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Role of the *Yersinia* YopJ protein in suppressing Interleukin-8 secretion by human polymorphonuclear leukocytes

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Abstract

Polymorphonuclear leukocytes, in addition to their direct bactericidal activities, produce cytokines involved in the activation and regulation of the innate and adaptive immune response to infection. In this study we evaluated the cytokine response of human PMNs following incubation with the pathogenic *Yersinia* species. *Y. pestis* strains with the pCD1 virulence plasmid, which encodes cytotoxic Yop proteins that are translocated into host cells, stimulated little or no cytokine production compared to pCD1-negative strains. In particular, PMNs incubated with pCD1-negative *Y. pestis* secreted 1,000-fold higher levels of interleukin-8 (IL-8 or CXCL8), a proinflammatory chemokine important for PMN recruitment and activation. Deletion of *yopE*, *-H*, *-T*, *-M* or *ypkA* had no effect on pCD1-dependent inhibition, whereas deletion of *yopJ* resulted in significantly increased IL-8 production. Like *Y. pestis*, the enteropathogenic *Yersinia* species inhibited IL-8 secretion by PMNs, and strains lacking the virulence plasmid induced high levels of IL-8. Our results show that virulence plasmid-encoded effector Yops, particularly YopJ, prevent IL-8 secretion by human PMNs. Suppression of the chemotactic IL-8 response by *Y. pestis* may contribute to the delayed PMN recruitment to the infected lymph node that typifies bubonic plague.

Keywords

Neutrophil; IL-8; CXCL8; YopJ; *Yersinia*; Plague

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Conflict of Interest

The authors declare no conflict of interest.

1. Introduction

Polymorphonuclear leukocytes (neutrophils or PMNs) are the most abundant circulating white blood cell of humans. PMNs are recruited to sites of infection and tissue damage by chemotactic molecules, such as N-formyl peptides, complement components, interleukin-8 (IL-8) and lipopolysaccharide (LPS) [1]. Recruited PMNs rapidly ingest microbes by a process known as phagocytosis and kill internalized organisms by releasing highly microbicidal reactive oxygen species (ROS) and potent antimicrobial components into the pathogen-containing vacuoles [1]. PMNs also secrete a number of cytokines, chemokines, and other effector molecules important in mediating inflammation [2,3]. Thus, PMNs are not only important for the elimination of pathogens through direct killing mechanisms but are also integrated in the activation and regulation of both the innate and adaptive immune systems [2,3]. In order to persist within the mammalian host and cause disease, pathogens likely make use of mechanisms that disrupt these broader immune functions of PMNs in addition to mechanisms that confer resistance to phagocytosis and killing.

The genus *Yersinia* includes three species pathogenic to humans. *Y. enterocolitica* and *Y. pseudotuberculosis* are transmitted by the fecal-oral route and usually cause self-limited mesenteric lymphadenitis and gastroenteritis. *Y. pestis* is typically transmitted through the bite of an infected flea or through exposure of open wounds to infected material and causes bubonic and septicemic plague. *Y. pestis* can also be transmitted through inhalation of aerosolized droplets containing *Y. pestis*, resulting in an uncommon but highly lethal pneumonic form of plague. All three pathogenic *Yersinia* species maintain a virulence plasmid necessary for pathogenesis, termed pCD1 in *Y. pestis* and pYV in the enteropathogenic *Yersinia*. The virulence plasmid encodes a type III secretion system (T3SS) that translocates six *Yersinia* effector proteins, termed YopE, -H, -T, -J (YopP in *Y. enterocolitica*), -M, and YpkA (YopO in *Y. enterocolitica*), into host cells. Once within the target cell, each of the effector Yops target different signaling molecules and function collectively to counteract multiple signaling responses in the infected host cell [4].

In murine models of bubonic plague, injection of *Y. pestis* into the dermis results in a rapid influx of large numbers of PMNs that surround and interact with the bacteria at the injection site [5]. Nevertheless, wild-type *Y. pestis* disseminates to the draining lymph node and multiplies to produce the bubonic stage of plague without stimulating a strong PMN response to that site [6–10]. In contrast, dissemination of pCD1-negative *Y. pestis* is accompanied by the influx of large numbers of PMNs to the lymph node, where the bacteria are eliminated without further spread [8,10]. Studies comparing the host response to virulence plasmid-positive and virulence plasmid-negative *Y. pestis* have in general demonstrated a virulence plasmid-dependent inhibition of certain proinflammatory cytokine responses [8,11–13]. A proposed role has been described for each individual Yop, except YpkA/YopO, in inhibition of cytokine production by a variety of cell types [4]. However, the cytokine response of human PMNs to *Y. pestis* and the enteropathogenic *Yersinia* has not been thoroughly investigated and is explored herein. The contribution of virulence plasmid-encoded factors, particularly the effector Yops, in alteration of cytokine production by human PMNs is also described.

2. Materials and Methods

2.1. Bacterial strains, plasmids and culture conditions

Strains and plasmids used are listed in Table I. Only attenuated *Y. pestis* strains which lack the Pgm locus (KIM5 and KIM6) or the pCD1 virulence plasmid (KIM6+ and KIM6) and are excluded from CDC Category A Select Agent regulations were used. KIM5 *ypkA* and KIM5 *yopE*, *-H*, *-T* and *-M* gene deletion strains were created using the lambda red recombinase-mediated knockout procedure system as described [14]. Gene deletion cassettes were generated by PCR using primers listed in Table 2. Gene deletions were verified by PCR. The *yopJ* deletion and the substitution of *Y. pestis yopJ* for *Y. enterocolitica yopP* (KIM5 *yopJ-yopP*) have been described [15].

The low-copy YopJ-complementation plasmid pWKS::*yopJ* was created by cloning the PCR-amplified YopJ open reading frame flanked by ~400 bp of upstream and downstream sequence into the low-copy plasmid pWKS130 using the *SacI* and *XbaI* restriction sites. The YopJ amplicon was also cloned directly into the high-copy plasmid pCR-XL-TOPO using a TOPO TA Cloning Kit (Life Technologies, Grand Island, NY). PCR primers used to amplify the *yopJ* cassette are listed in Table 2.

Yersinia were grown in brain heart infusion (BHI) broth, supplemented with 2.5 mM CaCl₂, with aeration at 21 °C overnight from frozen stocks. Cultures were transferred to 37 °C for 2 h prior to each assay to induce expression of the T3SS and effector Yop proteins. *Escherichia coli* was grown in Luria-Bertani (LB) broth with aeration at 37 °C overnight from frozen stocks. Where appropriate, kanamycin (50 µg/mL), chloramphenicol (10 µg/mL) or streptomycin (50 µg/mL) was added to the broth cultures at the indicated final concentration. Bacteria were washed in Dulbecco's phosphate-buffered saline (DPBS, Life Technologies, Grand Island, NY) and resuspended in RPMI 1640 medium with 10 mM HEPES (RPMI, Life Technologies). To prepare opsonized *Y. pestis* and *E. coli*, bacteria were resuspended in 20% autologous normal human serum (NHS) for 30 min at 37 °C, washed in DPBS and resuspended in RPMI. Heat-inactivation of *Y. pestis* was done at 56 °C for 1h. Bacteria were enumerated by Petroff-Hauser direct count and adjusted to the desired concentration. Virulence plasmid retention was assessed by PCR or plating cultures on low-calcium Congo red-magnesium oxalate agar (CRMOX, 20 mM Na oxalate, 20 mM MgCl₂) [16] and incubation at 37 °C for two days.

2.2. Preparation and use of human PMNs

Human heparinized blood was obtained from healthy individuals in accordance with a protocol approved by the Institutional Review Board for Human Subjects of NIAID. Donors were informed of the procedure risks and provided written consent prior to enrollment. Human PMNs were purified from heparinized venous blood as described [17] and preparations contained less than 1% contaminating lymphocytes.

PMNs (2×10^5) suspended in RPMI were added to serum-coated wells (20% NHS for 30 min at 37 °C) of a 96-well tissue culture plate. Bacteria were then added (5:1 bacteria-to-PMN ratio); replicate wells containing uninfected PMNs or media alone served as controls. Plates were incubated at 37 °C, 5% CO₂. For experiments where two strains of bacteria were

added to a single well, each strain was added at a 2.5:1 bacterium-to-PMN ratio to yield a final bacteria-to-PMN ratio of 5:1. The first strain was added and allowed to incubate with PMNs for 30 min prior to the addition of the second strain. The final volume per well, in all experiments, was 200 μ L. For all experiments, after 4 h of incubation at 37 °C, 5% CO₂, gentamicin (Sigma, Saint Louis, MO) (2.5 μ g/mL) was added and left for the remainder of the assay to limit growth of extracellular bacteria. Plates were incubated at 37 °C, 5% CO₂ for a total of 12 h or 24 h.

2.3. Secretion of cytokines and chemokines by PMNs

At the indicated time point, plates were centrifuged at 400 \times g for 10 min. Culture supernatants were then transferred into microcentrifuge tubes and centrifuged at 13,000 \times g for 10 min to pellet any remaining cells. Supernatants were collected and stored at -80 °C prior to analysis. Levels of IL-8 (CXCL8), G-CSF, GM-CSF, MIP-1 α (CCL3), MIP-1 β (CCL4), IL-1 β , IL-1ra, IL-6, IL-12, MCP-1 (MCAF, CCL2), IL-17 and TNF- α in the supernatant of each sample were simultaneously determined using a Bio-Plex Pro human cytokine assay plate and a Bio-Plex 200 System controlled by Bio-Plex Manager 4.1.1 (Bio-Rad Laboratories, Hercules, CA). Alternatively, levels of IL-8 in culture supernatants were analyzed by ELISA (Legend Max ELISA Kit, BioLegend Inc., San Diego, CA). With a few exceptions noted in the figure legends, experiments were performed in triplicate at least three times. Data were analyzed by one-way ANOVA with Tukey post-test using GraphPad Prism version 6.01 for Windows (GraphPad Software, San Diego, CA).

3. Results

3.1. Cytokine response of human PMNs following exposure to *Y. pestis*

As an initial screen to assess the cytokine response of human PMNs following exposure to *Y. pestis*, the levels of twelve cytokines/chemokines/growth factors (hereafter collectively referred to as cytokines) were measured in culture supernatants recovered 12 h or 24 h after addition of bacteria, using BioPlex multiplex immunoassay instrumentation. These twelve were chosen based on previous evidence that they can be produced by PMNs upon recognition of bacteria or bacteria-derived factors such as LPS [2,3]. *E. coli* and *Y. pestis* lacking the pCD1 virulence plasmid (KIM6) stimulated the secretion of several cytokines above the background levels observed from uninfected PMNs (Fig. 1). Three of them, the chemokine IL-8 and the cytokines IL-1 β and IL-1 receptor antagonist (IL-1ra) were consistently detected at elevated levels from all PMN donor samples at both 12 h and 24 h after infection with KIM6. By far the most pronounced response was IL-8 secretion, present at concentrations greater than 1 \times 10⁴ pg/mL, with IL-1 β and IL-1ra present at concentrations approximately one hundred-fold less than IL-8 (Fig. 1).

In contrast to *Y. pestis* KIM6 and *E. coli*, PMNs incubated with pCD1⁺ *Y. pestis* (KIM5) secreted less of nearly all cytokines tested (Fig. 1). Most prominently, PMNs incubated with KIM5 secreted 1,000-fold less IL-8 than PMNs incubated with the pCD1⁻ KIM6. KIM5 strains that were lacking one of the pCD1-encoded *yopE*, *yopH*, *yopT*, *yopM* or *ypkA* T3SS effector genes were all able to inhibit secretion of IL-8 as well as the parental KIM5 (Fig. 1). In contrast, KIM5 *yopJ*, the strain deleted of the pCD1-encoded YopJ effector protein gene,

was only partially able to inhibit secretion of IL-8. Levels of some other cytokines secreted following incubation with KIM5 *yopJ* were greater than PMNs incubated with KIM5, but were similar to or slightly less than PMNs incubated with KIM6 or *E. coli*. In particular, at 12 h, the amount of IL-8 secreted by PMNs incubated with KIM5 *yopJ* was approximately 100-fold greater than that of PMNs incubated with KIM5, but approximately 10-fold less than that of PMNs incubated with KIM6 (Fig. 1). This indicates that the pCD1 virulence plasmid encodes factors able to inhibit cytokines secreted by PMNs in response to *Y. pestis* and that YopJ plays a predominant role in this inhibition.

3.2. *Yersinia* pCD1-encoded factors actively inhibit IL-8 secretion by PMNs

Because the most abundant cytokine secreted by human PMNs within 12 h of incubation with *Y. pestis* KIM6 was IL-8, we performed specific ELISA assays to further characterize the apparent pCD1-dependent inhibition of IL-8 secretion. First, we ascertained whether factors encoded in the 102-kb chromosomal Pgm locus or on pCD1 other than the major effector proteins were involved in the ability of KIM5 to inhibit secretion of IL-8. PMNs were incubated with KIM6+, which unlike KIM5 and KIM6 contains a wild-type Pgm locus [18], and KIM6+ transformed with the plasmid pCD1 1234. This plasmid is a deletion derivative of pCD1 that lacks the genes for the major effector proteins YopE, YopH, YopT, YopJ, YopM and YpkA but still encodes the proteins necessary for assembly of a functional T3SS [19]. Incubation of either KIM6+ or KIM6+ (pCD1 1234) with PMNs resulted in high levels of IL-8, equivalent to levels resulting from incubation of PMNs with KIM6 (Fig. 2). Therefore, *Y. pestis* inhibition of IL-8 secretion by PMNs requires the major effector proteins encoded on pCD1 and factors not encoded on pCD1 do not play a detectable role.

To assess if pCD1-dependent inhibition of IL-8 secretion was due to active suppression of cellular signaling or to a lack of stimulation, PMNs were incubated with KIM5 prior to the addition of KIM6. Addition of KIM6 to PMNs that were preincubated with KIM5 did not induce secretion of IL-8 (Fig. 2C; KIM5/KIM6). Furthermore, heat-inactivated KIM5 was no longer able to inhibit IL-8 secretion (Fig. 2C; H. I. KIM5). These results suggest that the greatly reduced level of IL-8 secretion observed after infection with pCD1⁺ *Y. pestis* is due to active suppression of cellular signaling and secretory pathways within the PMN, rather than to a lack of stimulation.

3.3. YopJ of *Y. pestis* is a major factor involved in inhibition of IL-8 secretion by PMNs

IL-8 secretion by PMNs following incubation with KIM5 was compared by ELISA to KIM5 strains lacking *yopE*, *yopH*, *yopT*, *yopJ*, *yopM*, or *ypkA*. In keeping with results from the multiplex bead array (Fig. 1), KIM5 inhibited IL-8 secretion by PMNs, and incubation of PMNs with KIM5 *yopJ* resulted in an intermediate level of IL-8 secretion (Fig. 3A and B). Complementation of the KIM5 *yopJ* strain with low- or high-copy number plasmids containing *yopJ* and its native promoter (pWKS::*yopJ* and pCR::*yopJ*, respectively) restored IL-8 suppression to levels seen following incubation of PMNs with parental KIM5 (Fig. 3A and B). Complementation plasmids were also transformed into KIM6 (pCD1 1234) to determine if expression of YopJ in the absence of the other effector Yop proteins was sufficient for inhibition of IL-8 secretion by PMNs. KIM6 (pCD1 1234) containing the high-copy number pCR::*yopJ* was able to partially inhibit IL-8 secretion by PMNs, but IL-8

levels were about 10-fold higher than seen after infection with KIM5. Transformation of KIM6 (pCD1⁻ 1234) with the low-copy number plasmid pWKS::yopJ, however, was not sufficient to inhibit IL-8 secretion at all (Fig. 3A and B). As expected, transformation of KIM6, which completely lacks the T3SS, with pWKS::yopJ or pCR::yopJ had no effect. Taken together, these results indicate that YopJ is the major effector protein responsible for suppressing IL-8 production by PMNs. Complementation *in trans* was dependent on yopJ copy number, however, suggesting that other Yop effectors participate in IL-8 pathway inhibition.

3.4. Opsonization of *Yersinia* does not alter IL-8 secretion and YopJ/P of the enteropathogenic *Yersinia* also inhibits IL-8 secretion by PMNs

To determine if opsonization of *Y. pestis* alters IL-8 secretion by PMNs, unopsonized KIM5 and KIM6 were incubated with human PMNs. Unopsonized KIM6 stimulated equivalent levels of IL-8 secretion by PMNs (Fig. 4) as KIM6 that had been opsonized (Fig. 2 and 3). In addition, unopsonized KIM5 (Fig. 4) inhibited IL-8 secretion similarly to unopsonized KIM5 (Fig. 2 and 3). Since opsonization did not alter IL-8 secretion or inhibition and because enteropathogenic *Y. enterocolitica* is relatively more susceptible to killing by complement components from human serum than *Y. pestis* [20,21], experiments comparing *Y. pestis* to the enteropathogenic strains were performed using unopsonized bacteria. Wild-type (virulence plasmid-positive) *Y. enterocolitica* and *Y. pseudotuberculosis* were both able to inhibit IL-8 secretion by PMNs as well as *Y. pestis* KIM5 (Fig. 4). In contrast, high levels of IL-8 were secreted by PMNs within 12 h after infection with the two enteropathogenic *Yersinia* species that had been cured of their virulence plasmid (pYV⁻), similar to levels produced by PMNs incubated with the pCD1⁻ *Y. pestis* KIM6 (Fig. 4). A *Y. pestis* KIM5 strain in which the native yopJ on pCD1 had been replaced with the yopP gene from *Y. enterocolitica* inhibited IL-8 secretion to the same degree as the parental KIM5 strain (Fig. 4). Thus, IL-8 secretion by PMNs was similarly suppressed by the virulence plasmid-encoded T3SS of all three pathogenic *Yersinia*, independent of opsonization, and YopJ and YopP were equally capable of producing this inhibition.

4. Discussion

A delayed PMN response to *Y. pestis* infection correlates strongly with disease progression and virulence. In bubonic plague models, large numbers of PMNs respond to *Y. pestis* immediately after intradermal transmission [5]. Following this initial encounter at the dermal infection site, however, subsequent PMN recruitment to either the dermal infection site or the infected lymph node is not sustained in animals infected with wild-type *Y. pestis* [6–10]. Essential *Y. pestis* virulence factors including the virulence plasmid and the Ail outer membrane protein have been implicated in the delayed PMN response in the developing bubo, which is strongly correlated with virulence [8–10]. In sharp contrast to wild-type *Y. pestis*, intradermal infection with pCD1⁻ or ail⁻ *Y. pestis* strains results in marked recruitment of PMNs to the draining lymph node and resolution of the infection [8–10]. Thus, an ability to limit PMN recruitment to the infected lymph node appears to be a pivotal aspect of the anti-innate immunity pathogenesis strategy of *Y. pestis*.

Translocation of one or more of the virulence plasmid-encoded Yop effector proteins into human and murine PMNs has been shown to inhibit phagocytosis and ROS production and to alter apoptosis [15,22–25], and *in vivo* studies have noted that PMNs are a primary target cell for the *Y. pestis* T3SS [26,27]. Although most *Y. pestis* are killed following phagocytosis by PMNs, *in vitro* studies have shown that a certain percentage survives, and that this resistance is independent of the virulence plasmid [28]. Motivated by the fact that PMNs are not only efficient phagocytic cells capable of directly eliminating most invading pathogens but are also sources of cytokines and chemokines important for mediating the innate and adaptive immune response [2,3], we evaluated cytokine production by human PMNs incubated with *Y. pestis*. The most notable finding was that the pCD1⁺ KIM5 strain completely suppressed secretion of the proinflammatory chemokine IL-8, whereas extremely high levels (~10⁴ pg/ml) of IL-8 were secreted by PMNs incubated with the pCD1⁻ KIM6 strain. IL-8 (CXCL8) is rapidly produced and secreted in large amounts (ng/ml as opposed to pg/ml for most other cytokines) by human PMNs upon exposure to a foreign particle or an appropriate ligand [29]. IL-8 is also a key mediator for the recruitment of circulating PMNs to an infection site, because PMNs are also the primary target for this chemokine, to which they respond by chemotaxis, increased transmigration across endothelial barriers, release of granule enzymes, and respiratory burst [29]. Thus, suppression of IL-8 secretion by wild-type *Y. pestis* may be one reason for the limited PMN response in the developing bubo. The full effect of *Y. pestis* on IL-8 secretion occurred by the 12 h timepoint, indicating that it was not due to a delay in cytokine production but rather to rapid and permanent inhibition.

In the initial screen, other cytokines besides IL-8 were secreted at higher levels by PMNs incubated with pCD1⁻ *Y. pestis* or *E. coli* than with pCD1⁺ *Y. pestis* (Fig. 1). Compared to IL-8, however, the increased secretion was modest and was not detected in all of the donor samples. It is somewhat controversial as to which specific cytokines are produced by PMNs in response to different stimuli. A complicating factor is that small numbers of contaminating lymphocytes and monocytes in PMN preparations can produce the same cytokines, often in greater amounts than PMNs on a per-cell basis [2,3]. We did not detect large quantities of IL-6, IL-1 β , TNF- α , IL-12 or GM-CSF in our assay, and it is possible that some were produced by the <1% of contaminating lymphocytes or peripheral blood mononuclear cells (PBMCs) in our PMN preparations. In contrast to the other cytokines we screened, the release of large amounts of IL-8 is a well-established response of human PMNs to infection stimuli [29].

The contribution of each of the six known effector Yop proteins to the observed pCD1-dependent inhibition of IL-8 secretion was evaluated by incubating PMNs with individual Yop deletion mutant strains. We did not observe any differences in cytokine production by human PMNs incubated with the parental KIM5 strain or the *yopE*, *-H*, *-T*, *-M* or *ypkA/yopO* mutants. Only the *yopJ* mutant was significantly less inhibitory than KIM5, indicating that YopJ is the major effector involved in suppressing IL-8 production by PMNs. YopJ/P has previously been shown to specifically suppress IL-8 production by epithelial cells, [30,31], HeLa and HEK293 cells [32,33], and umbilical vein endothelial cells [34]. In addition to

YopJ/P, YopE, -H and -T have all been found to counteract production of IL-8 by epithelial cells and HeLa cells [30,35,36].

IL-8 levels secreted by PMNs incubated with the *yopJ* mutant, although ~10-fold higher than seen with the parental KIM5 strain, were still at least 2-fold lower than for pCD⁻ *Y. pestis*. KIM6 (pCD1⁻ 1234) (pCR::*yopJ*) was able to inhibit IL-8 secretion more so than KIM6 (pCD1⁻ 1234) (pWKS::*yopJ*), suggesting that inhibition is dependent in part on the amount of YopJ translocated into PMNs. In addition, whereas KIM5 *yopJ* complemented with YopJ expressed from either a low-copy (pWKS::*yopJ*) or high-copy (pCR::*yopJ*) plasmid was fully able to inhibit IL-8 secretion; KIM6 (pCD1⁻ 1234) (pWKS::*yopJ*) and KIM6 (pCD1⁻ 1234) (pCR::*yopJ*) were not able to inhibit IL-8 secretion as much as KIM5. Together, these results suggest that YopJ alone is not sufficient for the observed pCD1⁻ dependent complete inhibition of IL-8 secretion by human PMNs and that the other effector proteins likely potentiate YopJ-mediated inhibition and aid in disruption of PMN signaling pathways leading to IL-8 production. For example, YopH of *Y. pseudotuberculosis* has been shown to confer *in vivo* resistance to murine PMNs by disrupting signaling pathways [37]. It is noteworthy that YopJ was also the only individual effector required for suppression of all of the other cytokines secreted at elevated levels when exposed to the pCD⁻ KIM6 strain (Fig. 1), suggesting that YopJ is the most active T3SS effector against PMNs in general.

Because the T3SS has known antiphagocytic effects, decreased uptake of pCD1⁺ *Y. pestis* and a potentially decreased stimulation of the IL-8 synthesis pathway may account in part for our results. However, in the experimental conditions we used, PMNs rapidly phagocytize both pCD1⁺ and pCD1⁻ *Y. pestis*, with only about a 2-fold final difference in relative internalization [22]. Furthermore, no differences in phagocytosis by PMNs were observed between KIM5 and KIM5 *yopJ*, consistent with previous findings that YopH, -T, and -O, but not the YopJ homolog YopP, inhibit phagocytosis of *Y. enterocolitica* by human PMNs [24]. The most abundant cytokine secreted by human PMNs in response to infection with *Y. pestis* KIM6 was IL-8, and pCD1⁺ *Y. pestis*, particularly YopJ encoded on pCD1, was able to inhibit secretion of IL-8. This suggests that, similar to previous studies, differences in IL-8 production were not primarily due to differences in phagocytosis [32]. The previously described ability of YopJ/P to effectively inhibit both the MAPK and NFκB pathways [33] is a likely mechanism for inhibition of IL-8 secretion by human PMNs.

We also observed that IL-8 is highly produced by PMNs following incubation with the enteropathogenic *Yersinia* species lacking pYV, and that pYV⁺ strains inhibited IL-8 secretion. Arnold and König previously reported that *Y. enterocolitica* exhibits a pYV-dependent suppression of IL-8 production by human PMNs [38]. Furthermore, pYV⁺ *Y. enterocolitica* and *Y. pseudotuberculosis* inhibited IL-8 production similarly to pCD1⁺ *Y. pestis*. Our data indicate that expression of YopP either by *Y. enterocolitica* or in place of YopJ by *Y. pestis* (*yopJ-yopP* strain, Fig. 2C) cause equivalent inhibition of IL-8 secretion by PMNs.

In conclusion, pathogenic *Yersinia* species are able to inhibit production of the pro-inflammatory cytokine IL-8 by human PMNs and virulence plasmid-encoded factors, particularly YopJ/P, are necessary for this inhibition. Inhibition of IL-8, a potent

chemoattractant for PMNs, and other proinflammatory cytokines could be important for *Yersinia* disease progression by limiting the recruitment and accumulation of neutrophils and other immune cells. In mice and rats, infection with pCD1⁻ *Y. pestis* results in increased PMN recruitment and accumulation in the lymph node relative to pCD1⁺ *Y. pestis* [8,10]. YopJ is not essential for *Yersinia* pathogenesis in these murine models [13,39]; however, YopJ was found to be a virulence factor important for *Y. pseudotuberculosis* to establish a systemic infection [40] and for *Y. enterocolitica* persistence within the spleen [41]. As suggested by our findings, other effector proteins may compensate for the loss of YopJ to some extent, thus explaining a lack of an essential role for YopJ in disease progression in murine plague models. Alternatively, many differences between human and murine PMNs exist, notably the fact that murine PMNs do not produce IL-8 but rather the functional IL-8 homologs KC, MIP-2 and LIX. Since YopJ has such a dramatic inhibitory effect on IL-8 production by human PMNs, it is possible that YopJ plays a greater role in limiting a PMN response to *Yersinia* in humans than it does in murine models.

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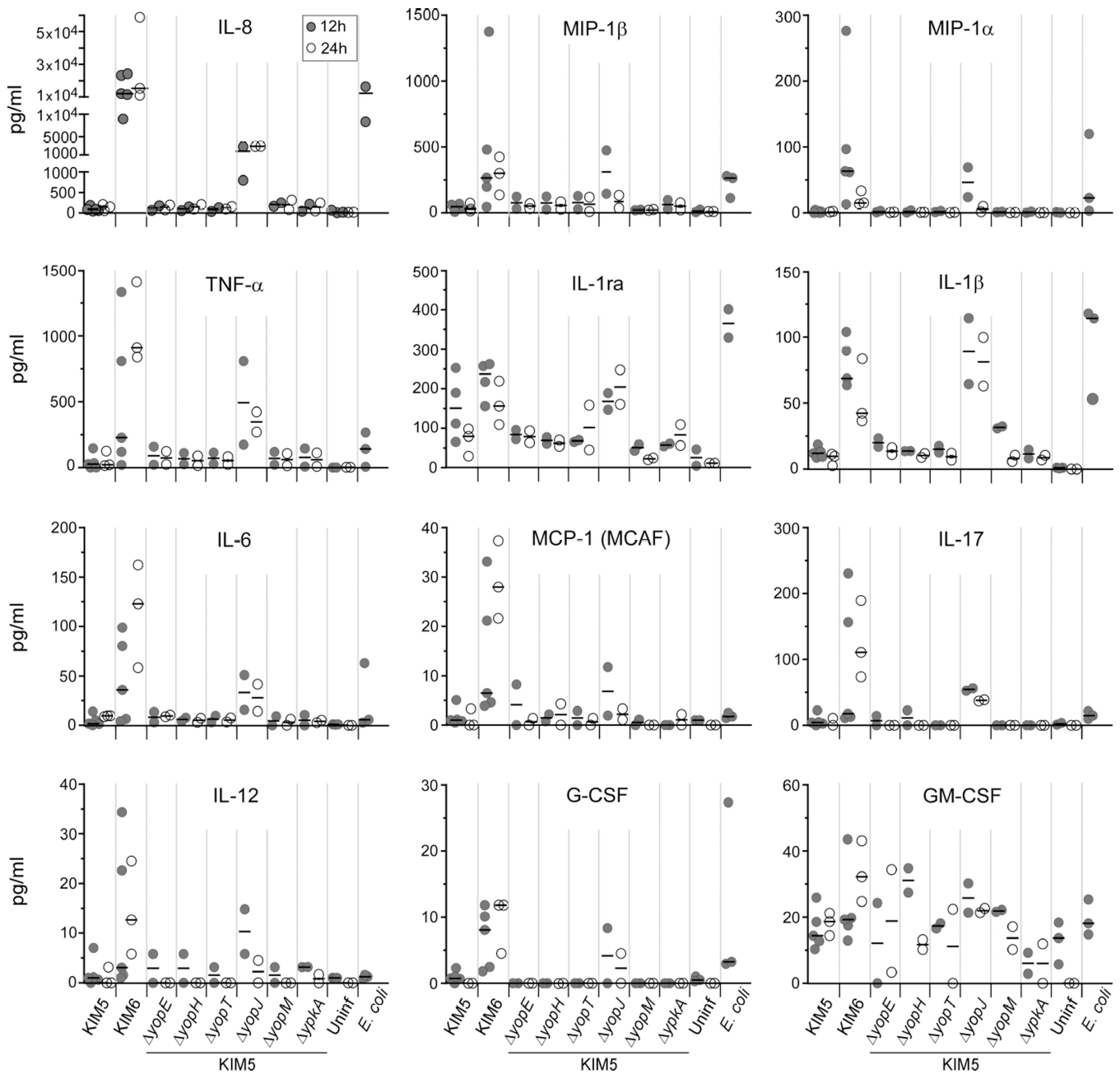


Fig. 1. *Y. pestis* pCD1-dependent inhibition of cytokine secretion by human PMNs. Human PMNs were incubated with *E. coli*, *Y. pestis* KIM5 (pCD1⁺), KIM6 (pCD1⁻), or KIM5 mutant strains deleted of *yopE*, *-H*, *-T*, *-J*, *-M* or *ypkA*. Uninfected PMNs served as unstimulated controls. Culture supernatant was collected after 12 h (gray circles) or 24 h (white circles) of incubation and assayed by multiplex bead arrays for the concentration of each indicated cytokine. The 24 h timepoint was not done for *E. coli*. Each circle represents the mean value of results from duplicate or triplicate wells of PMNs from a single donor; the horizontal line indicates the median of these values.

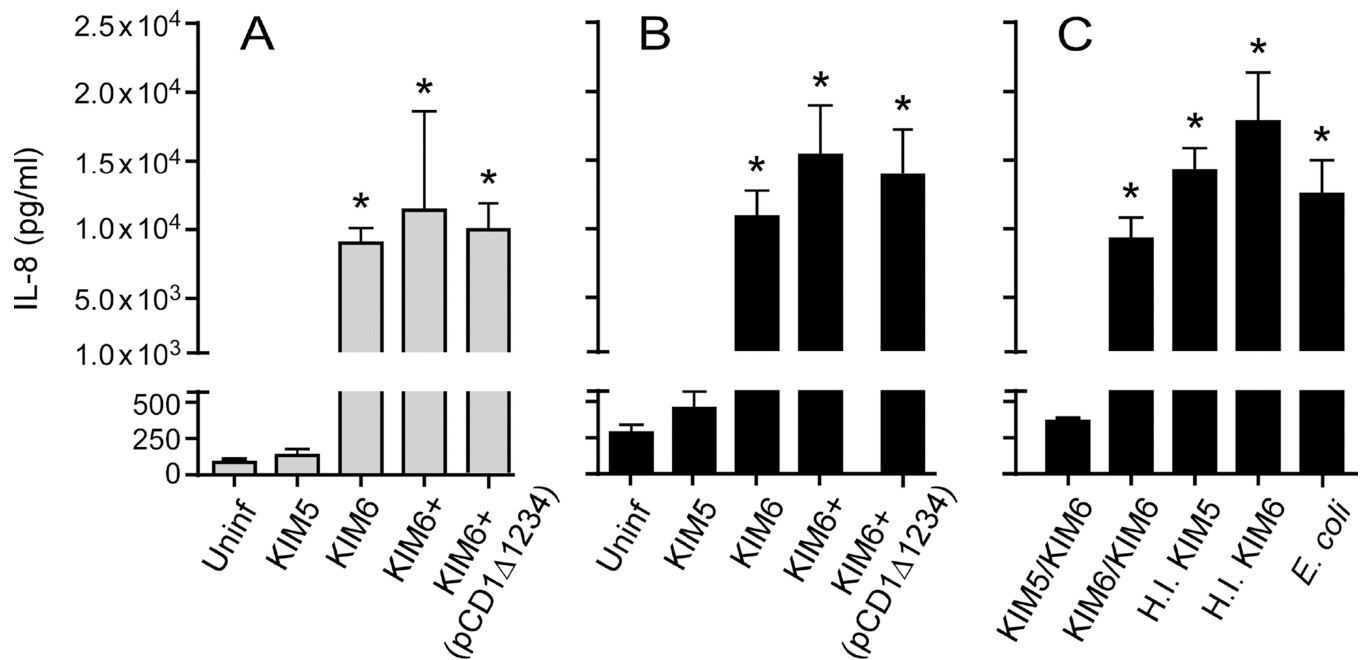


Fig. 2.

Y. pestis pCD1-dependent inhibition of IL-8 requires the effector Yop proteins. IL-8 secreted by human PMNs following incubation with *Y. pestis* containing the wild-type pCD1 virulence plasmid (KIM5), pCD1⁻ *Y. pestis* with (KIM6+) or without (KIM6) the chromosomal Pgm locus, or with KIM6+ transformed with pCD1 1234 (which lacks the six effector Yop genes but encodes the genes necessary for the T3SS) were compared (A, B). Alternatively, PMNs were incubated with KIM5 or KIM6 prior to the addition of KIM6 (KIM5/KIM6 and KIM6/KIM6, respectively) or with heat inactivated (H.I.) KIM5 or KIM6 (C). PMNs incubated alone or with *E. coli* were included as controls. Culture supernatant was collected after 12 h (A) or 24 h (B, C) of incubation and the concentration of IL-8 was determined by ELISA. The mean and SEM for at least three independent experiments (except for H.I. KIM6 and *E. coli*, which were tested only twice) are indicated. Data were analyzed by one-way ANOVA with Tukey post-test. *, $P < 0.05$ compared to KIM5.

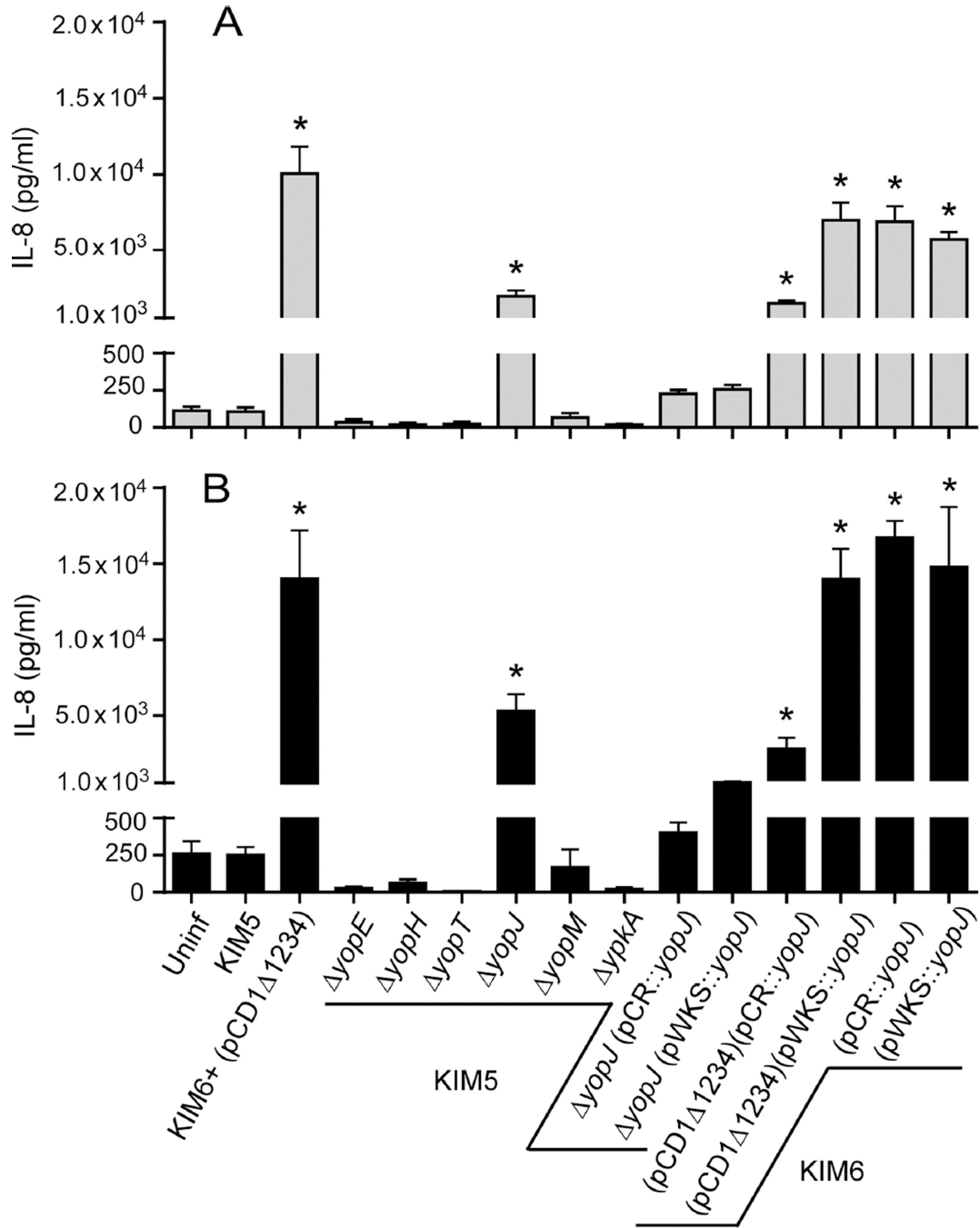


Fig. 3. YopJ and the T3SS are required for *Y. pestis* inhibition of IL-8 secretion by PMNs. IL-8 secretion by human PMNs incubated with *Y. pestis* lacking *yopE*, *-H*, *-T*, *-J*, *-M* or *ypkA* was compared to PMNs incubated with the parental KIM5 or with KIM6+ (pCD1 Δ1234). In addition, IL-8 secretion by PMNs incubated with the KIM5 *yopJ* or KIM6 strains that had been transformed with the low- or high-copy number complementation plasmids (pWKS::*yopJ* and PCR::*yopJ*, respectively) was determined. Culture supernatant was collected after 12 h (A) or 24 h (B) of incubation and the concentration of IL-8 was

determined by ELISA. The mean and SEM for at least three independent experiments are indicated. Data were analyzed by one-way ANOVA with Tukey post-test. *, $P < 0.05$ compared to KIM5.

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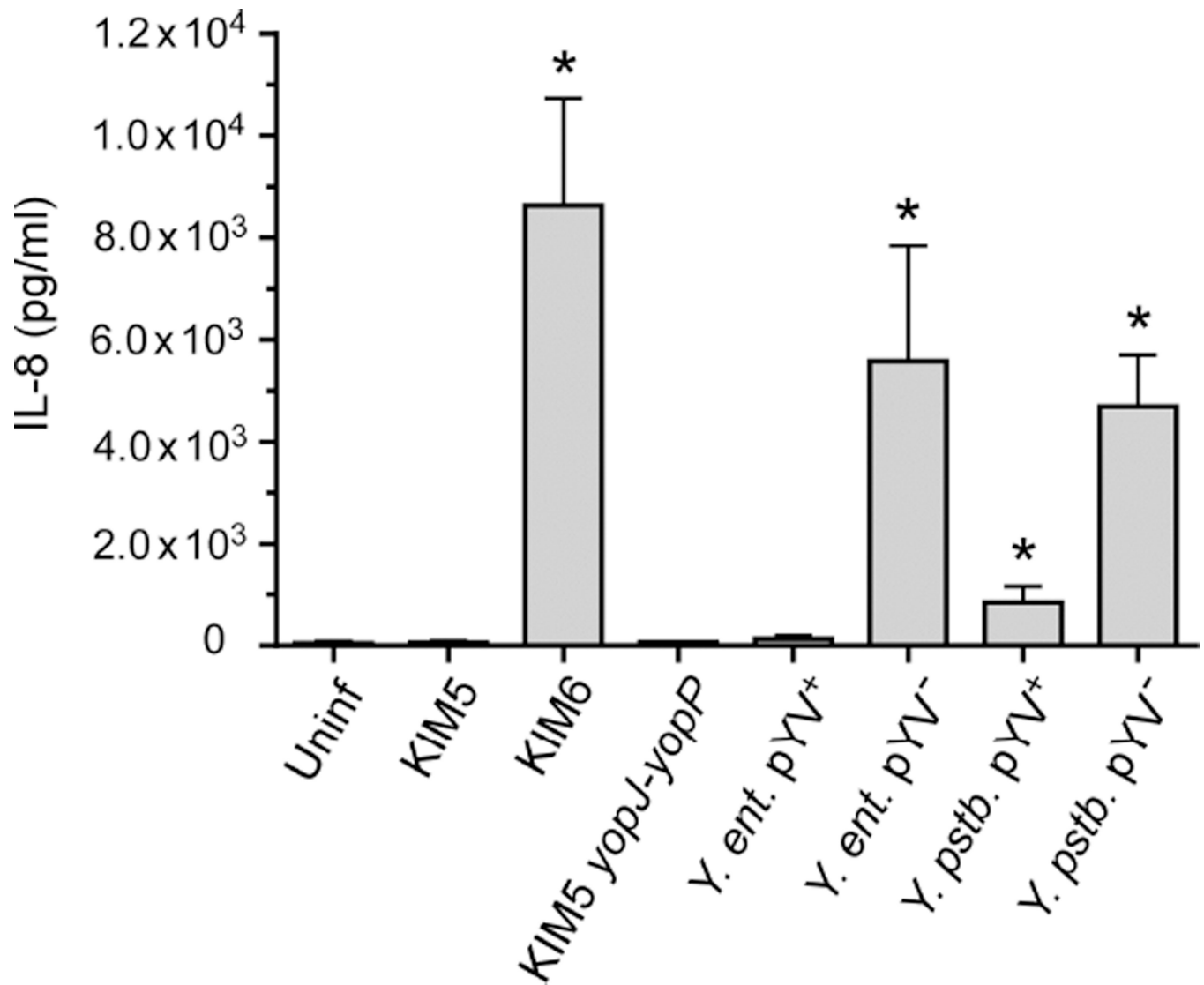


Fig. 4.

Virulence plasmid-dependent inhibition of IL-8 secretion by PMNs incubated with the enteropathogenic *Yersinia* species. *Y. enterocolitica*, (*Y. ent.*) and *Y. pseudotuberculosis* (*Y. pstb.*) strains, with (pYV⁺) or without (pYV⁻) their native virulence plasmid were incubated with human PMNs and the amount of IL-8 secreted was compared to PMNs incubated with pCD1⁺ (KIM5) or pCD1⁻ (KIM6) *Y. pestis*. *Y. pestis* engineered to express *yopP* instead of *yopJ* (*yopJ-yopP*) was also incubated with PMNs. Culture supernatant was collected after 12 h of incubation and the concentration of IL-8 was determined by ELISA. For these experiments the bacteria were not opsonized before addition to the PMNs. The mean and SEM for at least three independent experiments, except for the *yopJ-yopP* strain, for which only two experiments were realized, are indicated. Data were analyzed by one-way ANOVA with Tukey post-test. *, $P < 0.05$ compared to KIM5.

Table 1

Bacterial strains and plasmids used in this study

Strain or Plasmid	Description ^a	Source or reference
<u><i>Y. pestis</i> strains</u>		
KIM6+	pCD1-negative, Pgm ⁺	[18]
KIM6	pCD1-negative, Pgm ⁻	[18]
KIM5	pCD1-positive, Pgm ⁻	[18]
KIM5 <i>yopJ</i>	<i>yopJ</i> deleted from pCD1	[15]
KIM5 <i>yopJ-yopP</i>	<i>yopJ</i> replaced with <i>Y. enterocolitica yopP</i>	[15]
KIM5 <i>yopE</i>	<i>yopE</i> deleted from pCD1	This study
KIM5 <i>yopH</i>	<i>yopH</i> deleted from pCD1	This study
KIM5 <i>yopT</i>	<i>yopT</i> deleted from pCD1	This study
KIM5 <i>yopM</i>	<i>yopM</i> deleted from pCD1	This study
KIM5 <i>ypkA</i>	<i>ypkA</i> deleted from pCD1	This study
KIM5 <i>yopJ</i> (pWKS:: <i>yopJ</i>)	<i>yopJ</i> knockout with pWKS:: <i>yopJ</i> (kan)	This study
KIM5 <i>yopJ</i> (pCR:: <i>yopJ</i>)	<i>yopJ</i> knockout with pCR:: <i>yopJ</i> (kan)	This study
KIM6 (pCD1 1234)	KIM6 with pCD1 1234 (cam)	This study
KIM6 (pCD1 1234) (pWKS:: <i>yopJ</i>)	KIM6 (pCD1 1234) with pWKS:: <i>yopJ</i> (cam, kan)	This study
KIM6 (pCD1 1234) (pCR:: <i>yopJ</i>)	KIM6 (pCD1 1234) with pCR:: <i>yopJ</i> (cam, kan)	This study
KIM6 (pWKS:: <i>yopJ</i>)	KIM6 with pWKS:: <i>yopJ</i> (kan)	This study
KIM6 (pCR:: <i>yopJ</i>)	KIM6 with pCR:: <i>yopJ</i> (kan)	This study
<u><i>E. coli</i> strains</u>		
<i>E. coli</i>	CWG28 (O9a/K30 ⁻) (str)	[42]
<u><i>Y. enterocolitica</i> strains</u>		
<i>Y. ent.</i> pYV ⁺	8081v, pYV-positive	[43]
<i>Y. ent.</i> pYV ⁻	8081c, pYV-negative	[43]
<u><i>Y. pseudotuberculosis</i> strains</u>		
<i>Y. pstb.</i> pYV ⁺	IP32953, pYV-positive	[44]
<i>Y. pstb.</i> pYV ⁻	IP32953, pYV-negative	[44]
<u>Plasmids</u>		
pCD1 1234	plasmid encoding the genes required for the T3SS, no effector Yops (cam)	[19]
pCR:: <i>yopJ</i>	<i>yopJ</i> expressed under native promoter from the high-copy plasmid pCR-XL-Topo (kan, zeo)	This study
pWKS:: <i>yopJ</i>	<i>yopJ</i> expressed under native promoter from the low-copy plasmid pWKS130 (kan)	This study

^a antibiotic resistances where present are noted in parentheses: kan = kanamycin, str = streptomycin, cam = chloramphenicol, zeo = zeocin

Table 2

Primers

Primer Designation	Sequence ^a
<i>yopJ</i> complementation forward	5'-AGTTGAGCTCCCCTAAGCCTTGAGTTGATA-3'
<i>yopJ</i> complementation reverse	5'-AGTTTCTAGAGGATTGAGTTCCCTCAGTGAT-3'
<i>yopE</i> knockout forward	5'-ATGAAAATATCATCATTTATTTCTACATCACTGCCCGTGTAGGCTGGAGCTGCTTC-3'
<i>yopE</i> knockout reverse	5'-TCACATCAATGACAGTAATTTCTGCATCTGTTGCGCCATATGAATATCCTCCTTAG-3'
<i>yopH</i> knockout forward	5'-ATGAACTTATCATTAAAGCGATCTTCATCGTCAGGTAGTGTAGGCTGGAGCTGCTTC-3'
<i>yopH</i> knockout reverse	5'-TTAGCTATTTAATAATGGTCGCCCTTGTCTTCAGCCATATGAATATCCTCCTTAG-3'
<i>yopT</i> knockout forward	5'-ATGAACAGTATTCACGGACACTACCATATTCAACTAGTGTAGGCTGGAGCTGCTTC-3'
<i>yopT</i> knockout reverse	5'-TTAAACCTCCTTGGAGTCAAATGTAACTCTAAACATATGAATATCCTCCTTAG-3'
<i>yopM</i> knockout forward	5'-ATGTTTATAAATCCAAGAAATGTATCTAATACTTTTGTGTAGGCTGGAGCTGCTTC-3'
<i>yopM</i> knockout reverse	5'-CTACTCAAATACATCATCTTCAAGTTTGTCTGTAGTCATATGAATATCCTCCTTAG-3'
<i>ypkA</i> knockout forward	5'-ATGAAAAGCGTGAAAATCATGGAACTATGCCACCGTGTAGGCTGGAGCTGCTTC-3'
<i>ypkA</i> knockout reverse	5'-TCACATCCATTCCCCTCCAACCGTTTCAGTCGCTCCATATGAATATCCTCCTTAG-3'

^a Underlined sequence denotes restriction enzyme site addition