The *Yersinia pestis* autotransporter YapC mediates host cell binding, autoaggregation and biofilm formation

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YapC, a putative *Yersinia pestis* autotransporter protein, shows strong homology to the enterotoxigenic *Escherichia coli* adhesin TibA. As a potentially important surface protein of *Y. pestis*, we analysed YapC for several activities. When expressed in the non-pathogenic Fim[−] *E. coli* strain AAEC185, YapC mediated attachment to both murine-derived macrophage-like cells (RAW264.7) and human-derived epithelial-like cells (HEp-2). In addition, expression of YapC on the surface of *E. coli* led to autoaggregation in DMEM tissue culture medium, a phenomenon associated with virulence in *Yersinia* species. YapC also mediated formation of biofilm-like deposits by *E. coli* AAEC185. Deletion of *yapC* in *Y. pestis* strain KIM5 resulted in no change in adhesion to either RAW264.7 or HEp-2 cells, or in biofilm formation. Lack of a phenotype for the *Y. pestis* $\Delta yapC$ mutant may reflect the relatively low level of *yapC* expression *in vitro*, as assessed by RT-PCR, and/or redundant functions expressed *in vitro*. These data demonstrate several activities for YapC that may function during *Y. pestis* infection.

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INTRODUCTION

Yersinia pestis, the causative agent of plague, is a Gram-negative pathogen that evolved from the enteric pathogen *Yersinia pseudotuberculosis* as recently as 1500 years ago (Achtman *et al.*, 1999). Plague is one of the most deadly infectious diseases in history, killing ~25% of the population of Western Europe during the years 1347–1351 (Cantor, 2001; Perry & Fetherston, 1997).

To efficiently establish infection, bacteria express a variety of adhesins on their surface. While a number of *Yersinia* adhesins have been extensively studied, some of the best-characterized adhesins of *Y. pseudotuberculosis* and *Yersinia enterocolitica* are not expressed in *Y. pestis*. These include invasins (Isberg *et al.*, 1987; Rosqvist *et al.*, 1990) and YadA (Rosqvist *et al.*, 1990; Tamm *et al.*, 1993; Yang & Isberg, 1993), which have been mutated by IS100 element insertion and frameshift mutation, respectively (Deng *et al.*, 2002; Parkhill *et al.*, 2001).

Two known adhesins that are expressed by *Y. pestis* are pH 6 antigen and plasminogen activator. pH 6 antigen (encoded by *psaA*) was first described as a surface component of *Y. pestis* induced at temperatures $\geq 35^{\circ}\text{C}$ and pH ≤ 6.7 (Ben-Efraim *et al.*, 1961) that forms 4 nm thick fibrils on the bacterial surface (Lindler & Tall, 1993). In addition, pH 6 antigen acts as an adhesin for cultured cells and agglutinates red blood cells from several animal species (Bichowsky-Slomnicki & Ben-Efraim, 1963; Yang *et al.*, 1996). Plasminogen activator (Pla) also mediates *Y. pestis* adhesion to eukaryotic cells and extracellular matrix (Kienle *et al.*, 1992; Lahteenmaki *et al.*, 1998). Pla is localized on the outer membrane of *Y. pestis* and cleaves and activates plasminogen to facilitate bacterial dissemination from peripheral tissues to other organs (Beesley *et al.*, 1967; Sodeinde *et al.*, 1992; Welkos *et al.*, 1997). It has also been reported to be an invasin for *Y. pestis* (Cowan *et al.*, 2000; Lahteenmaki *et al.*, 2001).

One of the hallmarks of *Y. pestis* infection is the delivery of cytotoxic Yop proteins from the bacterium to the host cell cytoplasm via a type III secretion system (T3SS; Cornelis *et al.*, 1998), a process that requires adhesion to the host cell (Rosqvist *et al.*, 1990). The fact that adhesins in addition to the T3SS translocators, YopB and YopD of *Yersinia* species, are required for Yop delivery was

Abbreviations: ETEC, enterotoxigenic *E. coli*; FCS, fetal calf serum; T3SS, type III secretion system.

A supplementary table of primers is available with the online version of this paper.

demonstrated using an uncharacterized non-adherent derivative of *Y. pestis* strain EV76, in which Yop-mediated cytotoxicity was restored upon expression of the adhesins invasin or YadA of *Y. pseudotuberculosis* (Rosqvist *et al.*, 1990). However, *Y. pestis* strains defective for pH 6 antigen (Lindler *et al.*, 1990) or plasminogen activator (Brubaker *et al.*, 1965) can still mediate Yop delivery to host cells, as measured by maintenance of virulence. Thus, additional adhesins of *Y. pestis* are predicted to mediate the cell adhesion required for Yop delivery and other virulence-associated events.

To identify additional *Y. pestis* adhesins that are potentially important for virulence, we investigated the role of the annotated *Y. pestis* autotransporter protein YapC, a homologue (33.7 % identity) of TibA, an adhesin/invasin of enterotoxigenic *Escherichia coli* (ETEC) (Lindenthal & Elsinghorst, 2001).

Autotransporters are characterized by the presence of an N-terminal 'passenger' domain, which can possess an array of functions, and a C-terminal translocation domain that forms a β -barrel allowing secretion of the passenger domain to the cell surface (Henderson *et al.*, 2004). Some autotransporters remain intact on the cell surface, while others can be cleaved by a variety of mechanisms, including cleavage by an endogenous protease activity in the passenger domain (Hendrixson *et al.*, 1997), cleavage by a different protease in the bacterial cell (Egile *et al.*, 1997; Shere *et al.*, 1997) or a recently characterized intramembrane self-cleavage mechanism within the β -barrel structure (Dautin *et al.*, 2007). Neither TibA nor YapC appear to direct their own cleavage via a passenger-domain-mediated protease activity

(Elsinghorst & Weitz, 1994; Lindenthal & Elsinghorst, 2001; Yen *et al.*, 2007), and residues required for the newly discovered intramembrane self-cleavage mechanism are lacking in both TibA and YapC (Dautin *et al.*, 2007). YapC is also predicted to belong to the AT-1 family of monomeric autotransporters (Yen *et al.*, 2007).

TibA of *E. coli* is a glycosylated surface protein and glycosylation is required for its cell-binding activity (Sherlock *et al.*, 2005). In the case of TibA, the glycosylating enzyme, TibC, is encoded immediately upstream of *tibA* within the same operon. In the case of YapC, genome analysis revealed no neighbouring glycosylating enzyme homologue, although this does not exclude the possibility that YapC is glycosylated.

In addition to mediating adhesion and invasion of epithelial cells, TibA enhances biofilm formation and autoaggregation in ETEC (Sherlock *et al.*, 2005). We demonstrate here that YapC of *Y. pestis* mediates adhesion of recombinant *E. coli* to murine macrophages and human epithelial cells. YapC also facilitates *E. coli* autoaggregation and formation of biofilm-like deposits. Recent studies also found that YapC was surface localized when expressed in *E. coli* and that it could mediate autoaggregation (Yen *et al.*, 2007). These activities may be important for colonization, survival or transmission during *Y. pestis* infections.

METHODS

Bacterial strains, plasmids and tissue culture cells.

Characteristics and sources of the bacterial strains and plasmids used in this study are listed in Table 1. Gene deletions in *Y. pestis* KIM5

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or features	Reference or source
<i>E. coli</i>		
AAEC185	<i>supE44 hsdR17 mcrA mcrB endA1 thi-1 ΔfimB-fimH ΔrecA</i>	Blomfield <i>et al.</i> (1991)
<i>Y. pestis</i>		
KIM5-3001 (referred to as KIM5)	KIM5-3001, Pgm ⁻ , Str ^r	Laboratory collection
KIM5-3001-Δ <i>psaA</i>	KIM5-3001 Δ <i>psaA</i>	This study
KIM5-3001-Δ <i>yapC</i>	KIM5-3001 Δ <i>yapC</i>	This study
KIM5-3001-Δ <i>yapCΔpsaA</i>	KIM5-3001 Δ <i>yapCΔpsaA</i>	This study
KIM5-3001-Δ <i>cafI</i>	KIM5-3001 Δ <i>cafI</i>	This study
KIM5-3001-Δ <i>cafIΔyapC</i>	KIM5-3001 Δ <i>cafIΔyapC</i>	This study
KIM5-3001-Δ <i>cafIΔpsaA</i>	KIM5-3001 Δ <i>cafIΔpsaA</i>	This study
KIM5-3001-Δ <i>cafIΔyapCΔpsaA</i>	KIM5-3001 Δ <i>cafIΔyapCΔpsaA</i>	This study
KIM5-3001 pCD1 ⁻	KIM5-3001 pCD1 ⁻ , Pgm ⁻ , Str ^r	This study
KIM8-3002	KIM5 pPCP1 ⁻ (Pla ⁻) Pgm ⁻ , Str ^r	Laboratory collection
KIM8-3002-Δ <i>yapCΔpsaA</i>	KIM5 pPCP1 ⁻ (Pla ⁻) Δ <i>yapCΔpsaA</i>	This study
Plasmids		
pMMB207	9.1 kb, Cm ^r	Morales <i>et al.</i> (1991)
pMMB207- <i>psaABC</i>	13.1 kb, Cm ^r	This study
pMMB207- <i>yapC</i>	11.0 kb, Cm ^r	This study
pKD46	6.3 kb, Amp ^r , RED recombinase expression plasmid	Datsenko & Wanner (2000)
pKD4	3.3 kb, Km ^r , template plasmid	Datsenko & Wanner (2000)
pCP20	9.4 kb, Flp recombinase expression plasmid, Amp ^r Cm ^r	Datsenko & Wanner (2000)

were performed using PCR products and the method described by Yu *et al.* (2000) and Datsenko & Wanner (2000). Briefly, the pKD4 kanamycin-resistance cassette was amplified by PCR with primers having 5' extension sequences from regions flanking the gene(s) targeted for deletion. Input template DNA in the PCR reaction was digested with *DpnI*, and the *DpnI*-resistant PCR product was transformed into *Y. pestis* KIM5 that had been previously transformed with pKD46, encoding the λ -RED recombinase, and induced for 4–5 h with 10 mM arabinose before preparation of electrocompetent cells. Kanamycin-resistant colonies were selected and the deletion(s) confirmed by PCR. The strain was then transformed with pCP20, encoding the FLP recombinase, which resulted in excision of the kanamycin-resistance gene. Plasmids were removed from the strains by growth in heart infusion broth (HIB) without drug selection. All incubations were performed at 28 °C. HEp-2 cells (ATCC CCL-23) were cultured in MEM + 10 % fetal calf serum (FCS) and RAW264.7 macrophages (ATCC TIB-71) were cultured in Dulbecco's modified Eagle's medium DMEM (Gibco) + 10 % FCS.

Cloning of *Y. pestis* loci. For cloning, the genes encoding YapC or PsaABC were amplified from strain KIM5-3001 (referred to as 'KIM5' throughout this study) by PCR using the Expand High Fidelity PCR System (Roche) or *Pfu* Turbo Taq Polymerase (Stratagene), respectively. PCR products and plasmid pMMB207 (Morales *et al.*, 1991) were cut by appropriate enzymes, gel purified, and the PCR products were ligated into pMMB207. PCR products were designed to include the natural Shine–Dalgarno ribosome-binding site of the gene of interest, but lack the natural promoter. Expression was induced in *E. coli* with IPTG. *E. coli* DH5 α was transformed with the ligation products and chloramphenicol-resistant colonies were selected. Clones were confirmed by DNA sequencing. Plasmids were then purified and transformed into the Fim[−] *E. coli* strain AAEC185 (Blomfield *et al.*, 1991).

Outer-membrane preparations and SDS-PAGE. *E. coli* AAEC185 and derivatives were cultured overnight in LB medium supplemented with 10 μ g chloramphenicol ml^{−1} and 100 μ M IPTG at 28 °C or 37 °C and outer-membrane preparations were made by the method described by Hantke (1984). Briefly, 1 ml of a 3 ml overnight culture induced with 100 μ M IPTG was pelleted at 4 °C. Cells were resuspended in 1 ml 0.2 M Tris/HCl (pH 8.0) and centrifuged again at 4 °C for 5 min. Cells were resuspended in 50 μ l 0.2 M Tris/HCl (pH 8.0) and the following were added in order: 100 μ l 0.2 M Tris/HCl (pH 8.0)/1 M sucrose, 10 μ l 10 mM EDTA (pH 8.0), 10 μ l 2 mg lysozyme ml^{−1} and 320 μ l double-distilled (dd) H₂O. Preparations were mixed and incubated at room temperature for 10 min, then 10 μ l 1 mg DNase ml^{−1} was added and mixed, followed by 500 μ l 2 % Triton X-100/10 mM MgCl₂/50 mM Tris/HCl (pH 8.0). Preparations were mixed well and centrifuged at 4 °C in a microcentrifuge for 30 min at maximum speed. Supernatants were saved (referred to as 'soluble fraction' in Fig. 2) and pellets resuspended in 300 μ l ddH₂O. Preparations were then centrifuged at 4 °C for 15 min, washed twice more with ddH₂O and resuspended in 100 μ l SDS-PAGE sample buffer and boiled for 5 min. Isolated outer membranes were subjected to SDS-PAGE followed by Coomassie blue staining.

Western blotting. For cell fractionation experiments where extracts were probed with anti-PsaA or anti-RNA polymerase α -subunit (RNAP α) antibodies, cells were grown and processed as described for the outer-membrane preparations. In addition, the extent of shearing of pH 6 antigen (PsaA) filaments from the bacterial surface was assessed by Western blot analysis of culture supernatants. Western blots were probed with a 1:1000 dilution of a rabbit anti-PsaA antibody, kindly provided by Dr Susan Straley (Dept of Microbiology, Immunology & Molecular Genetics, University of Kentucky). Anti-RNAP α Western blots were probed with a 1:1000 dilution of mouse anti-RNAP α antibody generated against *E. coli* RNAP α (Neoclone). Blots were washed and

probed with anti-rabbit-horseradish peroxidase (HRP) or anti-mouse-HRP secondary antibodies, respectively (Zymed) prior to developing with Supersignal West Pico ECL substrate (Pierce).

Cytotoxicity assay. HEp-2 cells were grown to ≥ 80 % confluency in 24-well tissue culture plates (Falcon, Becton Dickinson) in minimal Eagle's medium (MEM) with Earle's salts and L-glutamine (Gibco) supplemented with 10 % FCS, 1 % non-essential amino acids and 1 % sodium pyruvate. RAW 264.7 cells were cultured until ≥ 80 % confluency in 24-well plates containing DMEM (high glucose, with L-glutamine and pyridoxine hydrochloride, without sodium pyruvate) supplemented with 10 % FCS.

Y. pestis KIM5, KIM5 pCD1[−] and different deletion mutants were cultured overnight at 28 °C in HIB (pH 7.0) with shaking. Cultures were diluted to OD₆₂₀ 0.15 in HIB pH 7.0 and incubated for a further 4 h at 28 °C with shaking. The cultures were pelleted and resuspended in tissue culture medium without serum to OD₆₂₀ 1.5. Plates were washed twice with 1 ml serum-free MEM or DMEM and 1 ml medium without serum was added to each well. Then 50 μ l of resuspended bacteria was added to achieve an m.o.i. of approximately 100 bacteria per cell. Cell morphology was observed for rounding under a phase-contrast microscope every hour for 4 h. Cell rounding is an indication of Yop-mediated cytotoxicity.

Adhesion assay. Twenty-four-well cell culture plates were prepared as described above. *E. coli* AAEC185 containing pMMB207, pMMB207-*yapC* or pMMB207-*psaABC* was cultured overnight (16 h) at 28 °C with shaking in Luria–Bertani (LB) medium supplemented with 10 μ g chloramphenicol ml^{−1} and 100 μ M IPTG. Bacterial cultures were centrifuged and the bacteria were resuspended in serum-free MEM or DMEM at OD₆₀₀ 0.6. Cells were then diluted 1:100 into serum-free MEM or DMEM and 100 μ l aliquots were used to infect 24-well plates seeded overnight with HEp-2 or RAW264.7 cells (after washing the cells twice with serum-free MEM or DMEM) in 500 μ l MEM or DMEM respectively (an m.o.i. of approximately 1–3 bacteria per cell). RAW264.7 cells were treated with 5 μ g cytochalasin D ml^{−1} in DMEM for 45 min prior to addition of bacteria to inhibit actin-dependent macrophage phagocytosis. Plates were incubated at 37 °C, 5 % CO₂ for 2 h. Wells were then washed twice with PBS followed by cell lysis in 0.5 ml ddH₂O containing 0.1 % Triton X-100 for 10 min. at room temperature. Wells were washed one more time with PBS and samples were pooled with the Triton X-100 lysis fraction of recovered bacteria. Duplicate wells were used to determine the total number of bacteria per well. For this purpose, all medium and washes were pooled. Tenfold dilutions in PBS were plated on LB agar plates containing 10 μ g chloramphenicol ml^{−1}. The plates were incubated at 37 °C overnight and the output c.f.u. were enumerated. Adhesion is expressed as the percentage of adherent bacteria relative to the total number of bacteria in a parallel infected well (total inoculum after 2 h).

Adhesion assays for *Y. pestis* strains were performed similarly, except that bacteria were grown in the absence of IPTG and chloramphenicol and cells were plated on HIB agar at 28 °C for 48 h prior to quantification.

Autoaggregation assay. *E. coli* AAEC185 containing the empty vector pMMB207, pMMB207-*yapC* or pMMB207-*psaABC* were grown overnight in LB with 10 μ g chloramphenicol ml^{−1} and 100 μ M IPTG. Cells were pelleted and resuspended at an OD₆₀₀ of 1.5 in 2 ml DMEM in glass test tubes. Tubes were incubated in a 37 °C water bath and OD₆₀₀ was read every 15 min, 30 min or 60 min as the assay progressed. As bacteria aggregated, they settled out of the DMEM solution, resulting in a decrease in OD₆₀₀.

Biofilm formation assay. Crystal violet staining was used to detect cells attached to polystyrene as described by O'Toole *et al.* (1999).

Briefly, overnight bacterial cultures were diluted 1:100 into LB plus 10 µg chloramphenicol ml⁻¹ and 100 µM IPTG in flat-bottomed polystyrene culture plates (Costar Corning) and incubated 24 h at 37 °C or 28 °C without shaking. The optical density of the cultures was read in a microplate reader (Perkin Elmer Lambda Reader) at 595 nm. Plates were washed twice with PBS, and 0.01 % crystal violet was added and incubated for 15 min at room temperature. Wells were washed three times with distilled water, and stained bacteria were solubilized with 80 % ethanol and 20 % acetone mixture. Absorbance of the mixture was measured by the microplate reader at 595 nm. Results were normalized for bacterial culture density. *Y. pestis* assays were performed similarly except that no IPTG or chloramphenicol was added and cells were grown in a defined medium, PMH2 (Gong *et al.*, 2001; Staggs & Perry, 1991).

RT-PCR. Expression of *yapC* under various *in vitro* growth conditions was assessed using reverse transcription (RT)-PCR. KIM5 or KIM5 $\Delta yapC$ was grown under three different conditions in HIB: 28 °C pH 7, 37 °C pH 6 and 37 °C pH 7. Bacterial cells were prepared for RNA extraction with Trizol according to the manufacturer's recommendations (Invitrogen). RNA samples were treated with DNase I for 10 min at 37 °C to degrade contaminating DNA followed by inactivation of DNase I with 2 mM EDTA and heating to 65 °C for 10 min. RNA was then precipitated with sodium acetate and ethanol and washed with 70 % ethanol prior to performing the RT-PCRs. RNA samples of 500 ng were used for reverse transcription, using random hexamer primers and Superscript II reverse transcriptase as described by the manufacturer (Invitrogen). PCR amplification was performed using the *yapC* or 16S rRNA primer pairs listed in Supplementary Table S1 (available with the online version of this paper). Thirty cycles of amplification were performed using an annealing temperature of 47 °C. Products were then run on a 2 % agarose gel, stained with ethidium bromide and imaged for visualization of appropriately sized PCR products. Negative control samples were processed in parallel, but no reverse transcriptase was added. A positive control PCR reaction was carried out using KIM5 chromosomal DNA.

Detecting YapC surface expression. YapC was detected on the surface of *Y. pestis* using an anti-YapC antibody generated in the laboratory of Dr Virginia Miller (Department of Molecular Microbiology, Washington University School of Medicine). *Y. pestis* KIM5 or the KIM5 $\Delta yapC$ mutant was grown overnight in HIB at 28 °C pH 7 (starting pH). Overnight cultures were diluted 1:50 into HIB and grown under three conditions: 28 °C pH 7, 37 °C pH 7 and 37 °C pH 6. Cells were grown to mid-exponential phase (4 h) and then cells were pelleted, washed once with 1 ml PBS and resuspended at an equivalent OD₆₂₀ concentration (OD₆₂₀ 0.424) in an Eppendorf tube. Cells were then fixed for 20 min at room temperature with 500 µl 4 % paraformaldehyde (with rolling), washed once with 1 ml PBS and blocked for 3 h at room temperature with 1 ml PBS + 1 % BSA (with rolling). Cells were then pelleted and resuspended in 500 µl PBS + 0.2 % BSA + 0.05 % Tween-20 + a 1:500 dilution of the rabbit anti-YapC antibody. Bacteria and antibody were co-incubated overnight at 4 °C with rolling. Cells were then pelleted and washed twice with 1 ml PBS (with resuspension and pelleting), after which 500 µl PBS + 0.2 % BSA + 0.05 % Tween-20 + 1:1000 goat anti-rabbit alkaline phosphatase-conjugated secondary antibody (Zymed) was incubated with the bacteria for 45 min at room temperature. Cells were then washed three times with 1 ml PBS and once with 1 ml 100 mM Tris pH 8.0, and reactions for detection of antibody binding were performed in 500 µl 100 mM Tris pH 8.0 + 4 mg ml⁻¹ of the alkaline phosphatase substrate PNPP (Fluka). Reactions were developed for between 19 and 27 min prior to spinning out the bacteria and transferring the supernatants to a 96-well plate for reading in an ELISA plate reader at 405 nm. Results were normalized to YapC surface expression of KIM5 grown overnight in HIB at

28 °C. A₄₀₅ readings were corrected for the time of PNPP development. *E. coli* samples were processed identically, except that they were induced with 100 µM IPTG during the 4 h growth in LB at 37 °C and exposed to PNPP for 8 min.

RESULTS

YapC of *Y. pestis* mediates adhesion to cultured cells

Adhesins can potentially enhance colonization of *Y. pestis* during infection or may play a role in Yop delivery to targeted host cells. To determine whether YapC can bind host cells, we analysed the ability of YapC to enhance attachment of the Fim⁻ *E. coli* strain AAEC185 (Blomfield *et al.*, 1991) to murine-derived macrophage-like cells (RAW264.7) or human-derived epithelial-like cells (HEp-2). Adhesion of *E. coli* expressing YapC to RAW264.7 macrophages and HEp-2 cells was analysed by a c.f.u. assay (see Methods). For these studies, the known *Y. pestis* adhesin pH 6 antigen (encoded by the *psaABC* locus) served as a positive control for efficient cell binding. *E. coli* expressing YapC showed a twofold increase in adhesion to RAW264.7 cells (28 %, corresponding to ~1 bacterium per cell using our m.o.i. of ~3) when compared to *E. coli* carrying the empty expression vector pMMB207 (Fig. 1a). The positive control, pH 6 antigen, provided 59 % adhesion (~2 bacteria per cell) to RAW264.7 macrophages. On HEp-2 cells (where background binding by *E. coli*

Fig. 1. Adhesion of *E. coli* AAEC185 expressing YapC to RAW264.7 macrophages (a) and HEp-2 cells (b). *E. coli* cells were grown overnight at 28 °C in LB containing 100 µM IPTG and tissue culture cells were infected at an m.o.i. of approximately 1–3 bacteria per cell for 2 h. Adhesion was quantified as the number of cell-associated c.f.u. divided by total c.f.u. in the tissue culture well (% bound out of total inoculum). Values are the means and standard deviations of three independent experiments with duplicate assays from each experiment. *, $P < 0.001$, **, $P < 0.00001$ as assessed by Student's *t*-test.

Fig. 2. Expression of YapC and pH 6 antigen in the outer membranes of *E. coli* AAEC185. (a) Outer membranes were subjected to SDS-PAGE and stained with Coomassie blue. A predicted 65 kDa band for YapC is indicated with an arrow. (b) Various cell fractions prepared from cells grown at 28 °C or 37 °C were subjected to SDS-PAGE and probed with an anti-PsaA antibody for Western blot analysis (* indicates an *E. coli* protein that is cross-reactive with anti-PsaA antibody). (c) Various cell fractions were subjected to SDS-PAGE and probed with an anti-RNA polymerase α -subunit antibody for Western blot analysis. 'Soluble fraction' indicates cell components extracted by Triton X-100, including inner membranes and cytoplasm (Hantke, 1984). (d) *E. coli* cells harbouring empty vector (pMMB207) or vector carrying *yapC* were induced for 4 h with 100 μ M IPTG in triplicate and analysed for surface expression of YapC with an anti-YapC antibody. Shown is a representative experiment from one day.

AAEC185 was lower), YapC showed a 3.3-fold increase (up to 2.1 % adhesion) in cell adhesion compared to *E. coli* with plasmid pMMB207 (0.65 % adhesion, Fig. 1b). pH 6 antigen conferred upon *E. coli* AAEC185 23.9 % adhesion for HEp-2 cells, demonstrating its strong adhesive ability. Giemsa staining of adhesion assays verified these findings, as *E. coli* expressing either YapC or pH 6 antigen showed higher numbers of bacteria bound per cell than *E. coli* carrying empty vector (data not shown).

We also assessed the effect of a $\Delta yapC$ mutation on *Y. pestis* adhesion. A KIM5 $\Delta yapC$ mutant had no defect in adhesion to either RAW264.7 macrophages or HEp-2 cells when pregrown at 28 °C or 37 °C (data not shown). Since the CafI capsule of *Y. pestis* (expressed at 37 °C) has been shown to interfere with the ability of some *Y. pestis* proteins to bind mammalian cells (Liu *et al.*, 2006) and capsule production interferes with the activity of the *E. coli* TibA protein (Sherlock *et al.*, 2005), we also constructed a $\Delta cafI\Delta yapC$ mutant of KIM5. The $\Delta cafI\Delta yapC$ mutant also had no cell-binding defect for RAW264.7 or HEp-2 cells (data not shown). The $\Delta yapC$ mutant also had no defect in delivery of cytotoxic Yop proteins (a process which requires adhesins; Rosqvist *et al.*, 1990) as measured by pCD1-dependent cell rounding (data not shown). The lack of an effect on cell binding in the $\Delta yapC$ mutant suggests that *Y. pestis* expresses multiple adhesins capable of host cell binding or that expression of YapC *in vitro* is low.

Expression of YapC in the outer membrane

To confirm the expression of YapC in the outer membrane of *E. coli*, we cultured strains carrying the pMMB207-*yapC* plasmid overnight at 28 °C or 37 °C in Luria-Bertani (LB) medium supplemented with 100 μ M IPTG to induce *yapC* expression and outer-membrane proteins were prepared according to the Triton X-100 extraction method of Hantke (1984). YapC was clearly visible as a predicted 65 kDa band (consistent with signal peptide processing) present in *E. coli* outer membranes (Fig. 2a, lanes 2 and 5), and its identity was confirmed by mass spectrometry (data not shown). Although pH 6 antigen was expressed, as evidenced by its ability to mediate cell adhesion (Fig. 1), it was poorly detected in our outer membrane preparations, when stained with Coomassie blue (Fig. 2a, lanes 3 and 6; PsaA is a predicted 15 kDa protein). Since fimbria-like structures are often shed or sheared from the surface of bacterial cells during processing (Hoschutzky *et al.*, 1989), we looked at the culture supernatants after spinning overnight cultures of bacteria at 7000 r.p.m. in a laboratory microcentrifuge. Anti-PsaA (the structural subunit of pH 6 antigen) Western blots performed on bacterial cell extracts or supernatants that were normalized to represent the amounts produced by similar numbers of bacteria demonstrated that the vast majority of PsaA filaments are sheared from AAEC185 during this initial bacterial pelleting step (Fig. 2b, lanes 12 and 24). PsaA was present

in the culture supernatants of both 28 °C and 37 °C preparations of AAEC185/pMMB207-*psaABC* (Fig. 2b). We were even able to visualize various polymerized forms (presumably incompletely denatured in our SDS-PAGE system) of pH 6 antigen (monomer to tetramer; Fig. 2b, lanes 12 and 24). It appears that only a small proportion of PsaA remains membrane-associated during our outer membrane preparation protocol, yet clearly PsaA-expressing *E. coli* cells have pH 6 antigen-mediated adhesive activity (leading to 24–59 % adhesion, depending on the cell line). Comparisons of RNA polymerase contamination from the cytosolic/inner-membrane Triton X-100-soluble fraction demonstrated minimal contamination when whole-cell lysates were compared with outer-membrane preparations (Fig. 2c).

There is an *E. coli* protein (denoted by an asterisk in Fig. 2b) that is cross-reactive with anti-PsaA antisera. Based on its fractionation, this protein is from either the cytoplasm or the inner membrane (Triton X-100-soluble fraction, Fig. 2b). The fact that we see some of this cross-reactive protein in supernatants from YapC-expressing *E. coli* suggests that YapC expression may be somewhat toxic to *E. coli*, causing some cells to lyse and release intracellular contents into the supernatant.

YapC surface expression in *E. coli* was also demonstrated using a recently generated anti-YapC antibody. AAEC185 carrying pMMB207 (empty vector) or pMMB207-*yapC* was grown for 4 h in LB with 100 μ M IPTG induction. Cells were washed, fixed and processed for anti-YapC surface labelling as described in Methods. Expression of YapC in *E. coli* gave a more than fourfold increase in anti-YapC antibody recognition (Fig. 2d).

YapC mediates *E. coli* autoaggregation in tissue culture media

Another phenotype associated with TibA (the YapC homologue) in ETEC is autoaggregation (Sherlock *et al.*, 2005). Autoaggregation in tissue culture medium is also associated with virulence in *Yersinia* species (Laird & Cavanaugh, 1980). Therefore, we investigated the ability of YapC to direct *E. coli* autoaggregation in DMEM tissue culture medium at 37 °C. These studies were performed in the Fim[−] *E. coli* derivative AAEC185 since the *E. coli* Fim system also mediates autoaggregation in tissue culture media (S. Felek & E. S. Krukoni, unpublished observations). Bacteria were induced for YapC or pH 6 antigen expression overnight at 28 °C with 100 μ M IPTG, pelleted, resuspended in DMEM at 37 °C at an OD₆₀₀ of 1.5 and monitored for aggregation by OD₆₀₀ measurements over time. Expression of YapC led to aggregation of *E. coli* AAEC185 within 15–30 min and a 50 % reduction in OD₆₀₀ occurred by approximately 45 min after resuspension in DMEM (Fig. 3). AAEC185 expressing the empty plasmid pMMB207 or pH 6 antigen showed no autoaggregation for up to 6 h (360 min) post-transfer to DMEM (Fig. 3).

Fig. 3. Autoaggregation of *E. coli* AAEC185 expressing YapC or pH 6 antigen in DMEM at 37 °C. Cells carrying empty vector (pMMB207) were used as control. Cells were monitored in a test tube for clearing over time. Results are the mean $\pm$ SD of three experiments on different days. $\blacklozenge$, pMMB207; $\bullet$, YapC; $\triangle$, PsaABC.

The loss of optical density that we see in DMEM is not due to cell death since we know from our adhesion assays that YapC-expressing *E. coli* maintain high c.f.u. counts for up to 2 h in DMEM (Fig. 1 and Methods).

A recent report by Yen *et al.* (2007) also demonstrated that expression of YapC in *E. coli* leads to autoaggregation, although in this case bacterial aggregation was tested in LB bacterial culture medium, not tissue culture medium.

Y. pestis also undergoes autoaggregation when grown at 28 °C. This phenotype is blocked by expression of the Caf1 protein capsule at 37 °C (S. Felek & E. S. Krukoni, unpublished observations). We tested our KIM5 $\Delta yapC$ mutant for clumping in several media, including HIB, LB, PBS and DMEM. There was no difference in autoaggregation in any media between KIM5 and KIM5 $\Delta yapC$ (data not shown). Subsequent investigations have revealed that a major determinant of KIM5 autoaggregation is a metabolic gene that is likely involved in LPS modifications (S. Felek & E. S. Krukoni, unpublished observations). Deletion of this locus results in a dramatic decrease in autoaggregation of KIM5.

YapC promotes biofilm-like deposits in *E. coli*

Since autoaggregation of bacteria is a critical step in biofilm formation, we assessed the ability of YapC to mediate formation of biofilm-like deposits in *E. coli*. Furthermore, the ETEC YapC homologue, TibA, has been shown to mediate biofilm formation in ETEC.

Biofilm assays were performed by incubating *E. coli* AAEC185 containing vector pMMB207 or pMMB207-*yapC* in LB medium in sterile 96-well polystyrene plates. YapC expression led to a 5.3-fold increase in AAEC185 biofilm formation (as measured by crystal violet staining) when the bacteria were grown at 37 °C and an 8-fold

increase when grown at 28 °C (Fig. 4). Thus, YapC has the potential to play a role in *Y. pestis* biofilm formation in both the human host and the flea vector.

Since YapC affected the formation of biofilm-like structures in *E. coli*, we investigated the role of this locus in *Y. pestis* biofilm formation. KIM5 or an isogenic $\Delta yapC$ mutant were grown overnight in PMH2 medium (Gong *et al.*, 2001; Staggs & Perry, 1991) at 28 °C or 37 °C in sterile 96-well polystyrene plates. We did not detect a defect in biofilm-like deposits in the *yapC* mutant at either temperature (data not shown).

Given that at 37 °C YapC may be masked by the F1 capsule of KIM5, we also tested an isogenic *caf1* mutant for biofilm formation in the presence or absence of YapC. Again, we saw no difference in biofilm-like deposits in a KIM5 $\Delta caf1$ mutant compared to the $\Delta caf1 \Delta yapC$ mutant (data not shown). It should be noted that KIM5 lacks the *hms* locus responsible for biofilm-dependent flea blockage by *Y. pestis* (Hinnebusch *et al.*, 1996). Thus, biofilm formation in the KIM5 assays is via a different mechanism and is considered *hms*-independent.

Expression profile of *yapC* under various growth conditions

Using RT-PCR, Yen *et al.* (2007) found that *yapC* of *Y. pestis* KIM10+ is expressed in HIB at 28 °C. Since the three activities of YapC – cell adhesion, autoaggregation and biofilm formation – may be important in either the human host or the flea vector, we assessed *yapC* expression in *Y. pestis* under various *in vitro* growth conditions.

Parental KIM5 cells or a $\Delta yapC$ mutant were grown overnight at 28 °C pH 7, diluted to OD₆₂₀ 0.2 and grown for 4 h at 37 °C pH 7, at 37 °C pH 6 (conditions which induce pH 6 antigen expression) or at 28 °C pH 7. RNA was isolated using a Trizol extraction protocol and

Fig. 4. Formation of biofilm-like deposits by *E. coli* at 37 °C (black bars) and 28 °C (white bars). Results are the mean $\pm$ SD crystal violet absorbance (A_{595}) divided by bacterial culture density (OD_{595} , prior to crystal violet staining) of three independent experiments with at least duplicate assays from each experiment. Results are normalized to set the AAEC185/pMMB207 28 °C control at 1.0. *, $P < 0.00001$ as assessed by Student's *t*-test.

RT-PCR was performed as described in Methods. Primers within the *yapC*-coding region led to amplification of a 113 bp product under all conditions and this product was absent in the $\Delta yapC$ deletion mutant (Fig. 5a). This amplification product was also dependent upon reverse transcriptase (only present in '+' lanes in Fig. 5). Thus, it was not due to DNA contamination. We also amplified the 16S rRNA transcript by RT-PCR and showed it was amplified equally well in both KIM5 and the $\Delta yapC$ derivative (resulting in a 125 bp band, Fig. 5b), indicating that the $\Delta yapC$ samples were competent for productive RT-PCR. Although these results indicate that *yapC* is expressed in *Y. pestis* under all three of the growth conditions we routinely use in the laboratory, such end-point (30 cycle) PCR reactions are non-quantitative. By comparing the

yapC and 16S rRNA reaction products, it appears that *yapC* is expressed less well (Fig. 5a, b). Upon further investigation, by examining PCR products after 50 rounds of amplification, using several different dilutions of the template cDNA, it was determined that *yapC* expression levels are about 1000-fold lower than that of 16S rRNA (data not shown).

In addition to RT-PCR, we determined the surface expression of YapC protein on KIM5 under various growth conditions. KIM5 or the $\Delta yapC$ mutant were grown overnight at 28 °C (starting pH 7) and then diluted 1:50 for 4 h growth under various conditions. YapC expression on the surface of KIM5 was assessed using an anti-YapC antibody (Methods). The only condition where we observed an increase in YapC surface expression compared to the $\Delta yapC$ isogenic control was when KIM5 was grown overnight to stationary phase at 28 °C (Fig. 5c; $P=0.1$). Thus, YapC appears to be poorly expressed *in vitro* (possibly induced in stationary phase), but may be expressed during infection. The fact that YapC is poorly expressed *in vitro* likely contributes to our inability to observe a strong *yapC* mutant phenotype in our various assays.

Fig. 5. Expression of *yapC* under various *in vitro* growth conditions. KIM5 and KIM5 $\Delta yapC$ were grown from an OD₆₂₀ of 0.2 to an OD₆₂₀ of 0.7–0.9. Cells were pelleted and RNA extracted. RT-PCR was performed using two sets of primers. (a) Amplification of a 113 bp *yapC*-encoded transcript. (b) Amplification of a 125 bp 16S rRNA transcript. Lane PC refers to a positive control sample using chromosomal DNA as a PCR template. (c) Surface expression of YapC protein was assessed for overnight (o/n) cultures of KIM5 (black bars) and KIM $\Delta yapC$ (white bars) grown in HIB at 28 °C pH 7, and for cultures diluted 1:50 from the overnight culture and grown for 4 h at 28° pH 7, 37° pH 7 and 37° pH 6. YapC protein on the surface of KIM5 was detected with an anti-YapC antibody. Shown is a representative experiment on cultures grown in triplicate. *, $P=0.1$ between KIM5 and KIM5 $\Delta yapC$ for the 28 °C overnight cultures (as assessed by Student's *t*-test).

DISCUSSION

Adhesins are important virulence factors that facilitate successful colonization and can affect the tissue tropism(s) of a particular micro-organism (Niemann *et al.*, 2004; Ofek *et al.*, 2003). Many bacterial pathogens maintain multiple adhesins to enable binding to various cell types. In some bacteria, more than ten adhesins have been described (Ofek *et al.*, 2003).

TibA is an ETEC glycoprotein that has been shown to mediate glycosylation-dependent binding to and invasion of several cell types (Elsinghorst & Kopecko, 1992; Elsinghorst & Weitz, 1994; Lindenthal & Elsinghorst, 2001). In this study, we found that YapC, a *Y. pestis* TibA homologue, can function as an adhesin. It promotes adhesion of *E. coli* AAEC185 to both RAW264.7 macrophages and HEp-2 cells (Fig. 1). We also confirmed the strong adhesive ability of the known *Y. pestis* adhesin, pH 6 antigen (Fig. 1; Yang *et al.*, 1996). A recent study by Yen *et al.* (2007) found that YapC expressed in *E. coli* was unable to mediate haemagglutination of sheep red blood cells. In those experiments, the starting dilution of bacterial cells was 1:10. We could detect weak haemagglutination activity in *E. coli* expressing YapC to a bacterial dilution of 1:4 (data not shown). It is also possible that RAW264.7 macrophages and HEp-2 cells express a YapC receptor that is poorly expressed on erythrocytes. It should be noted that in our HEp-2 and RAW264.7 adhesion studies, some component of the increased adhesion might be due to the autoaggregative activity of YapC in *E. coli* (Fig. 3). YapC expressed in *E. coli* also mediated a two- to fourfold increase in adhesion to two other human-derived cell lines:

THP-1 monocyte-like cells and A549 cells derived from lung epithelium (data not shown).

Another important bacterial process dependent on surface structures is biofilm formation. Biofilms are often important for bacterial virulence, facilitating colonization and protecting bacteria from host immune defence and antimicrobial treatments (Fux *et al.*, 2005; Stewart & William Costerton, 2001). Attachment of bacteria to a surface is the first step in biofilm formation, followed by replication to form microcolonies to produce a mature biofilm (Jefferson, 2004). Biofilm formation blocks the proventriculus of the flea and is important for transmission of bubonic plague (Darby *et al.*, 2002; Jarrett *et al.*, 2004). The HmsT and HmsP proteins have been shown previously to control biofilm production in *Y. pestis* at 28 °C; these proteins are thought to be critical only for the flea (ambient temperature) stage of the *Y. pestis* life cycle since HmsT is degraded at 37 °C (Kirillina *et al.*, 2004). We found that YapC mediates biofilm formation at both 37 °C and 28 °C in *E. coli* (Fig. 4). Thus, YapC may mediate biofilm formation during infection of mammalian hosts and/or the flea vector. YapC-mediated autoaggregation of bacterial cells (Fig. 3) may also contribute to biofilm formation. The ETEC YapC homologue, TibA, also induces biofilm formation in *E. coli* (Sherlock *et al.*, 2005). When tested in *Y. pestis*, a $\Delta yapC$ mutant of KIM5 had no defect in biofilm formation (data not shown). Autoaggregation in DMEM, perhaps an early step in biofilm formation, was also not affected in the $\Delta yapC$ mutant (data not shown). Thus, while we have shown that YapC possesses certain activities, there are apparently redundant proteins with similar activities in *Y. pestis*. In the case of biofilm formation, we know that expression of several annotated *Y. pestis* chaperone/usher systems also results in biofilm formation (Felek *et al.*, 2007) and we have recently identified a locus in *Y. pestis* that controls autoaggregation (S. Felek & E. S. Krukoni, unpublished). In addition, the expression of YapC *in vitro* appears to be fairly low (Fig. 5). The fact that YapC is poorly expressed *in vitro* likely contributes to our inability to observe a *yapC* mutant phenotype in our various assays.

It has been demonstrated that adhesins are required for Yop delivery in *Y. pestis* (Rosqvist *et al.*, 1990). Yop delivery leads to cytotoxicity in eukaryotic cells. Our results and previous studies by others demonstrate that *Y. pestis* has at least three adhesins: YapC (Fig. 1), pH 6 antigen (Fig. 1 and Yang *et al.*, 1996) and plasminogen activator (Kienle *et al.*, 1992; Lahteenmaki *et al.*, 1998). We tested various *Y. pestis* mutant strains for cytotoxicity in cultured cells. A $\Delta yapC$ or $\Delta psaA$ mutant of *Y. pestis* KIM5 showed wild-type levels of cytotoxicity for RAW264.7 macrophages and HEp-2 cells (data not shown), as assessed by cell rounding. As expected, a KIM5 pCD1⁻ (Yop-negative control) strain did not cause cytotoxicity (data not shown). In addition, a triple mutant KIM8 (plasminogen activator negative, *pla*) $\Delta yapC \Delta psaA$ strain was also able to deliver Yop proteins to eukaryotic cells with wild-type efficiency when pregrown

at 28 °C pH 7 (data not shown). It should be noted that a $\Delta psaA$ mutant shows a 1–2 h delay in Yop-mediated cytotoxicity when pregrown at 37 °C pH 6 (data not shown). This pH 6 antigen-dependent reduction in Yop delivery confirms the ability of pH 6 antigen to facilitate delivery of T3SS proteins, as was shown previously in a non-adherent mutant of *Pseudomonas aeruginosa*, where expression of pH 6 antigen established sufficient contact to allow type III secretion of the toxin ExoS (Sundin *et al.*, 2002).

The results discussed above suggest that there are redundant adhesins in *Y. pestis* for cell binding and Yop delivery. These could include the recently described adhesive autotransporters YapN and YapK (Yen *et al.*, 2007), or the chaperone/usher-dependent surface structure encoded by *Y. pestis* KIM locus *y0561* (Felek *et al.*, 2007). Alternatively, an uncharacterized adhesin may be specifically required for Yop delivery. It is important to note that the observation of redundant adhesins *in vitro* does not necessarily mean that the same adhesins are redundant *in vivo*. Environmental signalling and various forms of regulation may result in the expression of specific adhesins during different stages of *Y. pestis* infection. It may also be the case that Yop delivery to macrophages and neutrophils may be less dependent on a specific adhesin in addition to the YopB/D translocation complex, due to the phagocytic nature of these cells (Boyd *et al.*, 2000). In the closely related pathogen *Y. pseudotuberculosis*, at least two adhesins, invasin and YadA, have been shown to be able to mediate Yop delivery (Rosqvist *et al.*, 1990, 1994). This ability to deliver Yop proteins has also been shown to be dependent on adhesin length, with a minimum length required to establish proper contact for type III secretion (Mota *et al.*, 2005). However, neither invasin nor YadA is functional in *Y. pestis*, due to IS element insertion or frame shift mutation, respectively (Deng *et al.*, 2002; Parkhill *et al.*, 2001).

In summary, we have demonstrated that YapC, an annotated autotransporter of *Y. pestis*, can mediate cell adhesion to HEp-2 (human) cells and RAW264.7 (mouse) macrophages, can lead to autoaggregation and can facilitate biofilm formation. Each of these activities can play an important role in the efficiency of establishing an infection or overcoming the host innate immune response. The role of TibA in ETEC pathogenesis is not defined, but the various activities of TibA and its *Y. pestis* homologue, YapC, suggest that these molecules may play important roles in pathogenesis. While other factors of *Y. pestis* may also possess some of these activities, we hypothesize that YapC may be an important player in the infection process.

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