

Proteolytic processing of the *Yersinia pestis* YapG autotransporter by the omptin protease Pla and the contribution of YapG to murine plague pathogenesis

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Autotransporter protein secretion represents one of the simplest forms of secretion across Gram-negative bacterial membranes. Once secreted, autotransporter proteins either remain tethered to the bacterial surface or are released following proteolytic cleavage. Autotransporters possess a diverse array of virulence-associated functions such as motility, cytotoxicity, adherence and autoaggregation. To better understand the role of autotransporters in disease, our research focused on the autotransporters of *Yersinia pestis*, the aetiological agent of plague. *Y. pestis* strain CO92 has nine functional conventional autotransporters, referred to as Yaps for *Yersinia* autotransporter proteins. Three Yaps have been directly implicated in virulence using established mouse models of plague infection (YapE, YapJ and YapK). Whilst previous studies from our laboratory have shown that most of the CO92 Yaps are cell associated, YapE and YapG are processed and released by the omptin protease Pla. In this study, we identified the Pla cleavage sites in YapG that result in many released forms of YapG in *Y. pestis*, but not in the evolutionarily related gastrointestinal pathogen, *Yersinia pseudotuberculosis*, which lacks Pla. Furthermore, we showed that YapG does not contribute to *Y. pestis* virulence in established mouse models of bubonic and pneumonic infection. As *Y. pestis* has a complex life cycle involving a wide range of mammalian hosts and a flea vector for transmission, it remains to be elucidated whether YapG has a measurable role in any other stage of plague disease.

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INTRODUCTION

Bacteria of the genus *Yersinia* that are pathogenic to humans include *Yersinia pestis*, *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (Bottone, 1999; Naktin & Beavis, 1999; Rollins *et al.*, 2003). Whilst *Y. pseudotuberculosis* and *Y. enterocolitica* are enteric pathogens that infect humans through ingestion of contaminated food or water, *Y. pestis* is primarily a vector-borne pathogen that is the causative agent of plague. The life cycle of *Y. pestis* primarily involves a rodent host and a flea vector for transmission (Hinnebusch, 1997). However, humans can become accidental hosts, whereby *Y. pestis* infection results in an acute febrile disease with different clinical presentations

depending upon the route of inoculation. These forms of disease include bubonic, pneumonic and septicæmic plague. Bubonic plague occurs when humans are bitten by a flea infected with *Y. pestis*. Following the flea bite, bacteria disseminate to the proximal lymph nodes, where they proliferate and cause massive inflammation that results in a painful, swollen lymph node, or bubo (Sebbane *et al.*, 2005). Without antibiotic treatment, *Y. pestis* then disseminates into the bloodstream causing bacteraemia (leading to septicæmic plague) and subsequently colonizes the liver, spleen and in about 5% of human cases the lungs (Janssen *et al.*, 1958). Colonization of the lungs is known as secondary pneumonic plague, and may be transmitted from human to human by inhalation of respiratory droplets, which can result in primary pneumonic plague (Perry & Fetherston, 1997).

All pathogenic yersiniae share a highly conserved 70 kb low-calcium-response (Lcr) virulence plasmid (named

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Abbreviations: aTc, anhydrous tetracycline; i.n. intranasal; PD, passenger domain; p.i., post-infection; s.c., subcutaneous.

pCD1 in *Y. pestis*) (Wren, 2003). This plasmid encodes the Ysc type III secretion system (T3SS) and several effector proteins known as Yops, which contribute to *Yersinia* virulence by modulating host immune defences (Naktin & Beavis, 1999; Perry & Fetherston, 1997; Viboud & Bliska, 2005). In contrast to the enteric yersiniae, *Y. pestis* possesses two additional virulence plasmids, pPCP1 and pMT1. The multifunctional surface omptin protease/adhesin, Pla, is encoded on pPCP1 and has been demonstrated to cleave host plasminogen and components of the complement pathway and mediate adhesion to and invasion into human endothelial cells (Lähteenmäki *et al.*, 2001; Rakin *et al.*, 1996; Sodeinde & Goguen, 1989; Suomalainen *et al.*, 2007). Because this pathogen has adapted to the varying lifestyles within the flea, rodent and human, and has the capacity to infect by differing routes, it is likely that *Y. pestis* has evolved a set of 'host-specific' and 'route-specific' virulence factors that allow it to thrive in each niche. As rodents are a natural host for *Y. pestis* infection, murine models of bubonic and pneumonic infection have been developed that mimic the progression of human bubonic and pneumonic disease (Cathelyn *et al.*, 2006; Lathem *et al.*, 2005; Sebbane *et al.*, 2005). These models have proven to be invaluable for the identification and characterization of virulence factors in the fully virulent *Y. pestis* strain CO92.

The simplest mechanism of protein translocation across the inner and outer membranes in Gram-negative bacteria is the type V secretion system of autotransporter proteins (Dautin & Bernstein, 2007). The conventional autotransporter, or type Va, protein consists of three basic domains: an N-terminal signal sequence, a passenger domain (PD) and a C-terminal β -domain (Henderson *et al.*, 2004). The N-terminal signal sequence is required for secretion through the inner membrane via the Sec system. Once in the periplasm, many autotransporters have been shown to interact with periplasmic chaperones and the Bam (Omp85) complex for stabilization of the PD and proper insertion of the β -domain into the outer membrane (Bodelón *et al.*, 2009; Jong *et al.*, 2010; Ruiz-Perez *et al.*, 2009; 2010; Sauri *et al.*, 2009). The PD is then translocated through the periplasm and across the outer membrane via the C-terminal β -domain, which forms a pore in the outer membrane. Once the PD has traversed the outer membrane, it can (i) remain covalently attached to the β -domain, (ii) be proteolytically cleaved from the β -domain but remain non-covalently associated with the β -domain, or (iii) be cleaved from the β -domain and released from the cell. Cleavage of the PD can occur via an autocatalytic mechanism, as with the serine protease autotransporters of the *Enterobacteriaceae* (SPATEs) (Henderson *et al.*, 1998), or by outer-membrane proteases, such as the omptins OmpT and Pla (Lawrenz *et al.*, 2009; Lenz *et al.*, 2011). The sequences of autotransporter PDs vary greatly, and encode a number of different virulence-associated functions involved in adhesion, cytotoxicity, agglutination, motility, proteolytic degradation and complement resistance (Henderson & Nataro, 2001).

Within the *Y. pestis* CO92 genome, we recently identified genes encoding nine functional conventional autotransporters (referred to here as the Yaps, for *Yersinia* autotransporter proteins) (Lenz *et al.*, 2011). In examining the contribution of Yaps to plague pathogenesis, our laboratory found unique roles for YapE, YapJ and YapK. In particular, YapE is required for full virulence during bubonic but not pneumonic infection, and promotes adherence to human-derived lung epithelial cells in culture (Lawrenz *et al.*, 2009). The highly similar YapJ and YapK have an additive, non-redundant role in systemic *Y. pestis* infection (Lenz *et al.*, 2012). YapC is also known to mediate bacterial autoaggregation and adherence to murine-derived macrophage-like cells and human-derived epithelial cells when expressed in *Escherichia coli* (Felek *et al.*, 2008). Of the *Y. pestis* Yaps, YapG is unique in that its PD is cleaved at multiple sites by the omptin protease Pla, releasing three major fragments of the YapG PD to the extracellular milieu (Lenz *et al.*, 2011). In analysing expression of each of the *yap* genes by qRT-PCR, our previous work showed that *yapG* expression is modestly induced during bubonic and pneumonic plague in mice (Lenz *et al.*, 2011). Additionally, the production of YapG-specific antibodies was demonstrated during a sublethal rabbit model of bubonic plague using *in vivo*-induced antigen technology (Andrews *et al.*, 2010). Based on these data, and the observation that most characterized autotransporters are either implicated in virulence or have virulence-associated functions, it seems likely that YapG also contributes to *Y. pestis* pathogenesis. In this report, we further characterized the complex processing of YapG by Pla. In addition, we assessed the contribution of YapG to *Y. pestis* virulence using the established mouse models of bubonic and pneumonic plague.

METHODS

Bacterial strains, growth conditions and animal infections. All bacterial strains and plasmids used in this study are listed in Table 1. The fully virulent, WT *Y. pestis* CO92 is a clinical isolate from a pneumonic infection provided by the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA (Table 1) (Doll *et al.*, 1994). *Y. pestis* strains were routinely cultured on brain-heart infusion (BHI) agar (BD Biosciences) at 26 °C for 48 h or in BHI broth (BD Biosciences) with aeration at 26 or 37 °C as indicated. *Y. pseudotuberculosis* strains were routinely cultured on Luria-Bertani (LB) agar (BD Biosciences) at 26 °C for 48 h or in LB medium at 26 °C with aeration. *E. coli* DH5 α and DH5 α PRO were used for plasmid maintenance and propagation. *E. coli* strains were routinely cultured on LB agar at 37 °C for 16 h or in LB medium at 37 °C with aeration for 16 h. Antibiotic supplementation with carbenicillin (100 μ g ml⁻¹) or kanamycin (50 μ g ml⁻¹) was provided as necessary.

Strain and plasmid construction. The $\Delta yapG$ in-frame deletion strain (YP261) was constructed using the λ Red recombination system with modifications (Datsenko & Wanner, 2000; Lathem *et al.*, 2007). Briefly, ~500 bp fragments of the 5' and 3' regions of *yapG* were amplified by PCR using the DNA096/DNA097 and DNA098/DNA099 primer sets, respectively (Table 2). After gel purification, the resulting PCR products were mixed with a kanamycin resistance cassette (Kan^R) flanked by FLP recognition target (FRT) sites, which previously had been amplified from the template plasmid pKD13.

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description*	Reference/source
<i>Y. pestis</i> strains		
CO92	WT, pMT1 ⁺ , pPCP1 ⁺ , pCD1 ⁺ and <i>pgm</i> ⁺	Doll <i>et al.</i> (1994)
YP6	CO92, pCD1 ⁻	Cathelyn <i>et al.</i> (2006)
YPA45	CO92 Δ <i>pla</i> , pCD1 ⁻	Lawrenz <i>et al.</i> (2009)
YPA51	CO92 Δ <i>pla-2</i> , pCD1 ⁻	P. Price and W. Goldman (University of North Carolina, NC, USA)
YP261	CO92 Δ <i>yapG</i> , pCD1 ⁺	This study
YPA53	CO92 Δ <i>yapG</i> , pCD1 ⁻	This study
<i>Y. pseudotuberculosis</i> strains		
YPIII	WT	Bölin <i>et al.</i> (1982)
IP32953	WT	R. Isberg (Tufts University, MA, USA); Chain <i>et al.</i> (2004)
<i>E. coli</i> strains		
DH5 α PRO	<i>deoR endA1 gyrA96 hsdR17</i> (r _K ⁻ m _K ⁺) <i>recA1 relA1 supE44 thi-1</i> Δ (<i>lacZYA-argF</i>)U169 Φ 80d <i>lacZ M15 F⁻ λ⁻ P_{N25} tetR P_{lacI}^q lacI Spr</i>	Clontech
DH5 α	Φ 80d Δ (<i>lacZ</i>)M15 Δ (<i>argF-lac</i>)U169 <i>endA1 recA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>deoR thi-1 supE44 gyrA96 relA1</i>	Gibco
Plasmids		
pWL204	Plasmid carrying the λ <i>red</i> recombinase gene and <i>sacB</i> gene for sucrose counterselection; Carb ^R	Lathem <i>et al.</i> (2007)
pKD13	λ <i>red</i> recombinase system template plasmid; Kan ^R , Carb ^R	Datsenko and Wanner (2000)
pLH29	Plasmid carrying the FLP recombinase and <i>sacB</i> genes; Carb ^R	Lawrenz <i>et al.</i> (2009)
pMWO-005	Expression vector containing P _{LtetO-1} promoter, P _{N25} <i>tetR</i> repressor, pSC101 ori, and multiple cloning site from pWKS130; Kan ^R	Obrist and Miller (2012)
pLP-PROTet-6xHN	Expression vector that contains P _{LtetO-1} promoter; Carb ^R	Clontech
pLP-PROTet:: <i>yapG</i>	pLP-PROTet-6xHN containing <i>yapG</i>	This study
pMWO:: <i>yapG</i>	pMWO-005 containing <i>yapG</i> from pLP-ProTet:: <i>yapG</i>	This study
pMWO:: <i>yapG</i> SDM2	pMWO:: <i>yapG</i> containing KR ₅₄₈₋₅₄₉ AG mutations	This study
pMWO:: <i>yapG</i> SDM4	pMWO:: <i>yapG</i> containing KR ₅₉₄₋₅₉₅ AG mutations	This study
pMWO:: <i>yapG</i> SDM2 + 4	pMWO:: <i>yapG</i> containing KR ₅₄₈₋₅₄₉ AG and KR ₅₉₄₋₅₉₅ AG mutations	This study
pMWO:: <i>yapG</i> SDM1	pMWO:: <i>yapG</i> containing K ₅₁₂ A mutation	This study
pMWO:: <i>yapG</i> SDM2 + 3	pMWO:: <i>yapG</i> containing KR ₅₄₈₋₅₄₉ AG and K ₅₅₈ A mutations	This study
pMWO:: <i>yapG</i> SDM4 + 5	pMWO:: <i>yapG</i> containing KR ₅₉₄₋₅₉₅ AG and K ₆₀₄ A mutations	This study
pMWO:: <i>yapG</i> SDM3 + 5	pMWO:: <i>yapG</i> containing K ₅₅₈ A and K ₆₀₄ A mutations	This study
pMWO:: <i>yapG</i> SDM2 + 3 + 4 + 5	pMWO:: <i>yapG</i> containing KR ₅₄₈₋₅₄₉ AG, K ₅₅₈ A, KR ₅₉₄₋₅₉₅ AG and K ₆₀₄ A mutations	This study
pMWO:: <i>yapG</i> SDM1 + 4 + 5	pMWO:: <i>yapG</i> containing K ₅₁₂ A, KR ₅₉₄₋₅₉₅ AG and K ₆₀₄ A mutations	This study
pMWO:: <i>yapG</i> SDM1 + 2 + 3	pMWO:: <i>yapG</i> containing K ₅₁₂ A, KR ₅₄₈₋₅₄₉ AG and K ₅₅₈ A mutations	This study
pMWO:: <i>yapG</i> SDM1 + 2 + 3 + 4 + 5	pMWO:: <i>yapG</i> containing K ₅₁₂ A, KR ₅₄₈₋₅₄₉ AG, K ₅₅₈ A, KR ₅₉₄₋₅₉₅ AG and K ₆₀₄ A mutations	This study

*RBS, ribosome-binding site; Kan, kanamycin; Carb, carbenicillin; Sp, spectinomycin.

In a splicing-by-overhang-extension (SOE)-PCR, the three PCR fragments were joined together using primers DNA096 and DNA099. The resulting SOE-PCR product (*yapG*5'-FRT-Kan^R-FRT-*yapG*3') was gel purified and transformed into *Y. pestis* strain CO92 carrying pWL204, which previously had been cultured with 10 mM arabinose to induce expression of the λ Red recombinase gene. Recombinants were initially selected on BHI agar containing kanamycin and then passaged onto BHI agar containing 5% sucrose (w/v) to cure the λ Red recombinase plasmid, pWL204. The Kan^R was removed from the mutant strain using the FRT recombinase provided by a version of pLH29 that has been modified by replacing the chloramphenicol resistance gene with an ampicillin resistance gene (Lawrenz *et al.*, 2009). Kan^S and Carb^S recombinants were selected and the in-frame

deletion of *yapG* was verified by PCR. Additionally, the retention of major virulence determinants (pCD1, pMT1, pPCP1 and *pgm*) was confirmed by PCR.

For the YapG induction experiments, *Y. pestis* strains were cured of the pCD1 virulence plasmid by passage on BHI agar supplemented with magnesium oxalate (20 mM MgCl₂ and 20 mM Na₂C₂O₄) at 37 °C (Higuchi & Smith, 1961). Plasmid loss was confirmed by absence of PCR amplification of *yopH* and *yopT*. For induction of *yapG* expression, *yapG* was amplified from *Y. pestis* CO92 by PCR with gene-specific primers (Table 2) and initially cloned into the pLP-PROTet-6xHN plasmid (Clontech). Specifically, the *yapG* PCR product and pLP-PROTet-6xHN were digested with *KpnI* and

Table 2. Oligonucleotide primers used in this study

Primer name (description)	Primer sequence (5'→3')*
Mutant construction	
DNA096 (<i>yapG</i> 5' region forward)	AGGCTTTGCGTTCACAGATC
DNA097 (<i>yapG</i> 5' region reverse)	GAAGCAGCTCCAGCCTACACCATGATTTAGAGTTACCAAT
DNA098 (<i>yapG</i> 3' region forward)	GGTCGACGGATCCCCGGAATAATATCCATTGATAAACGATACCTGTGCC
DNA099 (<i>yapG</i> 3' region reverse)	CAATCGTGTAGYGATACAGCGTTTTATCC
P1Kan (<i>kan</i> of pKD13 forward)	GTGTAGGCTGGAGCTGCTTC
P4Kan (<i>kan</i> of pKD13 reverse)	ATTCCGGGGATCCGTCGACC
Cloning into pLP-PROTet	
JDL52 (<i>yapG</i> forward)	GGGGT <u>ACCCAT</u> GAAGAATTCAAATAGATCACCCAAAAAC
DNA269 (<i>yapG</i> reverse)	CGAAGCTTATAGGCACAGGTATCGTTTTATCAATGGATATTA
Site-directed mutagenesis	
DC7 (SDM2 and SDM4 forward)	CGTTCAGACCCAGCGGGCGCCAATGCCG
DC6 (SDM2 and SDM4 reverse)	CGGCATTGGCGCCCGCTGGGTCTGGAACG
MCL77 (SDM1 forward)	GCAGTCTTTTCAGCTGCATTAGCTGATTACGCGTTGCAAGCC
MCL76 (SDM1 reverse)	GGCTTGCAACGCGTAATCAGCTAATGCAGCTGAAAAGACTGC
MCL75 (SDM3 forward)	GCAGCCTTTTCAGCCGATTAGCTGATTACGCGTTGCAAACC
MCL74 (SDM3 reverse)	GGTTTGCAACGCGTAATCAGCTAATGCGGCTGAAAAGGCTGC
MCL73 (SDM5 forward)	GAAGCCTTTTCAGCCGATTAGCTGATTACGCGTTGCAAGCT
MCL72 (SDM5 reverse)	AGCTTGCAACGCGTAATCAGCTAATGCGGCTGAAAAGGCTTC

*Restriction sites are underlined.

*Hind*III and then ligated and transformed by electroporation into *E. coli* DH5 α PRO (Clontech), which harbours the tetracycline repressor. To allow for the induction of *yapG* in *Y. pestis*, the previously constructed pLP-PROTet plasmid was digested with *Bss*SI and *Hind*III to produce a fragment containing the inducible promoter/operator elements followed by the full-length *yapG* gene. The fragment was ligated into pMWO-005, a low-copy-number vector carrying origins compatible with *Y. pestis* and *E. coli* (Obrist & Miller, 2012), and transformed by electroporation into DH5 α . Upon verification of *yapG* insertion by PCR, the completed plasmid was isolated and transformed into *Y. pestis* CO92 (strain YP6, YPA45, YPA51 or YPA53).

Site-directed mutagenesis of the putative Pla cleavage sites of YapG was performed using the pMWO:: *yapG* double-stranded denatured plasmid template and *Dpn*I for selection of mutants (Sambrook & Russell, 2001). Point mutations were introduced into the overlapping regions of oligonucleotide primers DC7 and DC6 to replace either or both of the KR_{548–549} and KR_{594–595} codons with AG codons (Table 2). Additional site-directed mutations and combinations of site-directed mutations were similarly introduced using oligonucleotide primers MCL77/MCL76 (K₅₁₂A), MCL75/MCL74 (K₅₅₈A) and MCL73/MCL72 (K₆₀₄A) (Table 2). As each of these codons lies within the direct repeat regions of *yapG*, the desired site-directed mutations were confirmed by sequencing.

Induction of *yapG* expression, sample preparation and Western blot analysis. For induction experiments, *Y. pestis* cultures were grown overnight, diluted 1:25 in fresh BHI medium, and grown for 3 h at 26 °C. *yapG* expression was induced by the addition of 100 ng anhydrous tetracycline ml⁻¹ (Sigma), and cultures were grown for an additional 2 h. Whole-cell lysates and supernatants from induced and uninduced cultures were prepared for SDS-PAGE as described previously (Lawrenz *et al.*, 2009; Walker & Miller, 2004). Samples were boiled for 10 min, separated by SDS-PAGE and transferred to Immobilon-P membranes (Millipore) for Western blot analysis. Anti-YapG serum was generated in rabbits (Cocalico Biological) against the PD of YapG (aa 50–549) and pre-absorbed against *E. coli* lysates. Blots were incubated with a 1:2000 dilution of

anti-YapG serum, followed by incubation with a 1:50 000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma). The blots were developed using chemiluminescence according to the manufacturer's instructions (Amersham ECL; GE Healthcare Life Sciences).

Animal infections. All animal experiments were approved by the University of North Carolina Institutional Animal Care and Use Committee (protocol 11-128). Female C57Bl/6J mice and BALBc/J mice (4–6 weeks old; The Jackson Laboratory) were used for all *Y. pestis* infections. All mice were maintained in a barrier facility and allowed free access to sterilized food and water. For subcutaneous (s.c.) infections, *Y. pestis* CO92 WT (CO92) and Δ *yapG* (YP261) were cultured at 26 °C in BHI broth for 16 h with aeration. Subcutaneous inoculations were performed as described by Cathelyn *et al.* (2006) with doses of ~10² c.f.u. per mouse. For intranasal (i.n.) infections, *Y. pestis* CO92 WT (CO92) and Δ *yapG* (YP261) were cultured in BHI broth with aeration at 26 °C for 8–10 h prior to subculture into BHI broth containing 2.5 mM CaCl₂ for overnight growth at 37 °C. Cultures were then diluted in sterile PBS to achieve an inoculum of ~10⁴ c.f.u. per mouse, and inoculations were performed as described by Lathem *et al.* (2005). For all *Y. pestis* infections, groups of six to eight mice were euthanized by intraperitoneal injection with sodium pentobarbital [150 mg (kg body weight)⁻¹] at the time points indicated. At each time point, tissues were harvested, weighed and homogenized in sterile PBS, and serial dilutions were plated on BHI agar to determine bacterial load. The statistical significance of the data obtained in all independent challenge experiments was assessed using a Mann–Whitney test.

RESULTS AND DISCUSSION

Processing of YapG by the omptin protease Pla in *Y. pestis*

In a previous study conducted by our group, we demonstrated the secretion and localization of the

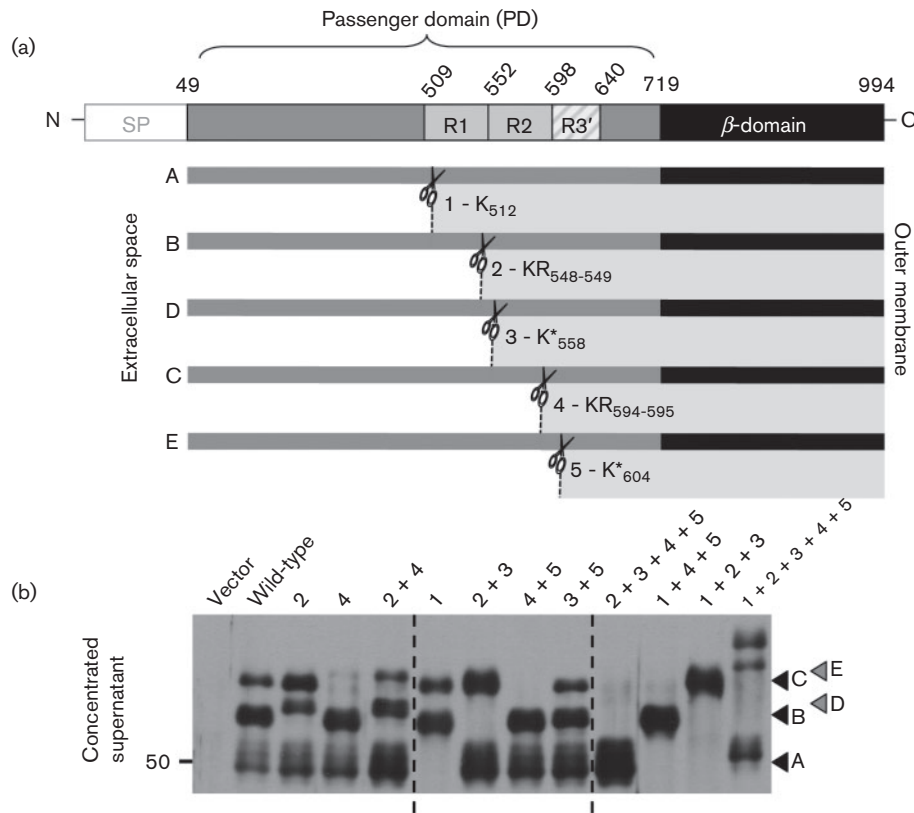


Fig. 2. Amino acid residues of YapG important for processing by the omptin protease Pla. (a) Schematic of the domain organization of YapG. The signal peptide (SP) is shown in white, the PD in grey and the β -domain in black. The basic Lys and Arg residues within the PD that are demonstrated to be important for Pla cleavage are located within two direct repeats (R1 and R2) and in a third partial direct repeat (R3'). Cleavage of sites 1–5 (K_{512} , $KR_{548-549}$, K_{558} , $KR_{594-595}$ and K_{604}) by Pla (illustrated with scissors) results in released fragments A–E. (b) Western blot demonstrating the secretion of WT and mutant YapG in the avirulent (pCD1⁻) *Y. pestis yapG* mutant. Bacteria were treated with aTC to induce *yapG* expression, and culture supernatants were probed with anti-YapG serum. The site-directed mutations (individual or multiple) examined for cleavage are indicated above the blot. The arrows to the right correspond to the predicted fragment sizes illustrated in (a). Sites 1 (K_{512}), 2 ($KR_{548-549}$) and 4 ($KR_{594-595}$) represent the major cleavage sites of YapG resulting in fragments A, B and C, respectively, whereas, sites 3 (K_{558}) and 5 (K_{604}) represent the secondary alternative cleavage sites of YapG resulting in fragments D and E, respectively. Molecular mass is indicated to the left of the blot in kDa.

57.6 kDa (indicated as fragments A–E in Fig. 2a, b), which are consistent with the masses of the three released fragments of YapG observed in Fig. 2(b). Because omptin proteases prefer to cleave between two basic amino acids, we initially decided to address whether the two KR residues are important for Pla cleavage by introducing site-directed mutations into the *yapG* gene by replacing either or both KR codons with AG codons to make these sites neutral and not basic. To determine whether one or more mutation(s) eliminated cleavage of YapG, the resulting *yapG* site-directed mutations were expressed in *Y. pestis*, and samples were collected and subjected to Western blot analysis using anti-YapG serum as described above. Site-directed mutations in site 2 ($KR_{548-549}AG$), site 4 ($KR_{594-595}AG$), and sites 2 + 4 ($KR_{548-549}AG/KR_{594-595}AG$) predominantly resulted in the release of two new fragments (D and E) of YapG with slightly larger molecular masses (Fig. 2b, left

panel). It is important to note that we also observed the same mass shift with a double-alanine ($KR_{548-549}AA$) mutation, suggesting that the altered cleavage of YapG by Pla is due to altered site specificity rather than a change in conformational flexibility of the protein structure due to the Gly substitution (data not shown). To identify the other residues of YapG that are targeted by Pla, we constructed the following additional site-directed mutations in *yapG* (individually or in combination with the original mutations): site 1 ($K_{512}A$), site 3 ($K_{558}A$), and site 5 ($K_{604}A$). The site 1 ($K_{512}A$) mutation resulted in loss of the smallest released fragment (A) of YapG (Fig. 2b, centre), suggesting that, in addition to site 2 ($KR_{548-549}$) and site 4 ($KR_{594-595}$), site 1 (K_{512}) is also a primary site of Pla cleavage. Furthermore, the sites 2 + 3 ($KR_{548-549}AG/K_{558}A$) and sites 4 + 5 ($KR_{594-595}AG/K_{604}A$) mutations resulted in loss of the middle (B and D) and largest (C and

E) released fragments of YapG, respectively (Fig. 2b, centre). Mutation of these two additional sites alone [sites 3 + 5 (K₅₅₈A/K₆₀₄A)] did not alter YapG cleavage specificity (Fig. 2b, centre panel), suggesting that site 3 (K₅₅₈) and site 5 (K₆₀₄) represent secondary alternate sites for YapG cleavage by Pla. By making combinations of mutations within the primary and secondary cleavage sites, we were able to isolate each of the major released fragments of YapG (Fig. 2b, right panel). Mutation of all primary and secondary sites in *yapG* did not entirely abolish cleavage of YapG. In fact, mutation of sites 1–5 resulted in three new released fragments of YapG, with slightly different molecular masses (Fig. 2b, right panel). Release of these three new YapG fragments in the site 1–5 mutant was determined to be Pla dependent (data not shown). In summary, through site-directed mutagenesis of *yapG*, we determined that sites 1 (K₅₁₂), 2 (KR_{548–549}) and 4 (KR_{594–595}) represent the primary cleavage sites of YapG resulting in fragments A, B and C, respectively, whereas sites 3 (K*₅₅₈) and 5 (K*₆₀₄) represent the secondary alternative cleavage sites of YapG resulting in fragments D and E, respectively.

Whilst the N terminus of the YapG PD (aa 49–508) has recently been shown to adopt the characteristic β -helical fold of conventional autotransporters, the extended linker of the C-terminal PD (aa 509–719) is predicted to have little to no secondary structure (M. L. Frazier, A. Pokorny, M. C. Lane, J. V. Lomino, W. K. Ballentine III, V. L. Miller, M. R. Redinbo, unpublished data). Indeed, this extended linker region is proline rich, being composed of 10% (21/210) prolines in comparison with 1.7% (8/459) prolines in the N-terminal PD region. It is tempting to speculate that this unstructured linker represents a flexible hinge that aids in the extension of YapG on the bacterial surface. This would allow YapG to be more accessible to Pla and may prevent negative interference from other outer-membrane structures, including the capsule, a common sterical barrier to other Gram-negative bacterial autotransporters (Schembri *et al.*, 2004). Despite the lack of a defined secondary structure, the major and minor Pla cleavage sites within this region appeared to be conserved among nearly all (11/12) the predicted *Y. pestis* protein sequences of YapG available from completed *Y. pestis* genome sequences in GenBank [with the exception of Angola (GenBank accession no. NC_010159), which appears to be missing a single adenine base in a homopolymeric track of nine adenines, which may be a result of a sequencing error rendering *yapG* non-functional]. In all, the presence of multiple and alternative Pla cleavage sites in YapG, and their high degree of conservation among YapG sequences of *Y. pestis*, suggest that processing of YapG by Pla may be evolutionarily significant to the function of YapG in *Y. pestis*.

Virulence of the *yapG* mutant in the mouse models of plague infection

Of the *Y. pestis* Yap autotransporters, YapE has been shown to be required for bubonic infection in mice but has no role

in primary pneumonic plague (Lawrenz *et al.*, 2009). YapI and YapK have both been shown to contribute in an additive manner to systemic infection after intraperitoneal inoculation of mice (Lenz *et al.*, 2012). Additionally, preliminary studies suggested that YapG may contribute to mammalian virulence, in that *yapG* expression is modestly induced during bubonic and pneumonic plague of mice (Lenz *et al.*, 2011), and that YapG-specific antibodies are produced following a sublethal injection in a rabbit model of bubonic plague (Andrews *et al.*, 2010). To determine whether YapG contributes to plague pathogenesis, we constructed an in-frame deletion of *yapG* in *Y. pestis* strain CO92, and compared differences in colonization of the WT and Δ *yapG* strains using the established mouse models of pneumonic and bubonic infection. Following i.n. inoculation of C57Bl/6 mice with either the WT or Δ *yapG* strain, no differences in colonization were observed in the lungs or spleen at 12, 24, 48 or 72 h post-infection (p.i.) (Fig. 3a). Because it appeared that the Δ *yapG* strain had a slight colonization defect in the lungs at 12 h p.i., we also compared the colonization of these strains at 6 h p.i. but were unable to detect any significant differences in either C57Bl/6 or BALB/c mice (data not shown). Because *Y. pestis* virulence factors vary in their contributions to pneumonic and bubonic plague, we also examined the role of YapG during bubonic infection. Following s.c. injection, no significant differences were observed between WT and Δ *yapG* colonization in the lymph nodes or disseminated organs (spleen and lungs) (Fig. 3b and data not shown). In addition, no differences in lymph node pathology were observed at 36 or 96 h p.i. in mice injected s.c. with the WT or Δ *yapG* strain (data not shown). Because *Y. pestis* most likely enters the dermis (and not subcutaneous space) after the bite of an infected flea (Sebbane *et al.*, 2006), we also determined whether YapG contributed to bubonic infection after intradermal injection. After intradermal injection of either WT or the Δ *yapG* strain (2×10^2 c.f.u. into the ear), we did not observe any differences in colonization of the lymph nodes or disseminated organs (spleen and lungs) (data not shown). Thus, using the established models of bubonic and pneumonic infection, no observable contribution of *yapG* to murine plague was detected.

Whilst autotransporter proteins in general have been shown to mediate a variety of virulence-related functions (Henderson & Nataro, 2001), few of these autotransporters have been shown to contribute directly to virulence in animal models of infection (Alamuri & Mobley, 2008; Alamuri *et al.*, 2010; Allsopp *et al.*, 2010; Dorsey *et al.*, 2005; Elder & Harvill, 2004; Noofeli *et al.*, 2011; Roy *et al.*, 2011). One possible explanation for this phenomenon is functional redundancy, whereby overlapping functions of multiple virulence factors might mask *in vitro* and *in vivo* phenotypes of single gene deletions. A prominent example of this includes the redundant contribution of an autotransporter, polysaccharide and fimbrial adhesins of uropathogenic *E. coli* to biofilm formation (Allsopp *et al.*,

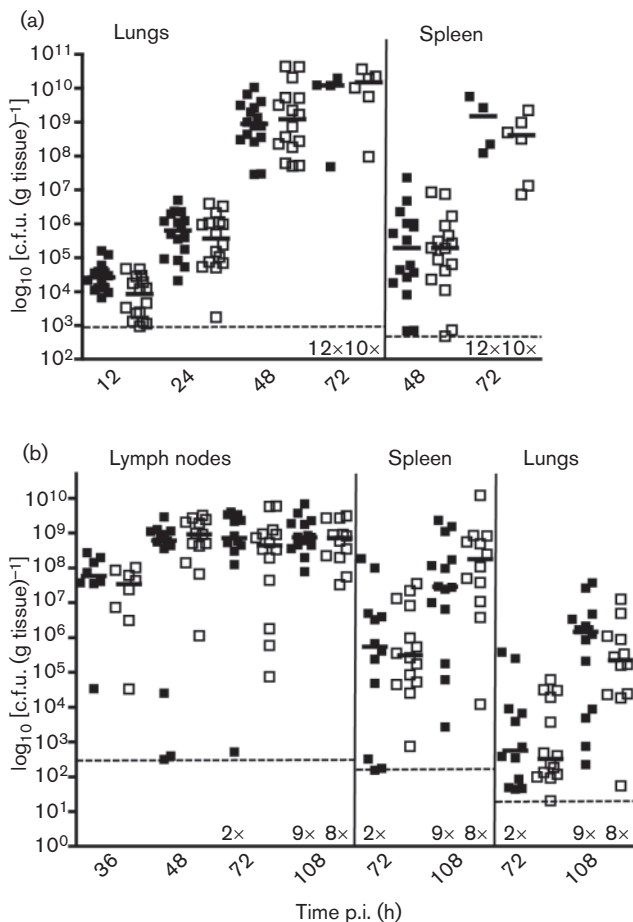


Fig. 3. Comparison of WT and *yapG* mutant colonization and dissemination during pneumonic (a) and bubonic (b) plague. (a) Mice were i.n. inoculated with $\sim 1 \times 10^4$ c.f.u. WT (■) or *yapG* mutant (□). (b) Mice were s.c. injected in the neck with $\sim 1 \times 10^2$ c.f.u. WT (■) or *yapG* mutant (□). For (a) and (b), data are representative of two independent experiments. Mice were sacrificed at various times p.i. (as indicated below the graphs), and organs were homogenized and plated to determine c.f.u. (g tissue) $^{-1}$. Each symbol represents bacteria recovered from an individual mouse. Horizontal bars denote the median of each population. Numbers with an 'x' below each population indicate the number of mice that were euthanized or found dead at that time point. The dashed lines indicate the limits of detection for each organ.

2010, 2012a, b; Kai-Larsen *et al.*, 2010; Melican *et al.*, 2011; Ong *et al.*, 2008; Spurbeck *et al.*, 2011; Ulett *et al.*, 2007a, b; Valle *et al.*, 2008; Wang *et al.*, 2004). Furthermore, *Y. pestis* is known to infect a wide spectrum of mammalian hosts (Perry & Fetherston, 1997). Whilst YapG does not appear to contribute to *Y. pestis* virulence in mice, it is possible that YapG might contribute to pathogenesis in another mammalian host or in fleas.

Conclusions

Upon translocation of autotransporter proteins to the outer membrane, PDs can either remain tethered to the

bacterial surface or are released following proteolytic cleavage. The mechanisms of PD release include cleavage by inter- or intramolecular autocatalytic mechanisms, or intermolecular cleavage by another outer-membrane protease or autotransporter (Dautin & Bernstein, 2007). Previous work and work presented in this study have demonstrated that the YapG autotransporter of the highly virulent *Y. pestis* strain CO92 is cleaved and released by the ompTn protease Pla. Moreover, we showed that Pla cleaves at multiple primary and secondary sites within the YapG PD, which are conserved among nearly all the predicted protein sequences of YapG obtained from available *Y. pestis* genome sequences. Altogether, these data suggest that YapG cleavage by Pla may be important for YapG function. In the evolutionarily related *pla*-negative pathogen *Y. pseudotuberculosis*, we demonstrated that YapG remained cell associated. Thus, the acquisition of Pla and release of YapG in *Y. pestis* may render a different function for YapG during the evolutionary process *Y. pestis* took to become a flea-borne pathogen. In contrast, it is possible that acquisition of Pla may render YapG non-functional. However, this explanation seems less likely, because, if function were to be lost, one would expect to observe an accumulation of inactivating mutations in the *yapG* gene in the different *Y. pestis* genomes, which is just not the case.

In an attempt to elucidate the function of YapG, we conducted extensive bioinformatic analyses and numerous *in vitro* assays by examining the functions of previously characterized autotransporters. In attempting to predict YapG function, we were unable to uncover any conserved domains using the CDD (Marchler-Bauer *et al.*, 2011), Pfam (Finn *et al.*, 2010), SMART (Letunic *et al.*, 2012) or InterPro (Zdobnov & Apweiler, 2001) databases, with the exception of the conserved autotransporter domain and some weak similarity to pertactin within the YapG PD N-terminal to the repeat region that is typically shared by conventional autotransporters. Despite a lack of strong functional predictions, we assayed YapG in both the WT and Δpla backgrounds of *Y. pestis* for common functions that are possessed by other autotransporters. Among the functions assayed, we examined cytotoxicity, adherence, autoaggregation, proteolytic activity of casein and lipase/esterase activity on emulsified tributyrin. However, under the conditions in which these assays were performed, YapG did not possess any of these functions (M. C. Lane, J. D. Lenz and V. L. Miller, unpublished data). These data do not imply that YapG does not have a defined function; indeed, there are a myriad of functional activities remaining that could be assessed. Without additional clues from homology models, *in vivo* studies, or a crystal structure, it becomes difficult to narrow down these infinite functional possibilities.

Although the function of YapG remains to be elucidated, we demonstrated here that YapG does not contribute to *Y. pestis* virulence in the mouse models of bubonic and pneumonic plague. Despite the lack of an *in vivo* phenotype in mice, we cannot definitively rule out a role

for YapG in mammalian disease, as (i) the lack of a phenotype in mice might be the result of functional redundancy with other *Y. pestis* virulence factors, or (ii) YapG may be important for virulence in other mammalian hosts. Alternatively, YapG may play a role during other stages of the *Y. pestis* life cycle, including flea colonization or the effective transmission of *Y. pestis* to the host.

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