Yersinia*. Only one polymorphism (group A; G183S) separated the consensus *Y. pseudotuberculosis* YPIII YopD sequence from all other YopD sequences sourced from *Y. pestis* isolates. This degree of conservation is impressive considering that all these *Y. pestis* isolates represent geographically diverse environmental reservoirs and also vary in their virulence potential. Moreover, it contrasts with LcrV findings; another translocon member coded for on the same polycistronic *lcrGVHyopBD* operon. Even among the *Y. pestis* isolates, LcrV sequence polymorphisms are comparatively common (Anisimov et al., 2010). This is consistent with it being a surface exposed and dominant immunogen, which may drive a greater selection pressure for diversification.

Comparisons between YopD sequences from the two enteropathogenic *Yersinia* sp. did reveal a greater diversity (groups B to E). We cannot determine if any of these polymorphisms impart unique structural constraints on YopD because a tertiary structure is unavailable. It is possible that some could alter YopD function, however on the basis of similar studies with LcrV, we consider this to be very unlikely. Even though *Y. enterocolitica* LcrV possesses 2 to 3 times more polymorphic loci than reported here for YopD, it was still fully capable of supporting both *in vitro* and *in vivo* T3SS function in *Y. pestis* lacking cognate LcrV (Miller et al., 2012). In view of this, changes in non essential regions of the translocon scaffold are obviously reasonably well-tolerated by the Ysc-Yop T3SS. This is well supported by the fact that the vast majority of identified polymorphisms either cluster to the N-terminus where a

1 high degree of variation can still maintain efficient YopD secretion (Amer et al., 2011) or to a region
2 between residues 150 to 227 that is known to be non-essential for YopD function (Olsson et al., 2004).

3 Ample evidence confirms that coiled-coil motifs in a variety of proteins from diverse origins are
4 inherent to protein function. Inspired by this, we examined YopD sequences for recognizable coiled-coil
5 motifs. In addition to a widely predicted coiled-coil at the C-terminus of most YopD-like sequences
6 (Bröms et al., 2003; Pallen et al., 1997), we took note of a consistently predicted (albeit with low
7 probability) motif present in the N-terminus of YopD sequences solely derived from pathogenic *Yersinia*
8 sp. We were curious to know if this contributed to a function of YopD that is unique to *Yersinia*. While
9 disruption of this segment did not reveal any obvious defect in T3S as measured by *in vitro* assays,
10 bacteria were attenuated for virulence in an oral infection mouse model. Hence, this region of YopD
11 imparts an influence on T3SS activity that is required for disease progression in orally infected mice. In
12 the absence of a YopD structure however, we cannot say for sure that this region is an actual coiled-coil.
13 Nevertheless, introduction of an I32P mutation into YopD induced subtle alterations in the chymotrypsin
14 digestion profile and reduced its propensity for oligomerization, which is suggestive that this N-terminal
15 sequence does confer some structural constraint on YopD. Interestingly, recently solved partial structures
16 of the YopB-like hydrophobic translocators IpaB and SipB from *Shigella flexneri* and *Salmonella*
17 *enterica* Typhimurium respectively, physically confirm the presence of an N-terminal coiled-coil (Barta
18 et al., 2012). Only with the solving of a solution structure can this also be verified for YopD.

19 At this stage, it is unclear what aspect of YopD function is limited by the I32K and I32P substitutions.
20 Coiled-coils motifs can facilitate host cell membrane association of the translocated T3S effector protein
21 SopB from *S. enterica* (Knodler et al., 2011). However, we do not believe that the putative N-terminal
22 coiled-coil of YopD functions to insert the translocator into membranes. This is based on the extent of
23 contact-dependent erythrocyte lysis induced by parent and mutant strains of *Yersinia* was
24 indistinguishable and results from carbohydrate osmoprotection studies clearly reflected that YopD-
25 dependent pores formed by native YopD and the point mutants were all of similar size. Further, these
26 observations are supported by independent *in vitro* translocation assays that indicate efficient

1 translocation of Yops. We interpret these data to indicate that fundamental protein-protein interactions
2 involving YopD, which ultimately dictate Yop effector translocation (Costa et al., 2010; Edqvist et al.,
3 2006; Francis et al., 2000; Hartland and Robins-Browne, 1998; Iriarte et al., 1998; Montagner et al., 2011;
4 Neyt and Cornelis, 1999a, 1999b; Sarker et al., 1998; Thorslund et al., 2011), remain intact in *Yersinia*
5 producing the YopD_{I32K} and YopD_{I32P} variants. However, we cannot rule out defects in other undisclosed
6 interactions or even alterations in interaction dynamics as reasons why the point mutants are less virulent
7 than the parental bacteria.

8 Critically, these data highlight the limitations of *in vitro* assays. While tissue culture models of
9 infection are an essential tool, individual cell lines are invariably riddled with undefined genetic
10 mutations, possess no growth control, lack typical morphological traits and *in vitro* culturing methods
11 cannot reproduce the physical, chemical and biological complexities associated with the whole animal *in*
12 *vivo* environment. Thus, wherever possible it is prudent to assess biological relevance with well-designed
13 *in vivo* studies. In this respect, the robust immune response generated by animals at the low inoculation
14 dose of $\sim 10^7$ CFU/ml was sufficient to halt systemic disease progression and clear infections by mutant
15 bacteria producing YopD_{I32K} and YopD_{I32P}, but not by the parental bacteria that rapidly progressed to a
16 fatal septicemic infection. On the other hand, at the higher inoculation doses of $\sim 10^8$ to 10^9 CFU/ml, the
17 balance favored infecting bacteria. In this scenario, the sheer numbers of mutant bacteria still enabled a
18 sub-optimal T3SS to nullify an over-whelmed immune response to confer rapid and fatal systemic spread.

19 Based on the very poor secretion of YopD _{Δ 23-47}, it was not surprising to find that bacteria producing
20 this variant were strongly attenuated *in vivo*, corroborating observed *in vitro* defects associated with our
21 pore-forming and tissue culture translocation assays performed in this study and also previously (Olsson
22 et al., 2004). It is unclear to us why secretion of this variant is so poor. We recently identified a YopD
23 secretion signal sequence in the extreme N-terminus that clearly lay upstream of this 23 to 47 region
24 (Amer et al., 2011). Yet, for some reason deletion of residues 23 to 47 must change the context of this
25 existing secretion signal. Future work is needed to understand this phenomenon. This is made all the more
26 curious because, at least *in vitro*, YopD_{I32K} and YopD_{I32P} retain capacity for efficient T3S by *Yersinia*.

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1 Naturally though, it remains a possibility that this region does truly contribute to optimal temporal
2 secretion of YopD, especially during animal infections where bacteria would be expected to encounter
3 authentic T3S inducing cues that guarantee a well-orchestrated substrate secretion program. While a type
4 III substrate secretion hierarchy – ensuring that translocators are secreted before effectors – has been
5 difficult to experimentally document in *Yersinia*, our earlier study provides some support for a unique
6 secretion signal in the first 20 residues of YopD that may permit its ordered secretion (Amer et al., 2011).
7 Hence, the N-terminal segment harboring a putative coiled-coil may in fact be an extension of this unique
8 secretion signal.

9 Along these same lines, YopD secretion is considered an essential component of the feedback
10 inhibitory loop governing Yop synthesis control (Cambronne and Schneewind, 2002; Wulff-Strobel et al.,
11 2002). It is therefore significant that bacteria producing YopD $_{\Delta 23-47}$ still maintain post-transcriptional
12 control of *yop*-regulation despite this variant being trapped in the cytoplasm. On this note, it is probably
13 not a coincidence that this strain also produced lower levels of Yops in T3S permissive growth medium.
14 In sum, this mutant offers a research tool that in time should give better clarity to the importance of YopD
15 secretion in the maintenance of *yop*-regulatory control, especially since it still engages with cognate LcrH
16 chaperone to form a functional regulatory complex (Francis et al., 2000; Francis et al., 2001).

18 **5. Conclusion**

19
20 We have identified an N-terminal structural element uniquely conserved in YopD sequences that
21 originate from human pathogenic *Yersinia* sp. Targeted disruption of this structure leads to the attenuation
22 of *Y. pseudotuberculosis* virulence in an oral infection model. This segment therefore fulfills a role for
23 YopD that is necessary for optimal T3SS activity in defense against *in vivo* killing by naturally activated
24 immune cells.

26 **Acknowledgments**

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18
19 8 experiments. MF and MSF provided critical reagents. TRC, AAA, AF and MSF analyzed data. TRC, AF
20
21 9 and MSF wrote the paper. All authors read, provided feedback and approved the paper. The funding
22
23
24 10 providers had no such involvement in any of these processes.
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28 12 **Appendix A. Supplementary data**

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33 14 Supplementary data associated with this article can be found online.
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Table 1

Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strain		
<i>E. coli</i>		
TOP10	F ⁻ , <i>mcrA</i> , Δ(<i>mrr-hsdRMS-mcrBC</i>), φ80 <i>lacZ</i> Δ <i>M15</i> , Δ <i>lacX74</i> , <i>recA1</i> , <i>araD139</i> , (Δ <i>ara-</i> <i>leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Sm ^R), <i>endA1</i> , <i>nupG</i>	Life Technologies
DH5	F ⁻ , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	Stratagene
S17-1λ <i>pir</i>	<i>recA</i> , <i>thi</i> , <i>pro</i> , <i>hsdR</i> ^{M+} , Sm ^R , <RP4:2- Tc:Mu:Ku:Tn7>Tp ^R	(Simon et al., 1983)
<i>Y. pseudotuberculosis</i>		
YPIII/pIB102	<i>yadA</i> ::Tn5, Km ^R (parent)	(Bölin and Wolf-Watz, 1984)
YPIII/pIB621	pIB102, YopD _{Δ4-303} , Km ^R	(Francis and Wolf-Watz, 1998)
YPIII/pIB605	pIB102, YopD _{Δ23-47} , Km ^R	(Håkansson, 1995)
YPIII/pIB60501	pIB102, YopD _{I32K} , Km ^R	This study
YPIII/pIB60502	pIB102, YopD _{I32P} , Km ^R	This study
YPIII/pIB155	pIB102, Δ <i>yopK</i> , Km ^R	(Holmström et al., 1997)
YPIII/pIB155D	pIB155, YopD _{Δ4-303} , Km ^R	(Francis and Wolf-Watz, 1998)
YPIII/pIB155-605	pIB155, YopD _{Δ23-47} , Km ^R	(Olsson et al., 2004)
YPIII/pIB155-60501	pIB155, YopD _{I32K} , Km ^R	This study
YPIII/pIB155-60502	pIB155, YopD _{I32P} , Km ^R	This study
Plasmids		
pCR4@-TOPO	TA cloning vector, Km ^R , Amp ^R	Life Technologies
pDM4	Suicide plasmid carrying <i>sacBR</i> , Cml ^R	(Milton et al., 1996)
pSF013	pDM4 containing a XhoI/XbaI 470 bp PCR product of the allele for YopD _{I32K} , Cml ^R	This study
pSF021	pDM4 containing a XhoI/XbaI 470 bp PCR product of the allele for YopD _{I32P} , Cml ^R	This study

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4 **Table 2**

5 **2** Amino acid identity to YopD from *Yersinia pseudotuberculosis* strain YPIII among the YopD family of translocator proteins and their predicted
6
7 coiled-coil segments
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Species ^a	Strain	Accession number ^b (YopD homologue)	Size (aa)	Percent identity to YPIII_YopD (% amino acid coverage)	Expect (E)-value	YopD type (number of database entries) ^c	Coiled-coil segments ^d
<i>Y. pseudotuberculosis</i>	YPIII*	AAA72322 (YPIII_YopD)	306	n/a	0	n/a	31-44 (unclear)
<i>Y. pseudotuberculosis</i>	IP32953	YP_068463	306	99.7 (100)	0	A (at least 2)	250-275
<i>Y. pestis</i>	PB1+ CO92* Kim10+ Pestoides F 91001 (biovar Microtus) UG05-0454 (biovar Antiqua) MG05-1020 (biovar Orientalis) K1973002 (biovar Mediaevalis)	YP_001874673 NP_395162 (YopD) NP_857754 (YopD) YP_001154618 (YopD) NP_995383 (YopD) ZP_02307382 (YopD) ZP_02314225 ZP_02318589	306	99.7 (100)	0	A (many)	31-44 (unclear) 250-275
<i>Y. enterocolitica</i>	W22703* (serotype 0:9) 105.5R(r)	AAD16812 (YopD) YP_004424912 (YopD)	306	98.0 (100)	0	B (at least 3)	31-44 (unclear) 250-275
<i>Y. enterocolitica</i>	09-856 (serotype O:5,27)	ACX69982 (YopD)	306	97.7 (100)	0	C (1)	31-44 (unclear) 250-275
<i>Y. enterocolitica</i>	8081 (serotype 0:8)	YP_001004066 (YopD)	306	97.4 (100)	0	D (1)	31-44 (unclear) 250-275
<i>Y. enterocolitica</i>	YE96 (serotype 0:9) A127/90* (serotype 0:8)	AAB31852 NP_783662	306	97.1 (100)	0	E (at least 3)	31-44 (unclear) 250-275
<i>A. veronii</i>	HM21* (biovar sobria)	ABP51935(AopD)	298	49.3 (90)	6E-84	n/a	84-111(unclear) 241-266
<i>A. hydrophila</i>	AH-1	AAR26342 (AopD)	299	47.3 (88)	1E-78	n/a	none
<i>A. salmonicida</i>	A449*	YP_001144286 (AopD)	298	45.7 (95)	2E-78	n/a	230-263
<i>Ph. asymbiotica</i>	ATCC 43949	YP_003039900 (PAU_01063)	297	46.2 (91)	3E-74	n/a	62-76(unclear) 243-265(unclear)
<i>Ph. luminescens</i>	W14*	AAO18056 (LopD)	294	46.5 (87)	2E-71	n/a	none
<i>P. aeruginosa</i>	PAO1*	NP_250400 (PopD)	295	41.3 (86)	2E-64	n/a	none
<i>V. harveyi</i>	HY01	ZP_01985143 (VopD)	334	29.2 (90)	2E-24	n/a	131-156 264-318
<i>V. parahaemolyticus</i>	RIMD 2210633*	NP_798035 (VopD)	334	31.3 (70)	3E-19	n/a	128-154 252-317
<i>Pb. damsela</i>	CIP 102761*	ZP_06157736 (VDA_000190)	286	27.8 (70)	3E-15	n/a	121-160 216-238 258-278(unclear)

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- 1 ^a *Y, Yersinia; A, Aeromonas; Ph, Photorhabdus; P, Pseudomonas; V, Vibrio; Pb, Photobacterium*
- 2 ^b NCBI Reference Sequence
- 3 ^c see Figure 1 for a *ClustalW* alignment of representative sequences
- 4 ^d Coiled coil regions identified using the unweighted method of Lupas and colleagues (Lupas et al., 1991) that is hosted by the Swiss Institute of
- 5 Bioinformatics web server http://www.ch.embnet.org/software/COILS_form.html. Unclear refers to those instances that a coiled coil region was
- 6 predicted, but only with a window size < 21 and/or with a probability of < 70%.
- 7 * Coiled- coil prediction output profiles of those marked are available for download and viewing online as Supplementary Fig. S1.
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4 **1 Figure legends**
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10 **3 Fig. 1. Amino acid sequence variation among YopD proteins.** A Blastp (protein-protein BLAST) using
11 YopD query sequence from *Y. pseudotuberculosis* YPIII (designated YPIII_YopD) was used to identify
12
13 4 all related alleles encoded by *Yersinia* (taxonomy identity: 629). This search identified five additional
14
15 5 groups (designated A to E) of different YopD sequences. Representatives of these sequences were then
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17 6 aligned with ClustalW. YopD produced by other *Y. pseudotuberculosis* strains and by *Y. pestis* are
18
19 7 identical with YPIII_YopD bar a sole substitution of Glycine at position 183 for Serine (sequence group
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21 8 A). In contrast, YopD produced by different *Y. enterocolitica* strains possess subtly more variation that
22
23 9 can be further divided into four additional sequence groups; group B (6 differences – K27G, I32V,
24
25 10 T113S, E172D, G183S and I185A), group C (7 differences – Q17R, K27G, I32V, T113S, E172D, G183S
26
27 11 and I185A), group D (8 differences – L18I, K27G, I32V, T111A, E172D, G183S, I185T and G186S) and
28
29 12 group E (9 differences – L18I, K27G, I32V, T111A, E172D, M179L, G183S, I185T and G186S).
30
31 13 Identical residues are represented by the asterisk (*) symbol. Residues predicted to form a putative
32
33 14 transmembrane domain are indicated with a bold black underscore. Predicted coiled coil regions in the N-
34
35 15 terminus and C-terminus are highlighted with a dashed line. Vertical filled bars indicate the heptad
36
37 16 periodicity of the individual segments with the predominately hydrophobic amino acids at the `a` (light
38
39 17 gray) and `d` (dark gray) positions highlighted. Moreover, the isoleucine at position 32 mutated in this
40
41 18 study to specifically disrupt the N-terminal coiled-coil is identified by an arrow. The C-terminal
42
43 19 amphipathic α -helix (Costa et al., 2010; Tengel et al., 2002) is emphasized with a solid gray underscore.
44
45 20 In several other T3SSs belonging to the Ysc-Yop clade (Troisfontaines and Cornelis, 2005), homologues
46
47 21 to YopD exist. Strictly conserved residues found in all analyzed YopD-like sequences are indicated by the
48
49 22 hash (#) symbol. Consult Table 2 for further details including individual bacterial names, NCBI accession
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51 23 numbers and sequence relatedness.
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Fig. 2. Limited chymotrypsin digestion of secreted YopD variants. Secreted YopD prepared from cleared culture supernatants of bacteria grown in BHI media depleted of calcium were incubated with (+) or without (-) 10µg/ml chymotrypsin (CT) for 30min prior to trichloroacetic acid precipitation. Samples were fractionated on a regular 18% acrylamide SDS-PAGE and YopD digestion products identified with polyclonal rabbit anti-YopD. Lanes: Parent (YopD_{wt}), YPIII/pIB102; YopD_{Δ23-47}, YPIII/pIB605; YopD_{I32K}, YPIII/pIB60501; YopD_{I32P}, YPIII/pIB60502. Note the absence of signal in the YopD_{Δ23-47} lane because this product is poorly secreted.

Fig. 3. Oligomeric state of secreted YopD. Secreted YopD prepared from cleared culture supernatants of bacteria grown in BHI media depleted of calcium were cross-linked with (+) or without (-) membrane permeable 0.5mM EGS. Samples were separated by 12% acrylamide SDS-PAGE and immunoblotted with polyclonal rabbit anti-YopD antiserum. The positions of the protein standards with their approximate molecular mass (kDa) are marked at left. Asterisks at right mark protein bands that represent higher order YopD oligomers. After chemiluminescence detection, the left panel indicates short exposure (15sec) and longer exposure (90sec) to X-ray film. Lanes: Parent (YopD_{wt}), YPIII/pIB102; YopD_{Δ23-47}, YPIII/pIB605; YopD_{I32K}, YPIII/pIB60501; YopD_{I32P}, YPIII/pIB60502. Note the absence of signal in the YopD_{Δ23-47} lane because this product is poorly secreted.

Fig. 4. YopD variants are stable and maintain Yops synthesis and secretion control. (A) *Yersinia pseudotuberculosis* was grown in BHI media under non-inducing conditions (+ Ca²⁺) at 37°C. At time point 0, chloramphenicol was added to stop *de novo* protein synthesis and aliquots were taken at different time points (0 to 60min). Resistance to degradation by endogenous proteases was determined by Western-

1 blot using a polyclonal anti-YopD antiserum. (B) Bacteria were grown *in vitro* in BHI media under Yops-
2 restrictive (+Ca²⁺) or Yops-permissive (-Ca²⁺) conditions. Synthesized Yop proteins associated with the
3 bacterial cell pellet (B) or secreted into the cleared culture supernatant (C) was analyzed by
4 immunoblotting using polyclonal rabbit anti-YopB, anti-LcrV, anti-YopD and anti YopE antiserum.
5 Lanes: Parent (YopD_{wt}), YPIII/pIB102; $\Delta yopD$ null mutant (YopD _{Δ 4-303}), YPIII/pIB621; YopD _{Δ 23-47},
6 YPIII/pIB605; YopD_{I32K}, YPIII/pIB60501; YopD_{I32P}, YPIII/pIB60502. The predicted molecular weight of
7 each identified Yop is specified in parentheses.

8
9 **Fig. 5. Contact dependent erythrocyte hemolysis and translocon pore formation by *Y. pseudotuberculosis*.** Erythrocyte lysis caused by *Y. pseudotuberculosis* parent and a series of *yopD*
10 mutants disrupted in the N-terminal putative coiled-coil domain was determined by hemoglobin release.
11 Horse red blood cells were infected in the absence (A) or presence (B) of carbohydrates of different sizes
12 (raffinose (1.2–1.4nm ϕ), dextrin 15 (2.2nm ϕ), and dextran 4 (3–3.5nm ϕ)). To increase the efficiency
13 of pore formation, the $\Delta yopK$ null mutant (YPIII/pIB155) (Holmström et al., 1997) was used as the parent
14 strain into which all *yopD* mutations were introduced. The extent of osmoprotection afforded by the
15 different size sugars is represented as the percentage of lytic activity occurring in the absence of sugars.
16 Strains: $\Delta yopK$ null mutant (parent) producing native YopD (YopD_{wt}), YPIII/pIB155; $\Delta yopK$ with full-
17 length $\Delta yopD$ null mutant (YopD _{Δ 4-303}), YPIII/pIB155D; $\Delta yopK$ with YopD _{Δ 23-47}, YPIII/pIB155-605;
18 $\Delta yopK$ with YopD_{I32K}, YPIII/pIB155-60501; $\Delta yopK$ with YopD_{I32P}, YPIII/pIB155-60502. Uninfected
19 refers to a mock infection in the absence of bacteria to assay for autolysis of red blood cells.

20
21
22 **Fig. 6. *Yersinia*-mediated cytotoxicity against epithelial cell monolayers.** HeLa cell monolayers were
23 infected with various *Y. pseudotuberculosis* strains. At 30, 60 and 120min post-infection, the eukaryotic
24 cell morphology was captured by a phase-contrast microscope containing a digital camera adaption.
25 Intoxication of HeLa cells by the translocation of the GTPase activating protein YopE cytotoxin causes a

1 morphological change in cell shape from oblong to rounded. HeLa cells not intoxicated with YopE show
2 normal cell morphology. Strains: Parent (YopD_{wt}), YPIII/pIB102; $\Delta yopD$ null mutant (YopD _{Δ 4-303}),
3 YPIII/pIB621; YopD _{Δ 23-47}, YPIII/pIB605; YopD_{I32K}, YPIII/pIB60501; YopD_{I32P}, YPIII/pIB60502.
4 Uninfected refers to a mock infection in the absence of bacteria to ensure that cell monolayer morphology
5 is not influence by any other parameter within the conditions assayed.

6
7 **Fig. 7. Disruption of YopD N-terminal coiled-coil does not compromise *in vitro* T3SS activity.** (A)

8 Protease protection and digitonin extraction of translocated *Yersinia* effector proteins present in the
9 cytosol of infected HeLa cell monolayers. Fractions were subjected to western blot analysis using rabbit
10 anti-YopE and goat anti-YopH antiserum and purified mouse anti-ERK1 (BD Pharmingen). Translocated
11 proteins reflected the difference between protein levels observed in digitonin-treated HeLa cells and non-
12 treated HeLa cells. The anti-YopH antiserum cross-reacts with unknown antigens of eukaryote cell origin.
13 The YopH protein also displays enhanced resistance to proteinase K digestion. (B) *Y. pseudotuberculosis*
14 strains were used to infect murine macrophage-like J774-1 cells. Bacterial cells with a compromised
15 T3SS were more rapidly phagocytosed and killed by these immune cells. Bacterial survival as measured
16 by CFU/ml was determined at 2h and 6h post-infection. The results are expressed as a mean of 5
17 independent assays \pm the standard deviation. Bacteria producing YopD_{I32K} and YopD_{I32P} show comparable
18 viability to parental bacteria after 6h. The asterisks (** or ***) indicate that YopD variants were
19 statistically less viable (two-tailed parametric Mann-Whitney *U* test, $P < 0.05$) than parental bacteria.
20 Strains: Parent (YopD_{wt}), YPIII/pIB102; $\Delta yopD$ null mutant (YopD _{Δ 4-303}), YPIII/pIB621; YopD _{Δ 23-47},
21 YPIII/pIB605; YopD_{I32K}, YPIII/pIB60501; YopD_{I32P}, YPIII/pIB60502.

22
23 **Fig. 8. An intact YopD N-terminus is essential for full *Yersinia* virulence.** Overnight cultures of *Y.*
24 *pseudotuberculosis* parent and mutant bacteria were harvested and then serially diluted to 10^9 , 10^8 and 10^7
25 CFU/ml in sterile tap water supplemented with 150mM NaCl and given *ad libitum* to all mice for 6 h to

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1 initiate an oral infection. (A) Mice were observed for a period of 14 days post-infection for survival and
2 (B) alterations in body weight. For an oral dose of 10^7 CFU/ml, mutants in YopD show either no tendency
3 (YopD $_{\Delta 23-47}$) or lower tendency (YopD $_{I32K}$ and YopD $_{I32P}$) to induce a terminal infection. This trend
4 corroborates body weight lost, which reflects general animal well-being. Strains: Parent (producing native
5 YopD), YPIII/pIB102; YopD $_{\Delta 23-47}$, YPIII/pIB605; YopD $_{I32K}$, YPIII/pIB60501; YopD $_{I32P}$,
6 YPIII/pIB60502.

Figure 1

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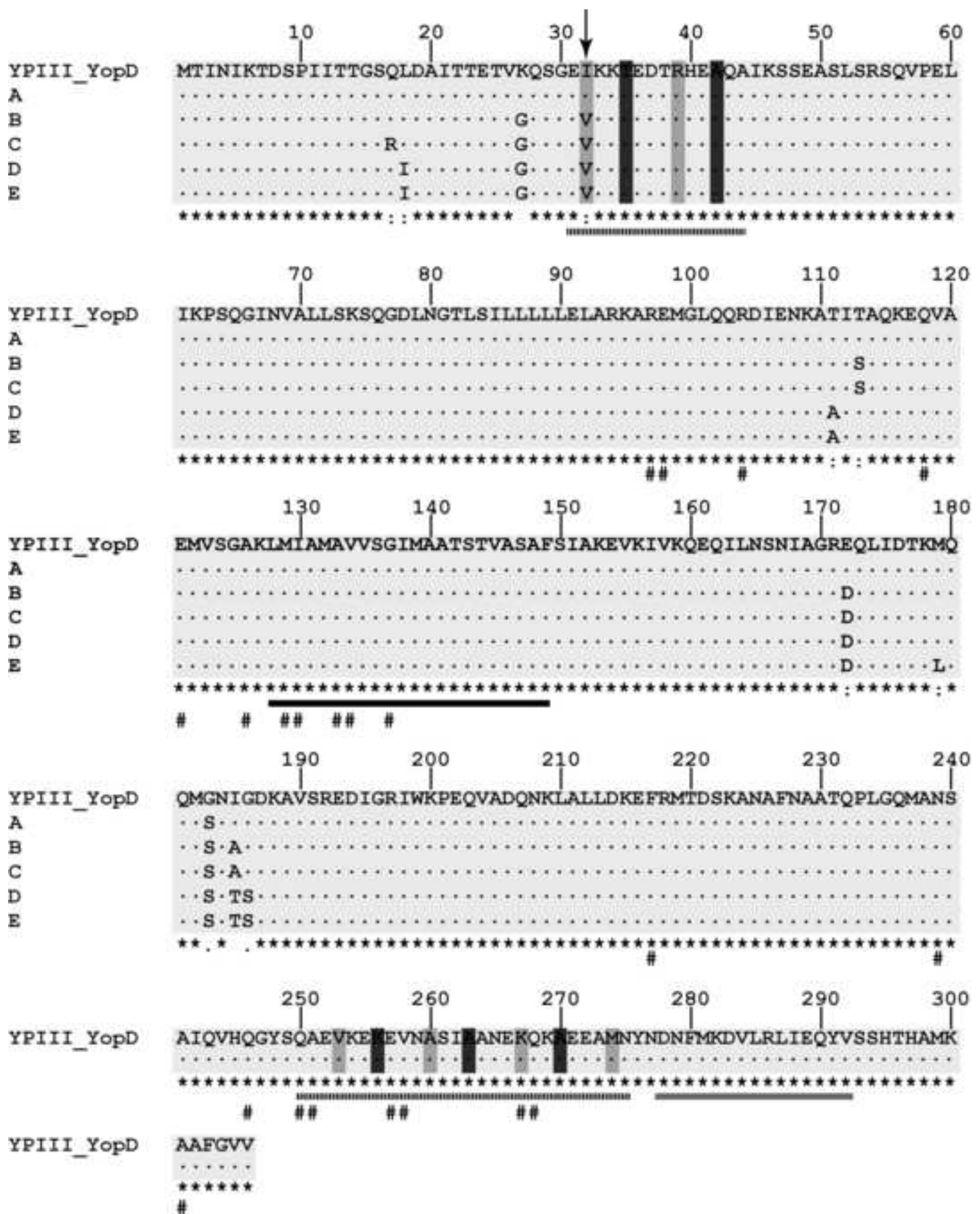


Figure 2
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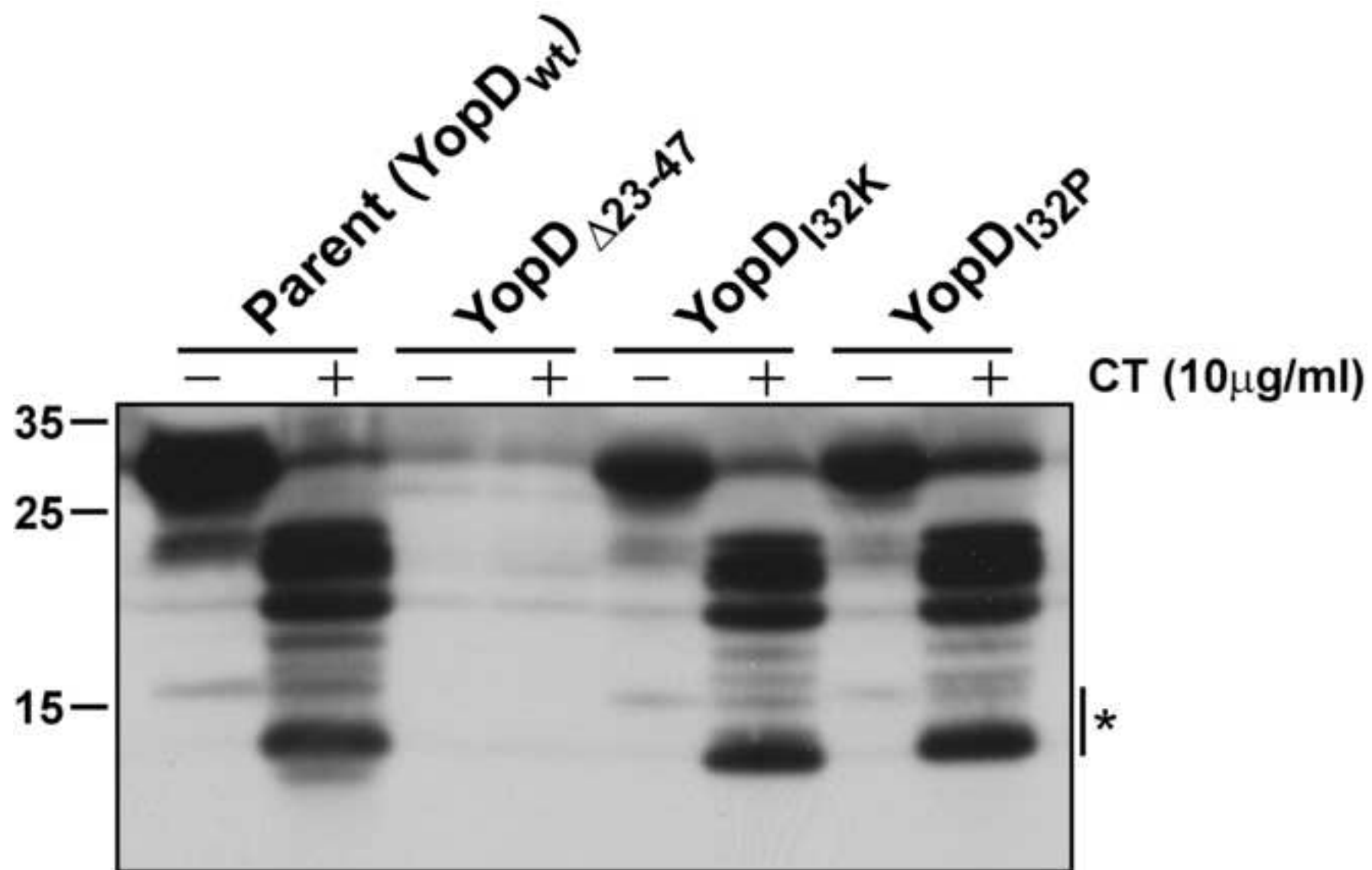


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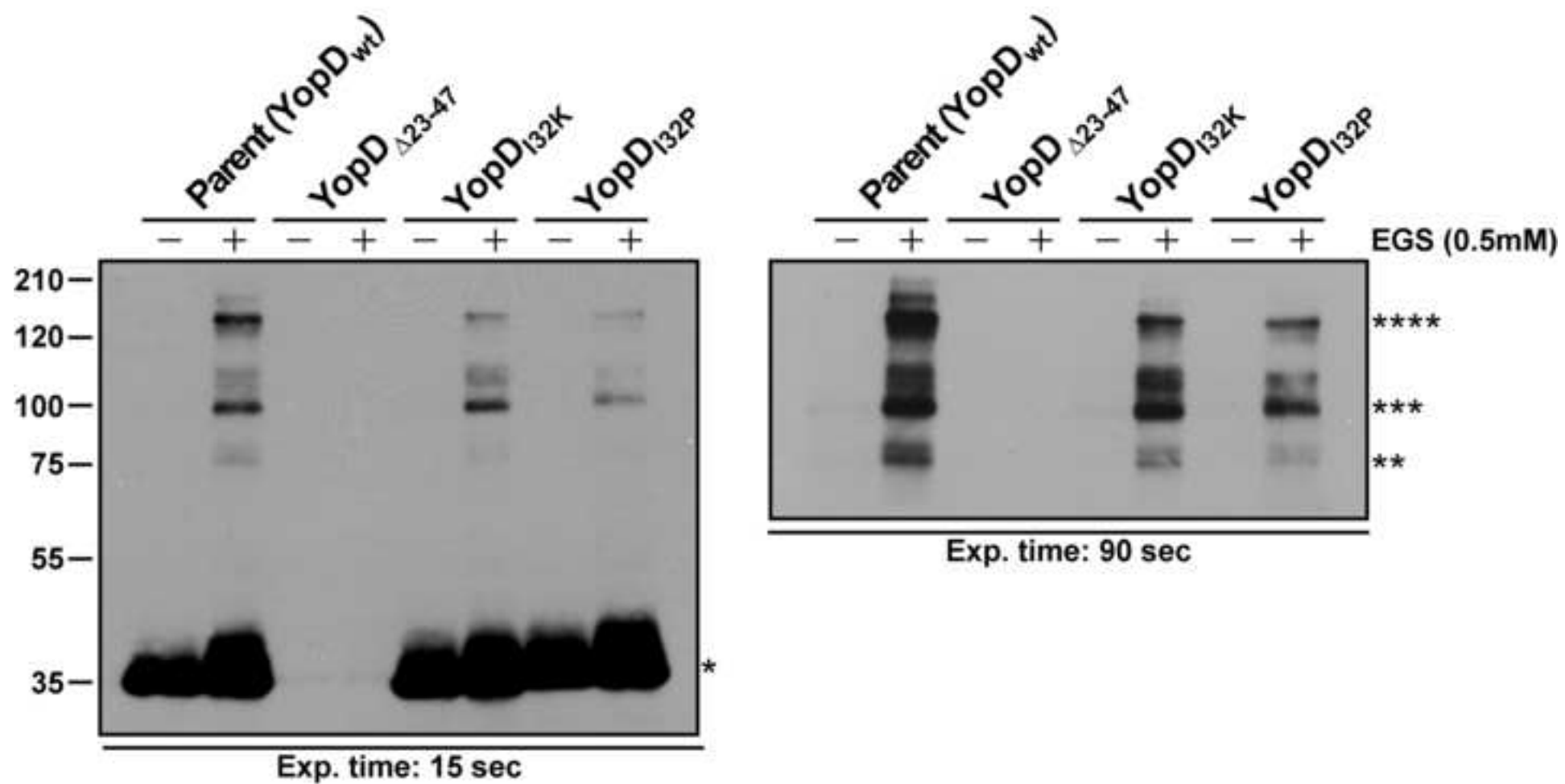
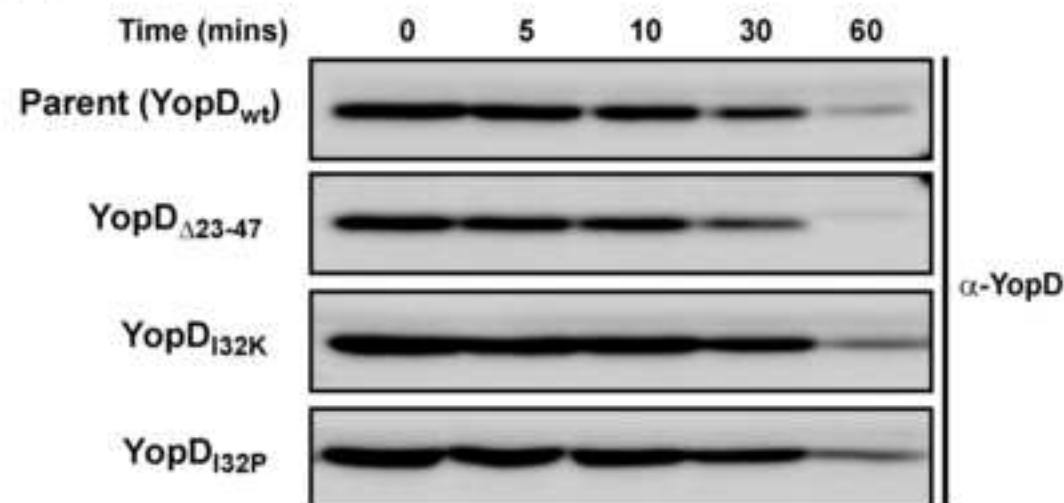
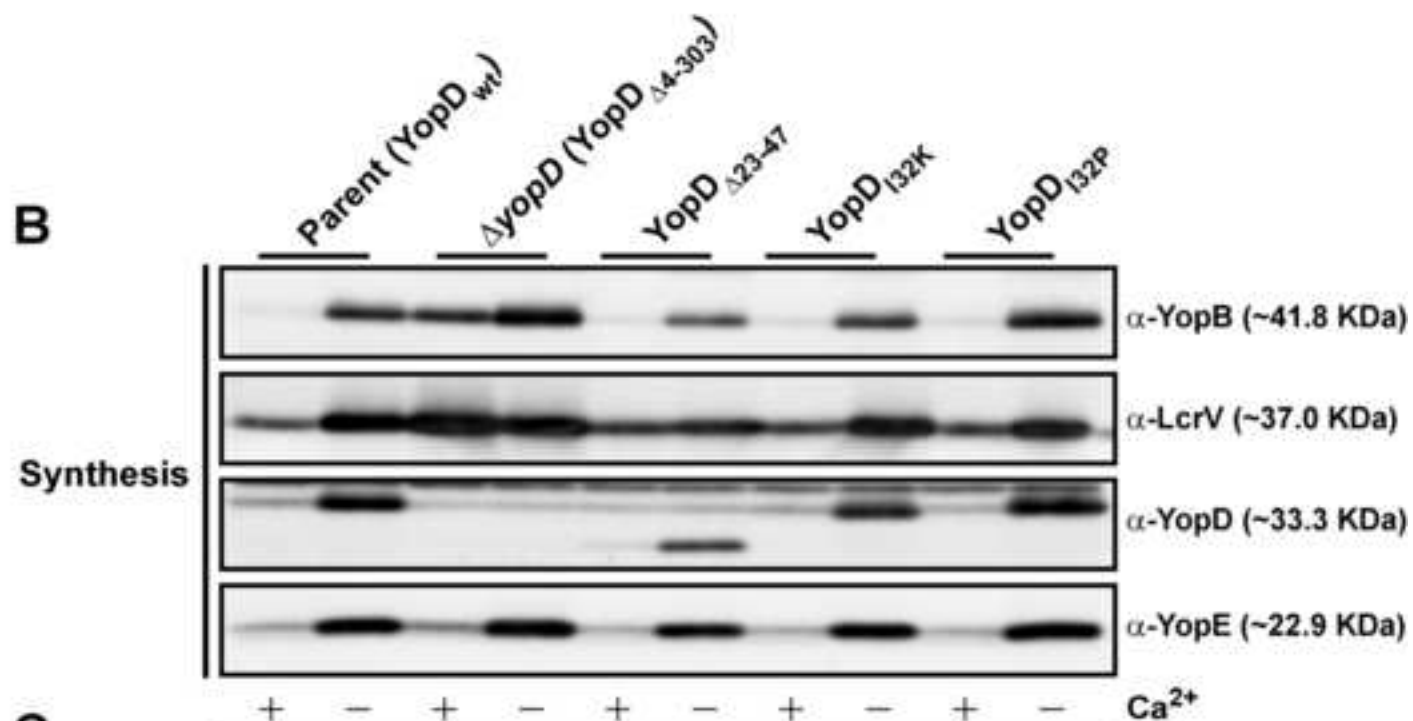


Figure 4
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A



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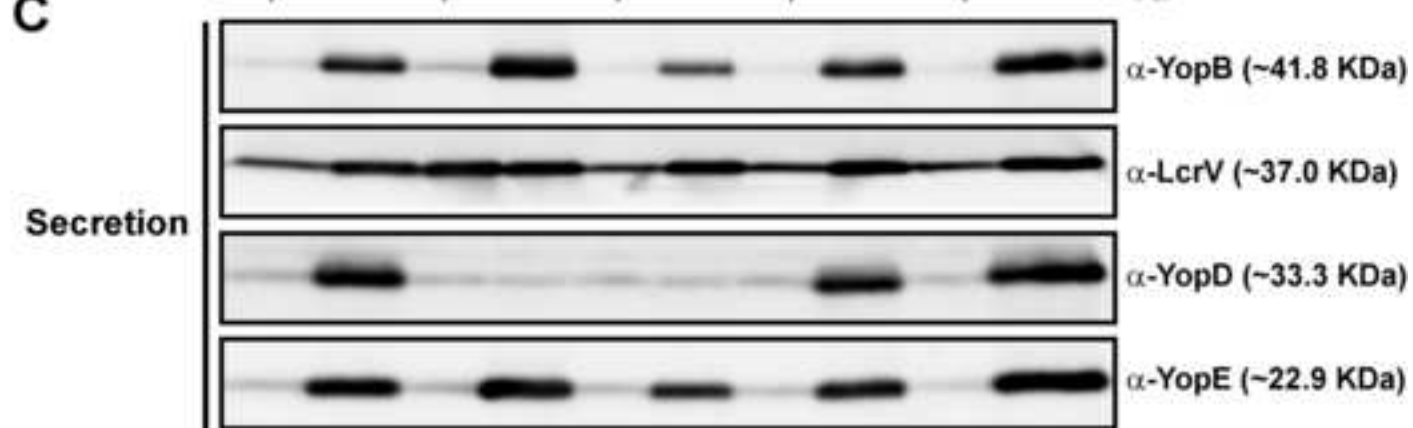


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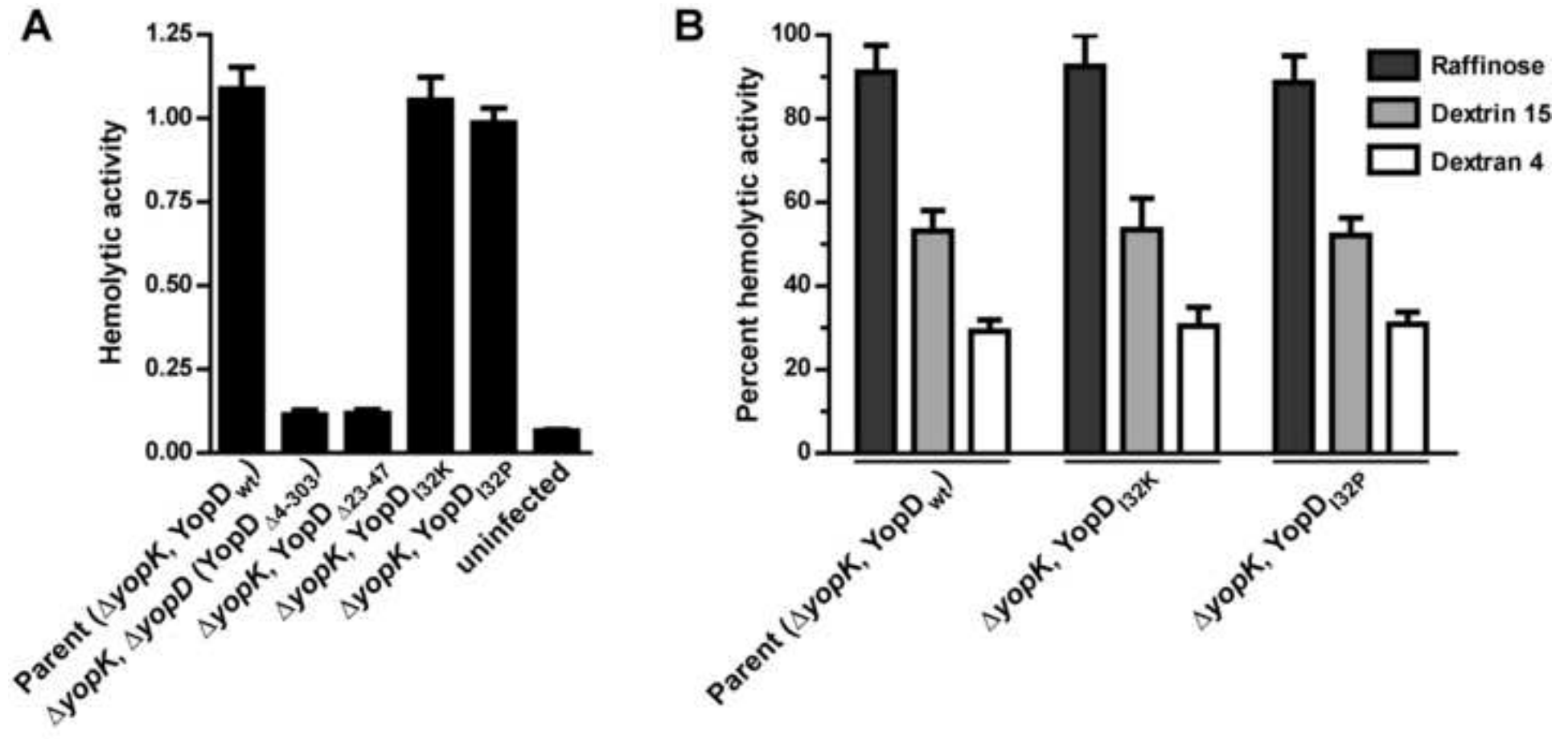


Figure 6
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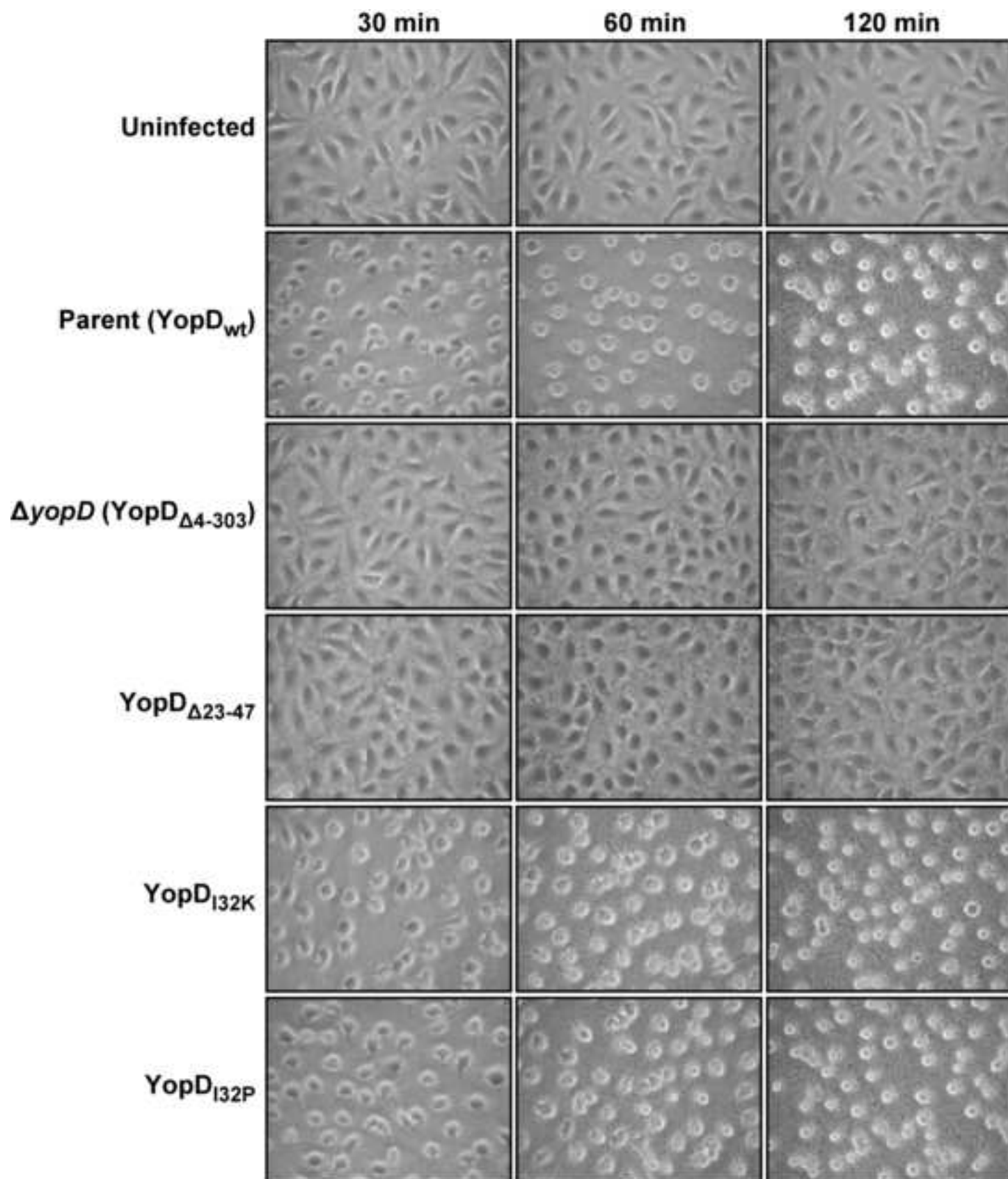


Figure 7
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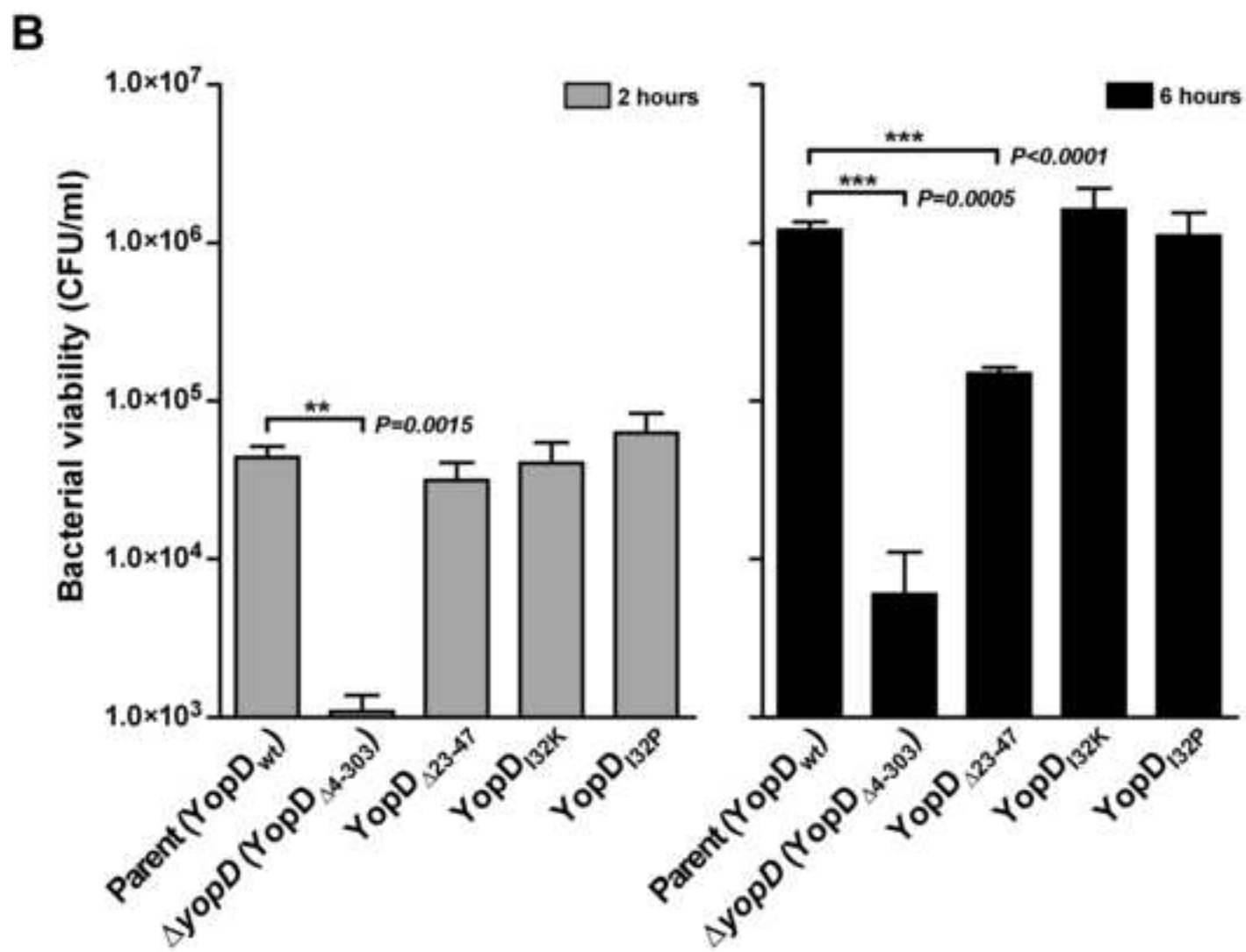
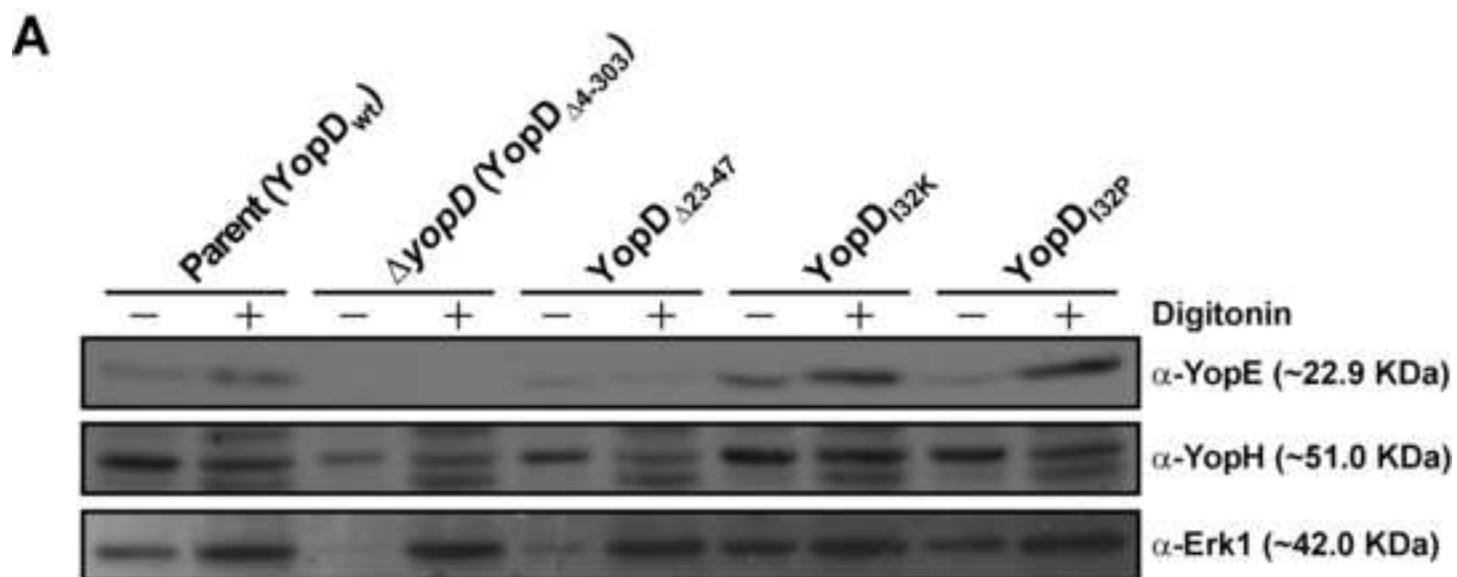
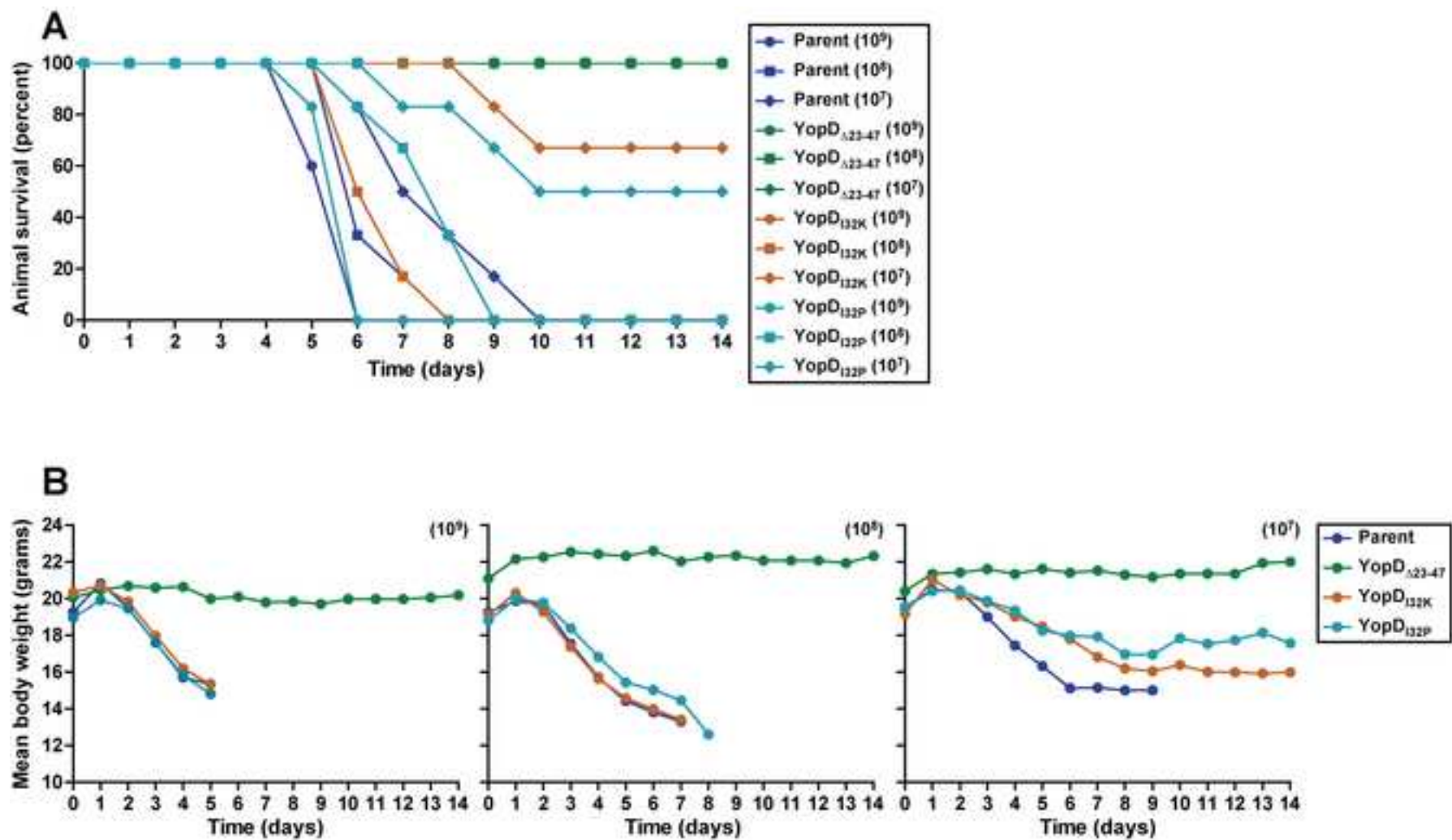


Figure 8
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APPENDIX A:

Contents

Tables:

Supplementary Table S1. Oligonucleotides used in this study

Figures:

Supplementary Figure S1. Coiled-coil prediction output profiles for selected sequences among the YopD family of type III secretion translocator proteins

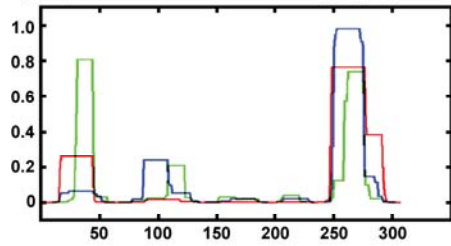
References:

Supplementary Table S2 Oligonucleotides used in this study

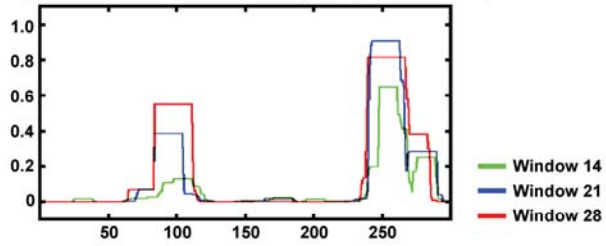
Purpose/vector	Oligonucleotide name and sequence
Mutant construction	
YopD _{I32K}	pXhoA3 5'- <i>ACGCTCGAGTACAACATAATAACGCTG</i> - 3' p(I32K)b 5'- GTCTTCTGTTTTTTTCTT CTCACCGCTTTGCTTGAC - 3' p(I32K)c 5'- GAGAAGAAAAAAAAACAGAAGACA - 3' pXbaD6 5'- <i>ACGTTCTAGATAAAGTACCATTAAGATC</i> - 3'
YopD _{I32P}	pXhoA3 5'- <i>ACGCTCGAGTACAACATAATAACGCTG</i> - 3' p(I32P)b 5'- GTCTTCTGTTTTTTTAGGCTCACCGCTTTGCTTGAC - 3' p(I32P)c 5'- GAGCCTAAAAAAAAACAGAAGACA - 3' pXbaD6 5'- <i>ACGTTCTAGATAAAGTACCATTAAGATC</i> - 3'

The nucleotide sequence in italics represents the incorporated restriction sites used for cloning of the PCR amplified DNA fragments. The base pair(s) underlined represent the amino acid substitution in each site-directed mutation.

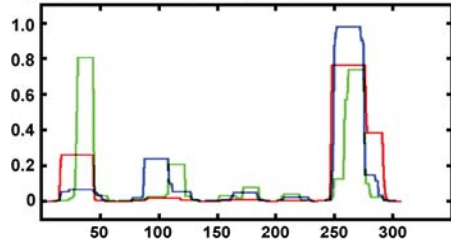
A) *Y. pseudotuberculosis* YPIII (YopD; AAA72322)



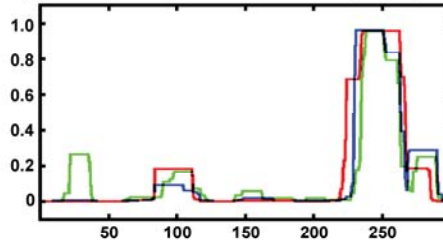
G) *Aeromonas veronii* HM21 (AopD; ABP51935)



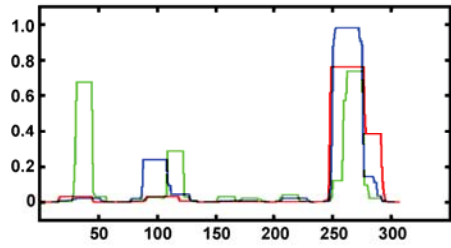
B) *Y. pestis* C092 (YopD; NP_395162)



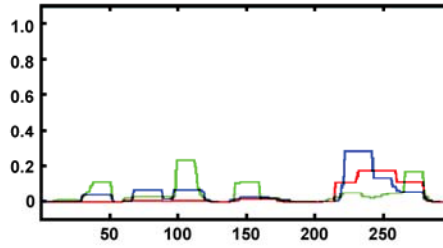
H) *Aeromonas salmonicida* A449 (AopD; YP_001144286)



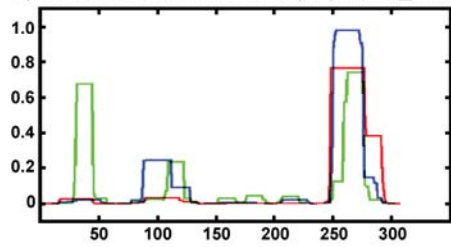
C) *Y. enterocolitica* W22703 (YopD; AAD16812)



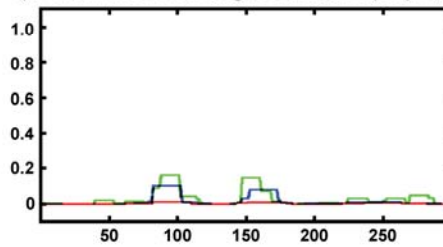
I) *Photobacterium luminescens* W14 (LopD; AAO18056)



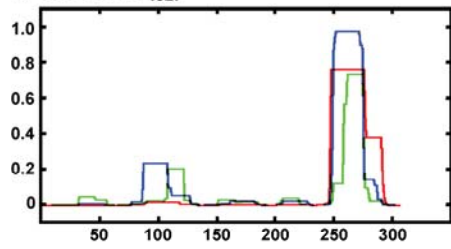
D) *Y. enterocolitica* A127/90 (YopD; NP_783662)



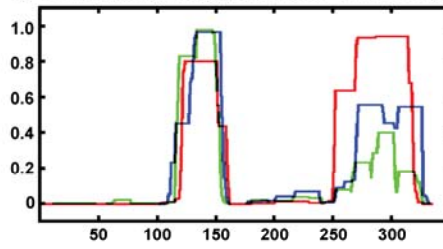
J) *Pseudomonas aeruginosa* HM21 (PopD; NP_250400)



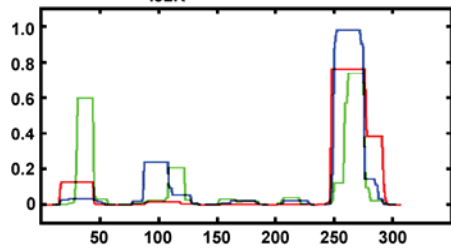
E) YPIII_YopD_{I32P}



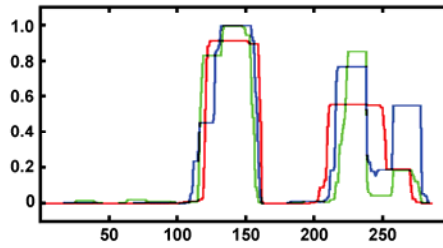
K) *Vibrio parahaemolyticus* RIMD 2210633 (VopD; NP_798035)



F) YPIII_YopD_{I32K}



L) *Photobacterium damsela*e CIP 102761 (VDA_000190; ZP_06157736)



Supplementary Figure S1. Coiled-coil prediction output profiles for selected sequences among the YopD family of type III secretion translocator proteins

Coiled coil prediction profiles were generated using the unweighted method of Lupas and colleagues (Lupas et al., 1991) that is hosted by the Swiss Institute of Bioinformatics web server http://www.ch.emblnet.org/software/COILS_form.html. Sequences are derived from the indicated bacterial species with the NCBI accession number displayed in parentheses. Evident is the heterogeneity among the coiled-coil prediction profiles. With a good degree of predictive certainty, all YopD sequences derived from pathogenic *Yersinia* sp. would be expected to possess a C-terminal coiled-coil (A to D). Additionally, a lower probability coiled coil is tentatively predicted for the extreme N-terminus. Given the unique positioning of this low-probability coiled-coil, a goal of this study was to investigate if this region is important for YopD function. To do this, two substitution mutations in *yopD* from *Y. pseudotuberculosis* YPIII were generated. The resultant variants produced YopD_{I32P} and YopD_{I32K}. While the sequence of the later modestly reduced the coiled-coil prediction (F), the former mutant would not at all be expected to produce a coiled-coil in this region (E). Interestingly, predicted coiled-coils commonly locate nearer to the C-terminus of YopD homologues from other diverse bacteria (G, H, K and L), suggesting these regions might be particularly important for function of this translocator family. Exceptions do exist however, for neither YopD homologues from *Photothabdus luminescens* (I) or *Pseudomonas aeruginosa* (J) appear likely to possess any coiled-coil structure. Functional comparisons between YopD family members with and without predicted coiled-coil regions could therefore be a useful approach to gain functional insight into this structure. Table 2 should be consulted for more information concerning the sequencing similarity within the YopD translocator family and predicted positioning of coiled-coil regions within individual homologues.

References

Lupas, A., Van Dyke, M., Stock, J., 1991. Predicting coiled coils from protein sequences. *Science*. 252, 1162-1164.