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Virulence Plasmid-Encoded YopK Is Essential for *Yersinia pseudotuberculosis* To Cause Systemic Infection in Mice

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The virulence plasmid common to pathogenic *Yersinia* species encodes a number of secreted proteins denoted Yops (*Yersinia* outer proteins). Here, we identify and characterize a novel plasmid-encoded virulence determinant of *Yersinia pseudotuberculosis*, YopK. The *yopK* gene was found to be conserved among the three pathogenic *Yersinia* species and to be homologous to the previously described *yopQ* and *yopK* genes of *Y. enterocolitica* and *Y. pestis*, respectively. Similar to the other Yops, YopK expression and secretion were shown to be regulated by temperature and by the extracellular Ca^{2+} concentration; thus, *yopK* is part of the *yop* regulon. In addition, YopK secretion was mediated by the specific Yop secretion system. In *Y. pseudotuberculosis*, YopK was shown neither to have a role in this bacterium's ability to resist phagocytosis by macrophages nor to cause cytotoxicity in HeLa cells. YopK was, however, shown to be required for the bacterium to cause a systemic infection in both intraperitoneally and orally infected mice. Characterization of the infection kinetics showed that, similarly to the wild-type strain, the *yopK* mutant strain colonized and persisted in the Peyer's patches of orally infected mice. A *yopE* mutant which is impaired in cytotoxicity and in antiphagocytosis was, however, found to be rapidly cleared from these lymphoid organs. Neither the *yopK* nor the *yopE* mutant strain could overcome the primary host defense and reach the spleen. This finding implies that YopK acts at a different level during the infections process than the antiphagocytic YopE cytotoxin does.

The human pathogenic species of the genus *Yersinia* include *Yersinia pestis*, the causative agent of bubonic plague. The two other human pathogenic species, *Y. pseudotuberculosis* and *Y. enterocolitica*, cause a systemic infection similar to plague in rodents, whereas in humans they only cause a self-limiting gastrointestinal disease. A 70- to 75-kb plasmid common to the three pathogenic *Yersinia* species is essential for virulence. This plasmid encodes a number of secreted proteins (28) called Yops (Yersinia outer proteins) (5, 18). By specific mutagenesis, several of the Yops have been identified as virulence determinants in animal infection models. YopE and YopH have a role in the antiphagocytic ability common to the pathogenic *Yersinia* species (7, 42). YopE is a cytotoxin with its target inside the eukaryotic cell (42–44). The function of YopE is unknown, but its activity leads to the disruption of the actin microfilaments, which can be visualized as a characteristic rounding of cultured epithelial cells (43). YopH has been shown to possess protein tyrosine phosphatase activity (24) and to mediate dephosphorylation of host proteins inside eukaryotic cells (3). This activity is thought to interfere with signal transduction pathways in phagocytic cells. In the mouse model, a *yopH* mutant was shown to be rapidly cleared from lymphoid organs such as the Peyer's patches after infection by the oral route (38). Thus, the antiphagocytic activity of YopH is likely to be of importance for yersiniae to overcome the primary host defense and establish a systemic infection. Another virulence factor with the potential to interfere with the signal transduction of eukaryotic cells is the Ser/Thr protein kinase YpkA (21), which displays homology with eukaryotic protein kinases. The molecular target of YpkA has not been identified, and it is not known whether this protein is also involved in the antiphagocytic activity of yersiniae. A third virulence plasmid-encoded protein with homology to a eukaryotic protein is YopM. This protein shows similarities with GPIbα, the α chain of the platelet receptor for the von Willenbrand factor (30). In vitro, YopM has been shown to prevent platelet aggregation by interacting with thrombin (31, 40). YopM is essential for virulence, since *yopM* mutants of *Y. pestis* and *Y. enterocolitica* have been shown to be avirulent for intravenously infected mice (31, 36). YopK and YopQ (Yop20) are additional plasmid-encoded virulence determinants of *Y. pestis* and *Y. enterocolitica*, respectively (36, 51). The role of YopK and YopQ in the infectious process is not known, but both *yopK* and *yopQ* mutants have been shown to be attenuated for intravenously infected mice (36, 52).

In vitro, *yop* expression is coordinately regulated by temperature and the extracellular Ca^{2+} concentration. Maximal expression is obtained in Ca^{2+}-depleted medium at 37°C (12, 53). A key element in the temperature regulation of *yop* gene expression is the transcriptional regulator VirF (LcrF) (13, 56). VirF belongs to the AraC family of transcriptional activators and has been shown to bind to DNA sequences upstream of promoters of several *yop* genes (29, 54). The calcium regulation involves the surface protein YopN, which in the presence of Ca^{2+} somehow senses and transmits a negative signal into the bacterial cytoplasm to lower the level of *yop* gene transcription (19). In vivo, however, *yop* expression is induced also in the presence of Ca^{2+}. In this case, the interaction between the pathogen and the eukaryotic cell seems to trigger increased *yop* gene expression (44).

During full induction in vitro, i.e., growth at 37°C in Ca^{2+}-depleted media, the Yops are secreted into the culture medium in large amounts (28). The secretion of Yops is mediated by a plasmid-encoded secretion system involving at least 20 genes (2, 33, 34, 42). Secretion of Yops does not involve processing of the proteins, but the amino termini of the Yops are essential for secretion (32). The Yop-specific secretion has been classified as a type III secretion pathway (45), and similar

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Source or reference
E. coli		
DH5α	*recA*, α complementation	26
SY327λ*pir*	Δ(*lac-pro*) *argE*(Am) *recA56 nalA* Rif^r (λ*pir*)	35
S17-1λ*pir*	RP4-2 Tc::Mu-Km::Tn*7* (λ*pir*)	48
Y. pseudotuberculosis		
YPIII	Virulence plasmid cured	4
YPIII(pIB102)	Wild type, *yadA*	6
YPIII(pIB23)	*yscJ* (*lcrK*) *yadA*	42
YPIII(pIB73)	*lcrF yadA*	14
YPIII(pIB102EL)	*yopE*::*luxAB yadA*	17
YPIII(pIB154)	*yopK*::*luxAB yadA*	This study
YPIII(pIB154, pAH9)	*yopK*::*luxAB yopK*$^+$ *yadA*	This study
YPIII(pIB522)	*yopE yadA*	20
Y. pestis		
KIM5	Wild type, pCD1, pPCP1, pMT1	S. C. Straley
KIM5-3131	pCD1::Mu dI1734-31, pPCP1, pMT1 (*yopK*)	S. C. Straley
Plasmids		
pACYC177	Ap^r Km^r	8
pCH257	Suicide plasmid used to construct a *yopK* mutant and to generate *luxAB* fusion	16
pGEX-KG	Fusion protein expression vector used to generate a GST-YopK fusion protein for immunization	25
pUC19	Ap^r	55
pIB572	12-kb *Bam*HI fragment 1 of pIB102 cloned into pBR322	15
pAH3	3.2-kb *Bgl*II fragment of *Bam*HI fragment 1 of pIB102 cloned into pUC19	This study
pAH9	1,072-bp PCR fragment containing *yopK* cloned into pACYC177	This study
pAKM1	152-bp internal fragment of *yopK* cloned into pCH257	This study
pFUSK	*yopK* cloned into pGEX-KG	This study

systems are also present in other gram-negative pathogens such as *Shigella* species and the plant pathogens *Pseudomonas fluorescens* and *Xanthomonas campestris* (2).

In this study, we show that *yopK* of *Y. pestis* is homologous to *yopQ* of *Y. enterocolitica*. We also identify the corresponding gene in *Y. pseudotuberculosis* (designated *yopK*), confirming that this gene is conserved in the pathogenic *Yersinia* species. Like the other *yop* genes, *yopK* of *Y. pseudotuberculosis* is regulated by temperature and Ca^{2+} concentration, but the level of expression is lower than those of most other *yop* genes. YopK is not involved in the antiphagocytic ability of *Y. pseudotuberculosis* but is essential for the pathogen to establish a systemic infection in mice.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria broth or on Luria agar plates. *Yersinia* strains were grown in the defined medium TMH (51) or in BHI (brain heart infusion; Oxoid) supplemented with either 2.5 mM $CaCl_2$ (TMH$^+$ and BHI$^+$) or 5 mM EGTA (TMH$^-$ and BHI$^-$) (plus 20 mM $MgCl_2$ for BHI). For solid media, *Yersinia* selective agar base (Difco) and blood agar base (Oxoid) were used. The blood agar base plates were supplemented with either 20 mM sodium oxalate, 20 mM $MgCl_2$, and 2% glucose (MOX plates) or 2.5 mM $CaCl_2$ (Ca^{2+} plates). Where appropriate, the antibiotics kanamycin (50 μg/ml), chloramphenicol (20 μg/ml), and carbenicillin (100 μg/ml) were used.

DNA methods. Preparation of plasmid DNA, restriction enzyme digests, ligations, and transformations into *E. coli* were performed essentially as described by Sambrook et al. (46). DNA fragments were purified from agarose gels by using Geneclean (Bio 101). DNA sequencing of *yopK* was performed by the method of Sanger et al. (47) with T7 DNA polymerase (Pharmacia AB), using pAH3 and pAH9 as the templates. Sequencing of the PCR-generated fragment from the junction of *yopK-lacZ* of *Y. pestis* KIM5-3131 was performed by using a Cyclist *Taq* DNA sequencing kit (Stratagene) according to the manufacturer's instructions.

Construction of a *yopK* mutant. A *yopK* insertion mutant of *Y. pseudotuberculosis* was constructed in a way which also generated a transcriptional fusion between *yopK* and the promoterless *luxAB* genes, thereby allowing *yopK* transcription to be monitored as luciferase activity. An internal fragment of the *yopK* gene was amplified by PCR from the virulence plasmid pIB102 by using the primers IMK1 and IMK2 (Table 2), which were tailed with sites for *Xba*I and

TABLE 2. Primer sequences

Primer	Sequence[a]	bp
K1	5′-GCT AG**T CTA GA**G *ATG TTT ATT AAA GAT GCT TAT AAC ATG C*-3′	964–991
K2	5′-GCG CT**G AGC TC**T *CAT CCC ATA ATA CAT TTT TGA TCG C*-3′	1512–1487
Rlac7[b]	5′-*CGT TGT AAA ACG ACG GCC AG*-3′	38–29
IMK1	5′-GCT AG**T CTA GA**C *TGT GCT ACT GCT CAT TTA CTG AGA AC*-3′	1071–1097
IMK2	5′-GCC AT**G AGC TC**C *ATG TTG CCA TTC GTA TAA GCG ATA TA*-3′	1222–1196
K3	5′-*GGA GGA ATT GAG TCG CG*-3′	1464–1480
A1	5′-GCA TGG AGC TC*T TAC AGG ATA TTG GCG ATA GAC TTC GC*-3′	2755–2739
LuxA	5′-*GTC GGT GGA CTC GAT AGA GTC TGG C*-3′	55–30
TK1	5′-*GCG TCA GAG TAC ATG TTG TAC ATT*-3′	582–605
TK2	5′-*GGC GTT GTT TCC CTT TGC GGC GAA*-3′	1758–1735

[a] Nucleotides in boldface letters correspond to restriction enzyme recognition sites. Nucleotides in italics are identical to positions given to the right. *Yersinia* sequences are derived from *Y. pseudotuberculosis* except for A1 and TK2, which are derived from *Y. enterocolitica* (accession number M33786 [X52753]).

[b] Derived from the *E. coli lacZ* gene (accession number V00296).

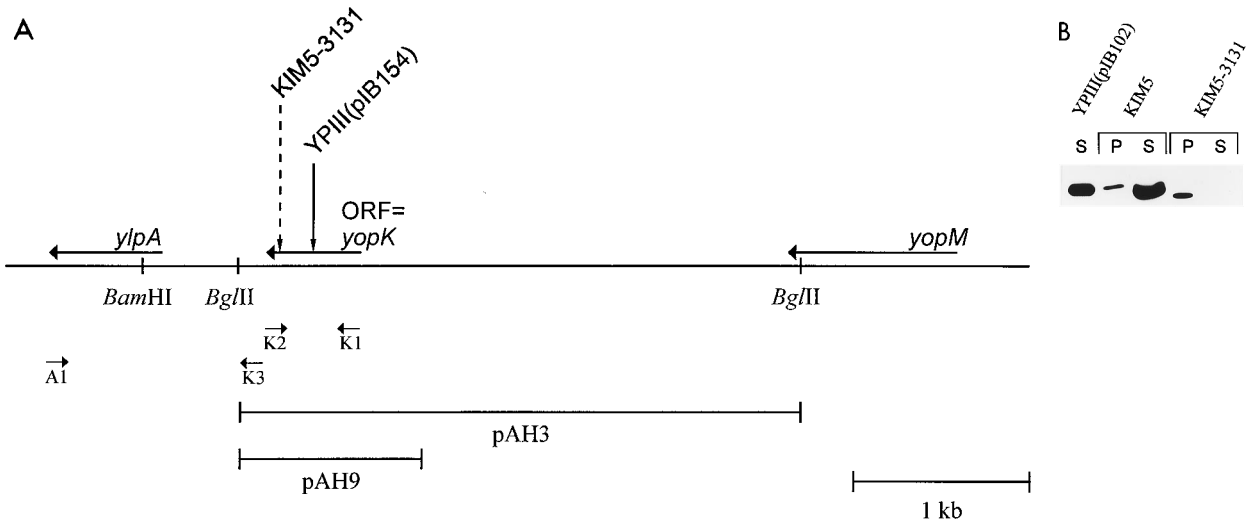

FIG. 1. (A) Physical map of the region of pIB1 of *Y. pseudotuberculosis* containing the *yopK* gene with flanking regions. The continuous vertical arrow shows the insertion point of the suicide plasmid in the *yopK* mutant, YPIII(pIB154), in *Y. pseudotuberculosis*. The dashed arrow marks the insertion point of Mu dI1743 in the *Y. pestis yopK* mutant, KIM5-3131. Small horizontal arrows indicate positions of primers used for characterization of the *yopK/ylpA* region in the pathogenic *Yersinia* species. pAH3 (pUC19) and pAH9 (pACYC177) are clones used for sequencing and transcomplementation, respectively. (B) Western blot analysis using anti-YopK antiserum of proteins from *Y. pseudotuberculosis* YPIII(pIB102) (wild type) and *Y. pestis* KIM5 (wild type) and KIM5-3131 (*yopK* mutant). Bacteria were grown in TMH^- as described in Materials and Methods. Yop proteins from the bacterial pellet (P) (for *Y. pestis*) and culture supernatant (S) were prepared as described in Materials and Methods.

*Sac*I, respectively. The PCR fragment was digested with *Sac*I and *Xba*I and cloned into the suicide vector pCH257 also cut with *Sac*I and *Xba*I to yield plasmid pAKM1. The cloning was performed in *E. coli* SY327λ*pir* (35). pAKM1 was subsequently transformed into *E. coli* S17-1λ*pir*, from which it was introduced by conjugation into the recipient strain YPIII(pIB102). Clones with pAKM1 integrated into pIB102 by a single recombination event were selected on plates containing chloramphenicol and kanamycin. The resulting mutant, YP III(pIB154) (Fig. 1A), was verified by PCR using primers IMK1 and LuxA, identical to nucleotides +55 to +30 of the *luxA* gene of *Vibrio harveyi* (10) (Table 2). In addition, YPIII(pIB154) was confirmed by restriction analysis and Western blotting (immunoblotting) (anti-YopK serum).

Cloning of the *yopK* gene. The *yopK* gene was cloned into the *Bam*HI site of the high-copy-number plasmid pUC19 as part of a 3.2-kb *Bgl*II fragment from *Bam*HI fragment 1 of the virulence plasmid of *Y. pseudotuberculosis* (Table 1). The resulting plasmid, pAH3, was used for sequencing of the *yopK* gene and its flanking regions. For transcomplementation of the *yopK* mutant, the entire *yopK* gene with flanking DNA was cloned into the medium-copy-number vector, pACYC177. The plasmid was constructed as follows. *yopK* with flanking DNA was amplified by PCR using pIB102 as the template DNA and oligonucleotides TK1 and TK2 as the primers (Table 2). Blunt ends were generated on the PCR fragment by the Klenow enzyme (Boehringer), and the fragment was digested with *Bgl*II and cloned into pACYC177, which was previously digested first with *Xho*I, for which the ends were filled to make blunt ends, and then with *Bam*HI. The DNA sequence of the *yopK* gene of the resulting plasmid, pAH9 (Fig. 1A), was confirmed. pAH9 was introduced into YPIII(pIB154) by electroporation (11).

Generation and purification of antibodies. The fusion protein expression vector pGEX-KG (25) was used to generate a glutathione *S*-transferase (GST)–YopK fusion protein for immunization of a rabbit. The entire *yopK* gene was amplified and cloned in frame downstream of the *gst* gene to yield a translational fusion. The primers used, K1 and K2, are described in Table 2. Expression and purification of the fusion protein were performed as described earlier (1, 25). The fusion protein was used as an antigen for intracutaneous immunization of a rabbit (New Zealand White, adult male). In the first immunization, 150 μg of the antigen was mixed 1:1 with Freund's complete adjuvant in a total volume of 1 ml. In the following immunizations, the antigen was mixed with Freund's incomplete adjuvant (1:1). For each booster injection, 100 μg of antigen was given every 2 to 3 weeks. Four booster injections were given. The anti-GST-YopK serum was affinity purified by immunoadsorption of the antibodies to nitrocellulose filters containing YopK as described earlier (19).

Luciferase assay. Overnight cultures of YPIII(pIB154) and YPIII(pIB102EL) grown at 26°C in BHI^+ and BHI^- were inoculated (1/20 dilution) into fresh medium. After 1 h of growth at 26°C, the cultures were shifted to 37°C. Control cultures were kept at 26°C. During a time period of 3 h, samples were collected and the cell density (optical density at 600 nm [OD_{600}]) and luciferase activity were monitored. The luciferase activity in 100-μl samples was measured in a Luminometer (1250 LKB Wallac), using *n*-decyl aldehyde as the substrate (100 μl of 0.1% *n*-decyl aldehyde in water solution). The luciferase activity was expressed as light units per OD_{600} and milliliter of sample.

Yop expression and analysis. Overnight cultures of *Yersinia* strains grown at 26°C were diluted (1/10) in fresh medium (TMH^-), grown for 1 h at 26°C, and then shifted to 37°C for 3 h. After measurement of OD_{600}, the cells were harvested and the culture supernatant was collected and filtered (0.22-μm-pore-size Sartorius filter). The proteins from the supernatant were precipitated with trichloroacetic acid as described earlier (15). The samples from the supernatant and from the whole bacteria were dissolved in sodium dodecyl sulfate (SDS) sample buffer, and the volume of the samples was adjusted in accordance to the OD_{600} values of the bacterial cultures. The proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed either by Coomassie brilliant blue R (Sigma) staining or by enhanced chemiluminescence Western blotting as instructed by the manufacturer (Amersham).

Virulence tests. Oral and intraperitoneal (i.p.) infections of male BALB/cJ mice (Bomgaard, Ry, Denmark) were carried out as described earlier (20, 42). The concentration of the bacterial suspension given to the mice in the oral infection was 5×10^9 bacteria per ml, and each mouse drank on average about 5 ml of the suspension. When infecting mice by the i.p. route, we used four dose groups with three animals in each. The doses ranged from 10^4 to 10^7 bacteria. The 50% lethal dose (LD_{50}) values were determined by the method of Reed and Muench (39). To study the kinetics of infection of different strains, two infected mice were sacrificed daily, starting 12 h after infection. From each mouse, the whole spleen and 7 to 10 Peyer's patches from the distal part of the small intestine were removed and homogenized. The number of bacteria in these organs was determined by plating serial dilutions of the samples on selective BAB plates.

Assays for cytotoxicity and phagocytosis. HeLa cells were cultivated and infected as described in detail previously (42). Briefly, monolayers of HeLa cells were seeded (0.5×10^5 cells) and grown to semiconfluency in a 24-well tissue culture plate in Leibovitz L-15 medium containing 10% heat-inactivated fetal calf serum [L-15 (10%)] and 100 IU of penicillin per ml at 37°C in a humid atmosphere. Before infection, the HeLa cells were washed in PBSA, and thereafter L-15 (10%) without antibiotics was added; 4×10^6 bacteria were added, and the tissue culture plate was centrifuged for 5 min at $400 \times g$ to facilitate contact between the bacteria and the HeLa cells. The infected cells were incubated at 37°C. The cytotoxicity assay was performed as described earlier (42). In the phagocytosis assay, cells from the mouse macrophage cell lineage J774A.1 (ATCC TIB 67) were seeded (2×10^5 cells) on coverslips and grown to semiconfluency in Ham's F10 containing 10% heat-inactivated calf serum [F10 (10%)] and 100 IU of penicillin per ml at 37°C in a humidified atmosphere of 5% CO_2 in air. Before infection, the macrophages were washed in PBSA (50 mM, pH 7.4), and thereafter F10 (10%) without antibiotics was added. Overnight cultures grown at 26°C were diluted 20-fold in fresh BHI containing 2.5 mM $CaCl_2$. The bacterial cultures were pregrown for 30 min at 26°C and then shifted to 37°C and incubated for an additional 2 h. The macrophages were infected with 10^6 bacteria and centrifuged for 5 min at $400 \times g$. The phagocytosis assay was

performed as described earlier (41), using the double-immunofluorescent antibody method to discriminate between intra- and extracellular bacteria.

Nucleotide sequence accession number. The GenBank/EMBL accession number of the *yopK* sequence of *Y. pseudotuberculosis* is U18804.

RESULTS

The *yopK*/*ylpA* region is conserved in the *Yersinia* virulence plasmids. The *yopK* gene of *Y. pestis* and the *yopQ* gene of *Y. enterocolitica* have been mapped adjacent to the *yopM* gene (30, 36, 51). To characterize the corresponding region of the virulence plasmid of *Y. pseudotuberculosis*, a 3.2-kb *Bgl*II fragment of pIB1 extending from *yopM* and further downstream was cloned (pAH3) (Fig. 1A) and partially sequenced. An open reading frame (ORF) of 549 bp encoding a protein of 21 kDa was identified 2.5 kb downstream of *yopM*. In total, 1,629 bp extending from the *Bgl*II site downstream of the ORF was sequenced. The ORF was 96% homologous at the nucleotide level with *yopQ* of *Y. enterocolitica*, but no significant homology to any other gene was found (GenBank/EMBL entries). However, the region upstream of the ORF showed strong homology (44% identity over 68 bp) to the region upstream of the *ypkA*-containing operon of *Y. pseudotuberculosis*. The transcriptional start point and the putative promoter for the *ypkA* operon have been mapped to this region (22). This finding indicates that the promoter regions of the ORF and of the *ypkA*-containing operon are highly homologous.

We next wanted to investigate if the ORF identified in *Y. pseudotuberculosis* corresponded to *yopK* of *Y. pestis*. Plasmid DNAs from both *Y. pestis* and *Y. pseudotuberculosis* were used as templates in PCR analysis using primers K1 and K2 (Fig. 1A; Table 2) derived from the sequence of the ORF in *Y. pseudotuberculosis*. Amplifications from both template DNAs resulted in fragments of similar sizes. No fragments were obtained when the same primers were used with the *Y. pestis yopK* mutant strain (KIM5-3131, *yopK*::Mu dI1734) as the template. This finding indicated that the ORF identified in *Y. pseudotuberculosis* corresponded to *yopK* of *Y. pestis*. To specifically localize the insertion of Mu dI1734 in the *yopK* mutant strain KIM5-3131, a PCR using one primer (Rlac7; Table 2) from the *lacZ* gene of the mini-Mud *lac* transposon and one primer from the ORF of *Y. pseudotuberculosis* (primer IMK1; Table 2) was performed. The amplified fragment was sequenced by using primers derived from the sequence of the ORF of *Y. pseudotuberculosis*. This analysis revealed that Mu dI1734 was inserted 95 bp from the end of the *yopK* gene of *Y. pestis* (Fig. 1A). The region sequenced upstream of the insertion point (395 bp) was 100% identical to the corresponding region of the virulence plasmid of *Y. pseudotuberculosis*. From these data, we conclude that the gene is highly conserved among the three *Yersinia* species and that *yopQ* of *Y. enterocolitica* is homologous to *yopK* of *Y. pestis*. Since the *yopK* gene of *Y. pestis* was identified before *yopQ* of *Y. enterocolitica* (51), we suggest that the gene of *Y. pseudotuberculosis* be designated *yopK*.

The YopK proteins of *Y. pseudotuberculosis* and *Y. pestis* were assayed for common antigenic sites by Western blotting (Fig. 1B). A secreted/cell-bound protein with a molecular mass of 21.5 kDa that cross-reacted with the *Y. pseudotuberculosis* YopK-specific antibodies (described in Materials and Methods) was detected in the *Y. pestis* wild-type strain KIM5. When the *yopK* mutant strain KIM5-3131 (*yopK*::Mu dI1734) was analyzed, no cross-reacting protein was detected in the supernatant, while a protein with a slightly lower molecular mass (20 kDa) was found in the bacterial fraction. This 20-kDa protein was most likely a truncated form of YopK, since the DNA sequence analysis described above showed that the transposon was inserted close to the very end of the *yopK* gene. The absence of the truncated product in the supernatant could be due to instability of the protein together with degradation by the *Y. pestis* protease, plasminogen activator, encoded by the *pla* gene of plasmid pPCP1 (50).

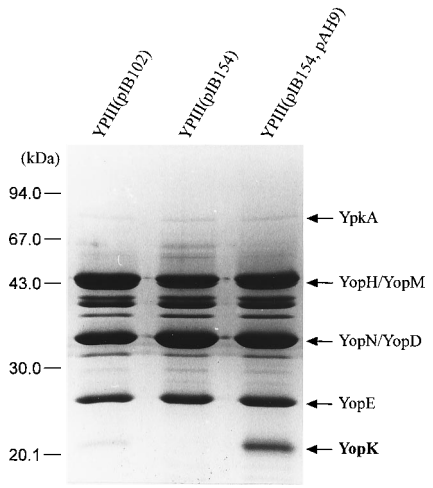

FIG. 2. SDS-PAGE analysis of the secreted proteins of the wild-type [YPIII (pIB102)], *yopK* mutant [YPIII(pIB154)], and transcomplemented *yopK* mutant [YPIII(pIB154, pAH9)] strains. The positions of some Yops are indicated by arrows. The strains were grown in TMH^{-} (for details, see Materials and Methods); protein samples from the supernatant were run on an SDS–14% polyacrylamide gel, which was subjected to Coomassie brilliant blue staining.

A gene encoding a lipoprotein, YlpA, has been mapped to the region downstream of *yopQ* in *Y. enterocolitica* (9). *ylpA* is encoded by a monocistronic operon transcribed in the same orientation as *yopQ* (Fig. 1A). Using PCR with primers K3 and A1 (Fig. 1A; Table 2), we found that the *yopK*/*ylpA* region is conserved in the virulence plasmids of virulent *Yersinia* species, since the sizes of the amplified DNA fragments were indistinguishable when DNAs from the different *Yersinia* species were used as templates (data not shown).

YopK is a secreted protein, and the *yopK* gene is part of the *yop* regulon. The Yops so far characterized are coordinately regulated, with maximal expression at 37°C in the absence of Ca^{2+} ions. During these conditions, the Yops are secreted in large amounts to the culture medium. To conclusively identify YopK in *Y. pseudotuberculosis*, we constructed a *yopK* insertion mutant by forcing the integration of a suicide plasmid containing an internal fragment of *yopK*, pAKM1, into the virulence plasmid. The resulting *yopK* mutant strain, YPIII(pIB154), was tested for growth characteristics in vitro, and the growth rate was found to be indistinguishable from that of the wild-type strain (data not shown). The *yopK* mutant was also Ca^{2+} dependent for growth at 37°C to the same extent as the wild-type strain (data not shown). The proteins secreted to the culture supernatant from different strains grown in Ca^{2+}-depleted media at 37°C were analyzed by SDS-PAGE. When the protein profiles of the wild-type [YPIII(pIB102)] and *yopK* mutant [YPIII(pIB154)] strains were compared, a faint band at 21.5 kDa (Fig. 2) was missing in YPIII(pIB154). The *yopK* mutant strain was transcomplemented by using plasmid pAH9, which harbors a 1,070-bp fragment containing the *yopK* gene cloned into pACYC177. This strain, YPIII(pIB154, pAH9), was found to overproduce a protein of the same size. The majority of other Yops were found to be secreted in larger amounts compared with YopK. One secreted protein with a similar expression level was the Ser/Thr kinase YpkA (Fig. 2).

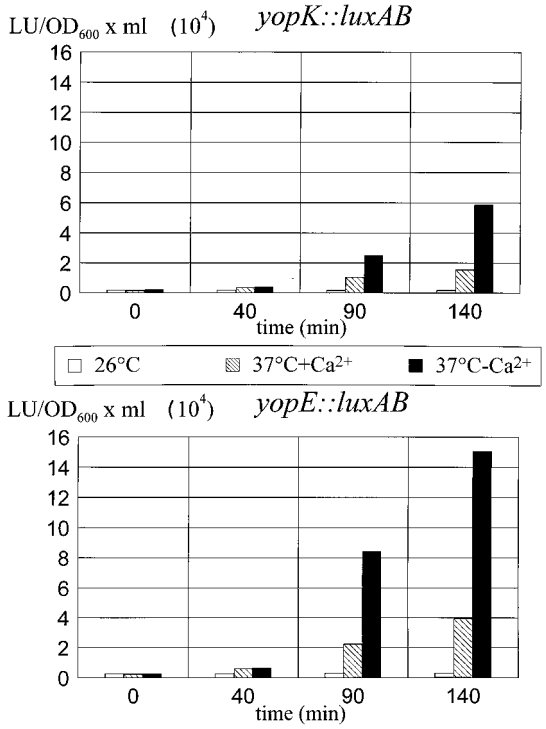

FIG. 3. Analysis of *yopK* transcription [YPIII(pIB154; top) and *yopE* transcription [YPIII(pIB102EL; bottom), using the *luxAB* reporter system. The two strains were grown as described in Materials and Methods. At different time points, samples were taken for luciferase activity and OD_{600} measurements. Activity is expressed as LU (light units) per OD_{600} and milliliter of sample.

Since our studies of YopK secretion indicated that YopK is expressed at lower levels than most other Yops, we wanted to investigate if the pattern of *yopK* expression is similar to that of other *yop* genes. *yopK* transcription was monitored as luciferase activity in strain YPIII(pIB154), since integration of the suicide plasmid generated a *yopK*::*luxAB* fusion. For comparison, strain YPIII(pIB102EL) (17), which contains a *yopE*::*luxAB* fusion, was used. Transcriptions of both fusions were regulated by the Ca^{2+} concentration and by temperature, with maximal expression at 37°C in medium depleted of Ca^{2+} (Fig. 3). When comparing the expression of the *yopK* fusion with the expression of the *yopE* fusion, we found that the kinetics of induction after the temperature shift from 26 to 37°C were similar, but the level of expression of *yopK* was lower than that of *yopE*.

YopK expression was also analyzed by Western blot analysis using monospecific antibodies against YopK. Samples from cultures grown at 37°C in the presence or absence of Ca^{2+} were subjected to SDS-PAGE followed by immunodetection. When strain YPIII(pIB102) was grown in TMH^- (depleted of Ca^{2+}), YopK was found both in the culture medium (Fig. 4A) and in the bacterial fraction. When the wild-type strain was grown in Ca^{2+}-containing medium, YopK could be recovered only from the bacterial fraction. The transcomplemented strain, YPIII(pIB154, pAH9), was found to overproduce the protein in TMH^- as well as in TMH^+.

A key regulator of *yop* expression is the DNA-binding transcriptional activator LcrF (29). The *lcrF* mutant strain, YPIII (pIB73), failed to produce detectable amounts of YopK (Fig. 4B). The *yscJ* mutant strain, YPIII(pIB23), is blocked in the secretion of Yops and derepressed for expression of *yop* genes

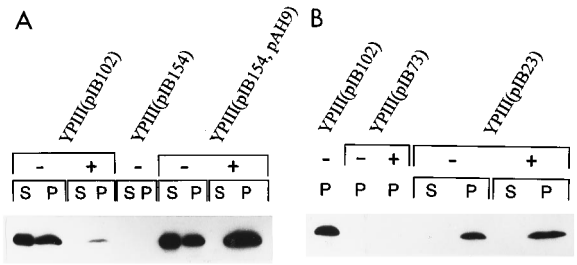

FIG. 4. Western blot analysis of YopK expression. Bacteria were grown in TMH^- (−) and TMH^+ (+). Yop proteins from the bacterial pellet (P) and culture supernatant (S) were prepared as described in Materials and Methods. Protein samples from the indicated strains were separated on SDS–12% polyacrylamide gels, which were subjected to immunodetection with an anti-YopK antiserum.

at 37°C independently of Ca^{2+} concentration. When samples from this secretion mutant were analyzed, equal amounts of YopK were found in the bacterial fraction of cells grown in TMH^- or TMH^+, whereas no YopK could be recovered from the culture supernatants. Together, these results show that YopK expression and secretion are regulated by temperature and Ca^{2+} concentration, and thus the *yopK* gene is part of the *yop* regulon.

A *yopK* mutant is fully cytotoxic and inhibits phagocytosis by macrophages. An important activity which allows pathogenic *Yersinia* species to overcome the primary defense of the host is the ability to resist uptake by macrophages. Two proteins known to be involved in this process are YopE and YopH. Mutants of *yopE* and *yopH* are less able to induce cytotoxicity in HeLa cells (42) and have decreased ability to inhibit phagocytosis by macrophages (41, 42). The *yopK* mutant, however, was found to resist phagocytosis by the mouse macrophage cell line J774A.1 and to induce cytotoxicity in HeLa cells to a similar extent as the wild-type strain (data not shown). These results indicate that YopK is not involved in the inhibition of phagocytosis.

YopK is essential for virulence. To study the importance of *yopK* in *Y. pseudotuberculosis* infections, the *yopK* mutant strain and the wild-type strain were evaluated in the mouse infection model. The *yopK* mutant strain, YPIII(pIB154) was found to be avirulent for orally infected mice (Table 3). When mice were challenged orally with the transcomplemented strain, YPIII(pIB154, pAH9), virulence was restored since only one of three mice survived the infection (Table 3). The importance of YopK was also tested for i.p.-infected mice. The LD_{50} values for the *yopK* mutant strain, YPIII(pIB154), and the virulence plasmid cured strain, YPIII, were at least 40-fold less than the LD_{50} value for the wild-type strain, while the

TABLE 3. Virulence of various strains of *Y. pseudotuberculosis* after infection of BALB/cJ mice by different routes

Strain	No. of mice surviving oral infection[a]	LD_{50} in mice infected i.p.
YPIII(pIB102)	0	5.1×10^5
YPIII(pIB154)	3	$>2 \times 10^7$
YPIII(pIB154, pAH9)	1	4.9×10^5
YPIII	ND	$>1.9 \times 10^7$

[a] Mice (three per group) were deprived of water for 18 h and then allowed to drink ad libitum water containing 5×10^9 bacteria per ml. Each mouse drank about 5 ml of the bacterial suspension. ND, not determined.

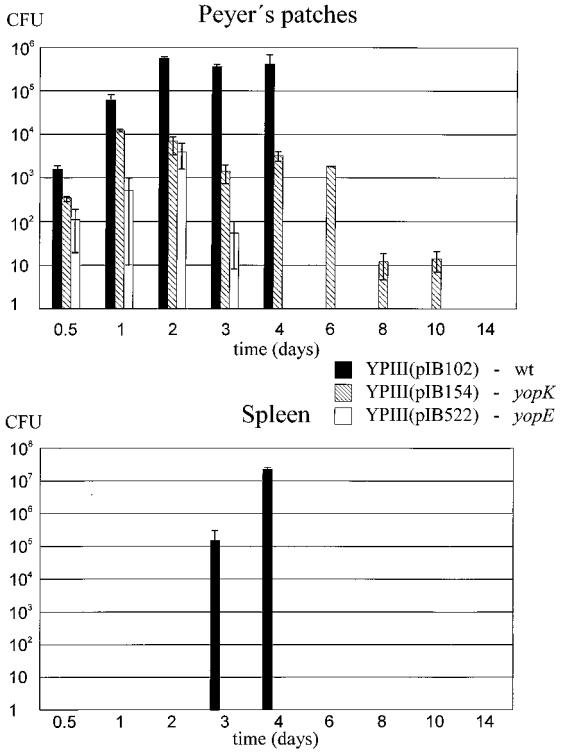

FIG. 5. Kinetics of infection in Peyer's patches and spleen of orally infected mice. BALB/cJ mice were infected with the indicated *Y. pseudotuberculosis* strains. Two mice were sacrificed daily, whole spleens and 7 to 10 Peyer's patches from the distal part of the small intestine were taken from each mouse, and the number of CFU per organ was determined. Values for Peyer's patches are given as CFU per Peyer's patch. Mice infected with the wild-type (wt) strain died 5 days after infection. Values are mean CFU per organ obtained from two mice.

transcomplemented strain in this case regained full virulence (Table 3).

Upon oral administration, *Y. enterocolitica* has been shown to pass the epithelial barrier by preferentially entering through the M cells to colonize the underlying Peyer's patches (23, 27). To further characterize the role of YopK of *Y. pseudotuberculosis* during infection, we investigated the kinetics of infection of Peyer's patches and of the spleen of mice infected by the oral route (Fig. 5). The wild-type strain, YPIII(pIB102), was found to colonize the Peyer's patches at high levels 24 h after infection. This high level was maintained throughout the infection. In contrast, the spleen was not colonized until 3 days after infection, but the bacterial numbers in the spleen increased further and death occurred 5 days after infection. The *yopK* mutant strain, YPIII(pIB154), was also found to colonize the Peyer's patches, but the numbers of bacteria were lower than those of the wild-type strain; nevertheless, the *yopK* mutant persisted in these organs for up to 10 days. The *yopK* mutant was, however, unable to colonize the spleen. The YopE cytotoxin has been shown to be involved in the antiphagocytic ability of *Y. pseudotuberculosis*. Strain YPIII(pIB522) (*yopE*) was also able to colonize the Peyer's patches but was completely cleared from these lymphoid organs 4 days after infection. As expected from the clearance of these bacteria from the Peyer's patches, the *yopE* mutant was unable to colonize the spleen of the infected mice. Together, these results show that YopK is important for establishing a systemic infection in mice but acts at a different level during the infectious process than the cytotoxin YopE does.

DISCUSSION

In this study, we show that YopK is essential for *Y. pseudotuberculosis* to cause a systemic infection in mice. A *yopK* mutant strain can colonize and persist in the Peyer's patches of orally infected mice. In contrast, a *yopE* mutant strain was rapidly eliminated from these lymphoid organs. The infection caused by the *yopK* mutant strain is, however, limited to the intestine, since the bacteria are unable to reach the bloodstream and cause a bacteremia. Thus, YopK is essential for the virulence of *Y. pseudotuberculosis* but acts by a different mechanism during the infection than the cytotoxin YopE does.

The *yopK* mutant strain was also found to be avirulent for i.p.-infected mice. Previously, *yopK* (*yopQ*) mutants of *Y. pestis* as well as *Y. enterocolitica* have been shown to be attenuated for intravenously infected mice (36, 51, 52). Therefore, it appears that YopK is of importance for virulence irrespective of the route of infection. This again is different from findings for YopE, which was shown to be less important for virulence in intravenously infected mice than in mice infected i.p. (20).

YopE and YopH have been shown to act in concert to resist phagocytosis (42), an activity essential for the virulence of *Yersinia* species (7, 41). This finding is also in agreement with the observation that yersiniae mainly multiply extracellularly during infection (27, 49). We show here that a *yopE* mutant of *Y. pseudotuberculosis*, in a finding similar to what was recently found for a *yopH* mutant (38), is rapidly eliminated from the Peyer's patches of orally infected mice. This rapid elimination from the lymphoid organ is most likely due to the inability of these mutants to resist uptake by the phagocytic cells in these organs. In contrast, the *yopK* mutant was found to inhibit uptake by phagocytes to the same extent as the wild-type strain and also to persist in the Peyer's patches. This is similar to what was shown for an *ypkA* mutant of *Y. pseudotuberculosis*, which also colonized and persisted in Peyer's patches after oral infection (22). YpkA has a Ser/Thr protein kinase activity, but it has not been shown that YpkA is involved in antiphagocytosis, and the fact that an *ypkA* mutant persists in lymphoid organs indicates that the target cell type for YpkA could be other than phagocytic cells. Similar to YopK, YpkA is required for *Y. pseudotuberculosis* to overcome the host defense and cause a lethal infection in mice (22).

The *yopK* gene was first identified in *Y. pestis* (51) and was described to be part of an operon with an additional gene, *yopL*, downstream of *yopK*. In *Y. enterocolitica*, the *yopQ* (*yop20*) gene was identified and mapped to the same region of the virulence plasmid as *yopK* of *Y. pestis* (36). YopQ was, however, shown to be encoded by a monocistronic operon, and downstream of *yopQ*, the *ylpA* gene, encoding a lipoprotein with homologies to TraT, was identified (9). Therefore, it was not clear whether *yopQ* and *yopK* were homologs of the same genes in *Y. enterocolitica* and *Y. pestis*, respectively. Our studies show that the *yopK* gene is highly conserved in the three species and that the *ylpA* gene is located downstream of the *yopK* gene in all three pathogenic species. It is still possible, however, that there is an additional gene downstream of *yopK* in *Y. pestis*, since there is about 500 bp between *yopK* and *ylpA*, enough to encode a protein of 15 kDa, the size reported for YopL. The final identification of the YopL-encoding gene must await sequencing of the region downstream of *yopK* in *Y. pestis.*

Our results show that YopK expression in *Y. pseudotuberculosis* is lower than the expression of most other Yops, consistent with the expression levels previously found for the secreted YopQ and YopK of *Y. enterocolitica* and *Y. pestis*, respectively (36, 37). Similar to other Yop proteins, YopK was

secreted only when the bacteria were grown in the absence of Ca^{2+} at 37°C. YopK was not secreted in an *yscJ* mutant, indicating that secretion of this protein is mediated by the Yop-specific secretion system. Here, we show that *yopK* is coordinately regulated with the other *yop* genes. The induction kinetics after a temperature shift from 26 to 37°C in Ca^{2+}-depleted media and the absolute requirement of the LcrF protein for transcription show that *yopK* is part of the *yop* regulon. The key to the lower expression levels can probably be found in the homologies identified in the promoter regions of the *yopK* gene and the *ypkA* operon. The expression level of YopK is similar to that of YpkA (21), and as discussed above, the *yopK* mutant has a phenotype in the mouse model similar to that found for the *ypkA* mutant (22). Therefore, it is tempting to speculate that YopK and YpkA act at the same stage during infection and that the two genes are coordinately expressed in the host.

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