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Adhesive Properties of YapV and Paralogous Autotransporter Proteins of *Yersinia pestis*

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Yersinia pestis **is the causative agent of plague. This bacterium evolved from an ancestral enteroinvasive *Yersinia pseudotuberculosis* strain by gene loss and acquisition of new genes, allowing it to use fleas as transmission vectors. Infection frequently leads to a rapidly lethal outcome in humans, a variety of rodents, and cats. This study focuses on the *Y. pestis* KIM *yapV* gene and its product, recognized as an autotransporter protein by its typical sequence, outer membrane localization, and amino-terminal surface exposure. Comparison of *Yersinia* genomes revealed that DNA encoding YapV or each of three individual paralogous proteins (YapK, YapJ, and YapX) was present as a gene or pseudogene in a strain-specific manner and only in *Y. pestis* and *Y. pseudotuberculosis*. YapV acted as an adhesin for alveolar epithelial cells and specific extracellular matrix (ECM) proteins, as shown with recombinant *Escherichia coli*, *Y. pestis*, or purified passenger domains. Like YapV, YapK and YapJ demonstrated adhesive properties, suggesting that their previously related *in vivo* activity is due to their capacity to modulate binding properties of *Y. pestis* in its hosts, in conjunction with other adhesins. A differential host-specific type of binding to ECM proteins by YapV, YapK, and YapJ suggested that these proteins participate in broadening the host range of *Y. pestis*. A phylogenic tree including 36 *Y. pestis* strains highlighted an association between the gene profile for the four paralogous proteins and the geographic location of the corresponding isolated strains, suggesting an evolutionary adaption of *Y. pestis* to specific local animal hosts or reservoirs.**

Yersinia pestis is a Gram-negative, facultative intracellular bacterium responsible for bubonic, systemic, or pneumonic plague in humans. *Y. pestis* enters mammalian hosts by one of three methods. When an infected flea injects *Y. pestis* into a host's skin, the bacteria use the lymphatic system to reach a local lymph node, possibly hitchhiking with polymorphonuclear leukocytes or dendritic cells (1, 2). Local multiplication with the ensuing inflammatory response leads to the typical swollen lymph node or bubo that characterizes bubonic plague. Unconstrained bacteria can cross into the blood, leading to a more deadly bacteremic form of plague, whereby the bacteria colonize the lungs, causing secondary pneumonic plague, or disseminate to further organs, resulting in septicemic plague. More rarely, fleas deliver the pathogen directly into a blood capillary, consistent with cases of septicemic plague in patients lacking a bubo (3). When systemic spreading of the bacteria leads to colonization of the lungs, aerosol transmission to new hosts can result in cases of primary pneumonic plague.

Various bacterial surface molecules are involved in the adherence and colonization of *Y. pestis* in the lungs. Work in our laboratory has revealed that the Psa fimbria is a dominant *Y. pestis* adhesin that mediates binding of bacteria to pulmonary epithelial cells even in the presence of the capsular antigen F1 (4). Mutants lacking Psa, F1, and Pla, the cell surface plasminogen activator protease that was reported to have adhesive and invasive properties (5, 6), still bound to and invaded pulmonary epithelial cells, hinting at the existence of additional *Y. pestis* adhesins and invasins.

Although the *yadA* and *inv* genes of enteropathogenic *Yersinia* express invasins, the corresponding orthologs are pseudogenes in *Y. pestis*. Information on the genomic sequences of several *Y. pestis* strains highlighted the presence of potential new adhesins and invasins, particularly by targeting predicted surface proteins (7). In addition to the identification of several fimbriae with known or potentially relevant adhesive functions (4, 8, 9), adhesive and invasive properties have been characterized for a variety of predicted nonfimbrial outer membrane proteins. The *Y. pestis* Ail protein was identified as another major adhesin (10–13), whereas several autotransporter proteins (14), such as YapC (15), YapE (16, 17), and the YadA-like oligomeric autotransporter proteins (18, 19), were also found to have adhesive properties.

The "autotransporter" designation was given to specific outer membrane proteins based on the early assumption that they extrude their N-terminal end or passenger domain through a channel formed by their membrane-embedded C-terminal β-barrel domain (20). More recent work indicates that the Bam proteins and possibly TAM (translocation assembly module) proteins participate in this process (21–23). Even though the translocated pas-

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TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or characteristic(s)[a]	Reference or source
Strains		
E. coli		
SE5000	MC4100 *recA56* (Fim$^-$)	59
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r$_B^-$ m$_B^-$) *dcm gal* (DE3)	Novagen
Y. pestis		
KIM5	*pgm*	60
KIM6	KIM5 pCD1$^-$	60
DSY50	KIM6 pPCP1$^-$ (*pla*) Δ*caf* Δ*psa*	4
DSY51	KIM6 pPCP1$^-$ (*pla*) Δ*yapK*::*aphA*	This study
DSY52	KIM6 pPCP1$^-$ (*pla*) Δ*yapK*::*aphA* Δ*yapV*::*cat*	This study
DSY53	KIM6 pPCP1$^-$ (*pla*) Δ*yapV*::*aphA*	This study
Plasmids		
pMAL-p2X	*ori* pMB1; Ap^r	NEB
pET22b(+)	*ori* pMB1; Ap^r	Novagen
pCS319	pMAL-p2X-*yapK*	This study
pCS320	pMAL-p2X-*yapV*	This study
p1741	pMAL-p2X-*yapJ*	This study
pCS326	pCS320-*yapV*(ΔIle$_{145}$-Ala$_{602}$)	This study
pCS327	pCS320-*yapV*(ΔAsp$_{277}$-Ile$_{317}$)	This study
pCS328	pCS320-*yapV*(ΔArg$_{517}$-Thr$_{1023}$)	This study
pCS329	pET22b-*yapK*(Cys$_{56}$-Ala$_{759}$)-6×His	This study
pCS330	pET22b-*yapV*(Asn$_{53}$-Ala$_{749}$)-6×His	This study
p1779	pET22b-*yapJ*(Pro$_{59}$-Leu$_{753}$)-6×His	This study
pKD3	Template plasmid; *cat* flanked by FRT sites	31
pKD4	Template plasmid; *aphA* flanked by FRT sites	31
pKD46	Red recombinase expression plasmid	31

[a] FRT, FLP recombination target.

senger domain of some autotransporter proteins is cleaved off (17, 24), a defining characteristic of the type V protein secretion system (T5SS), a number of them remain surface associated by noncovalent bonds (25). Passenger domains typically endow the bacteria with new virulence properties by serving as adhesins, invasins, proteases, or toxins. Surface exposure (or secretion of the passenger domain) of several autotransporter proteins of *Y. pestis* strain CO92 was confirmed *in vitro*, and transcription of their genes was detected in the lymph nodes and lungs of mice using models of bubonic or pneumonic plague (26). Interestingly, two of these proteins, YapK and YapJ, were shown in *Y. pestis* strain CO92 to share a high level of sequence identity that was extended to the corresponding autotransporter proteins in *Yersinia pseudotuberculosis*.

A recent study described a variety of structural and export properties of a *Y. pestis* KIM strain-specific autotransporter protein, designated YapV, including its capacity to recruit mammalian neural Wiskott-Aldrich syndrome protein (N-WASP) (27). Here, we characterized new adhesive properties of YapV and analyzed them in the context of its paralogous proteins YapK and YapJ.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* was routinely grown at 37°C in Luria-Bertani (LB) medium (Difco, BD Diagnostics, NJ). *Y. pestis* strains were grown overnight in brain heart infusion (BHI) broth (Difco) at 26°C, diluted 1:20 in fresh BHI broth containing 2.5 mM $CaCl_2$, and cultured overnight at 37°C. Appropriate antibiotics were used when required, at the following concentrations: 200 μg ml^{-1} ampicillin, 45 μg ml^{-1} kanamycin, and 35 μg ml^{-1} chloramphenicol. Maintenance of plasmid pMT1 in the mutants was checked by agarose gel electrophoresis.

Autotransporter orthology and paralogy identification. All the *Yersinia* autotransporter proteins were identified by using the OMPdb database (http://aias.biol.uoa.gr/OMPdb/). All the outer membrane proteins were manually scanned for an N-terminal extracellular domain ("passenger domain") and a C-terminal membrane-embedded domain (β-barrel domain) by using Conserved Domain Database v2.29 (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and the InterPro release v18.0 database (http://www.ebi.ac.uk/Tools/pfa/iprscan/), respectively. The nucleotide sequences of all the hits were further analyzed by using a BLASTN search with a cutoff value of 0.1 to identify potentially missed genes and pseudogenes for autotransporter proteins within 55 representative *Yersinia* genomes (see Table S2 in the supplemental material).

Phylogenomic tree. A phylogenomic tree was prepared from 40 *Y. pestis* and *Y. pseudotuberculosis* genomes, which were obtained from the NCBI (http://www.ncbi.nlm.nih.gov/bioproject/12302) and PATRIC (Pathosystems Resource Integration Center) (http://patricbrc.vbi.vt.edu/portal/portal/patric/Taxon?cType=taxon&cId=629). To identify all homologous (orthologous and paralogous) genes for full or partial autotransporter proteins shared by *Yersinia* spp., the predicted encoded protein sequences were retrieved from the GenBank and RAST databases (28). Pairs of proteins with >45% identity, 70% alignment coverage, and an E value of $<1 \times 10^{-20}$ were considered homologous. To construct the phylogenomic tree, 35 highly conserved orthologous housekeeping genes (with most being involved in gene regulation and transcription) were chosen (*atpD*, *cinA*, *cpdP*, *dnaN*, *engD*, *ftsX*, *glpB*, *hflX*, *kdpA*, *lpxH*, *metK*, *modE*, *mreC*, *mukF*, *mutH*, *nicO*, *nlpE*, *nqrD*, *nuoB*, *ompW*, *pcbC*, *ptsP*, *rarA*, *recO*, *rexX*, *rph*, *rplS*, *rsmB*, *tyrR*, *vacJ*, *via*, *ydgD*, *yibK*, *yraN*, and *ystK*). For this, the 35 genes were concatenated into a nucleotide sequence of ~30 kb. The 44 concatenated sequences were aligned by using ClustalW with default parameters to produce an alignment in MEGA format (29). Phylogenomic tree construction was done by using the maximum likelihood method with a bootstrap value of 1,000 (30).

Phylogenetic tree for the *Yersinia* autotransporter proteins. All 73 identified homologous autotransporter proteins in the 40 *Y. pestis* and *Y. pseudotuberculosis* strains with an intact reading frame, and the corrected y3427/y3428 gene, were used to perform multiple-sequence alignments with ClustalW (default parameters) and to construct a phylogenetic tree (neighbor-joining method with 1,000 bootstrap replicates; Jones-Taylor-Thornton model). Representatives of the 13 known autotransporter proteins YapA (YPO2886), YapB (y1345), YapC (YPO2796), YapE (YPO3984), YapF (YPO0606), YapG (YPO0587), YapH (YPO1004), YapJ (YPO1672), YapK (YPO0309), YapL (YPO1672), YapM (YPO0823), YapV (y3429), and YapX (YPK_0763) were analyzed by the same method to produce a *Yersinia*-specific autotransporter protein phylogenetic tree.

Construction of *Y. pestis* mutants. Mutants and plasmids are listed in Table 1. Strain DSY50 [KIM6 pPCP1$^-$ (*pla*) Δ*caf* Δ*psa*] was described previously (4). Mutant strains DSY53, DSY51, and DSY52 were constructed by using the lambda red recombination method (31). Briefly, to create strain DSY53, the kanamycin resistance marker of the red template plasmid pKD4 was PCR amplified by using primers y3428-29-RED (see Table S1 in the supplemental material), which target DNA flanking the y3429 gene (*yapV*) with the upstream pseudogene y3428 on the annotated KIM genome sequence (32). The PCR product was introduced into electrocompetent DSY50 cells expressing λ red recombinase from pKD46. Kanamycin-resistant colonies were selected, and the deletion of *yapV* was confirmed by PCR. The recombinant strain was cured of pKD46 by growth of the bacteria overnight at 37°C. The same approach was used to delete the y0567 gene (*yapK*) from strain DSY50 and to generate strain DSY51, using primers y0567-RED (Table 1) (32). Similarly, to generate *yapV yapK* mutant strain DSY52, primers designed for y3428-29 and pKD3 were used to amplify a chloramphenicol resistance marker, which

was introduced into strain DSY51. *In vitro* growth curves of mutants (or constructs) and their parental strains were not significantly different.

Cloning of *yapV*, *yapK*, and *yapJ*. The genes encoding YapV (y3429), YapK (y0567), and YapJ (y1833-y1834; one open reading frame [ORF] in KIM5) (33) were amplified by PCR using genomic DNA from strain KIM5 as the template and primers designed from the genome of strain KIM (32) and flanked by NdeI and XbaI restriction sites. Our gene designations are from the GenBank sequence of parental strain KIM, which contains an erroneous frameshift in the *yapJ* DNA (32). Our KIM5 *yapJ* gene lacked the frameshift, as determined by sequencing, and corresponded to the one in strain KIM D27 (33). The three genes were inserted into the corresponding sites of pMAL-p2x (New England BioLabs) to create pCS219, pCS320, and p1741, respectively. The three constructs were sequenced to confirm PCR accuracy. Three different in-frame deletions were constructed in *yapV* by digesting pCS320 with MfeI, ClaI, or SmaI. For each digest, the longer fragment was isolated by agarose gel electrophoresis and religated, resulting in plasmids pCS326, pCS327, and pCS328, respectively.

Purification of His-tagged YapV, YapK, and YapJ passenger domains and antibody production. y3429-His, y0567-His, and y1833-y1834-His primers (see Table S1 in the supplemental material) were used to amplify DNA fragments from strain KIM5 encoding the passenger domains of YapV (amino acids [aa] 53 to 748), YapK (aa 56 to 761), and YapJ (aa 59 to 753), respectively. The PCR products were digested with NdeI and XhoI and inserted into the corresponding sites of pET22b (Novagen, Gibbstown, NJ), resulting in plasmids pCS330, pCS329, and p1779, respectively. The inserted DNA was sequenced to confirm PCR accuracy. The recombinant proteins were expressed by induction with isopropyl-β-D-thiogalactopyranoside (IPTG) in *E. coli* BL21(DE3) and purified by metal chelation chromatography, as described previously (34). The resulting proteins did not have their signal sequences and contained 6-histidine tags at their C-terminal ends. Specific polyclonal antisera against YapV-His and YapK-His were prepared in rabbits by using a conventional immunization protocol (Cocalico Biologicals Inc., Reamstown, PA). Both antisera reacted strongly against the three His-tagged Yap proteins but not against His-tagged Psa and bovine serum albumin (BSA), as shown by enzyme-linked immunosorbent assays (ELISAs), indicating comparable antibody affinities for the three Yap proteins (see Fig. S1 in the supplemental material). For fluorescence microscopy and quantitative Western blot analyses, the antisera were adsorbed twice with *E. coli* BL21(DE3)/pMAL-p2X. Briefly, 1 ml antisera and 0.06% sodium azide were incubated with bacterial pellets from 10-ml cultures grown overnight for 18 h at 4°C; the adsorbed sera were filtered (0.02-μm-pore-size filter) before use.

Analysis of YapV, YapK, and YapJ expression. To examine YapV, YapK, and YapJ expression, cells of *E. coli* SE5000 carrying plasmid pCS320, pCS319, p1741, pCS326, pCS327, or pCS328 were grown overnight in LB broth at 37°C; diluted 1:100 in fresh medium; and grown for 4 h at 37°C to an optical density at 600 nm (OD_{600}) of 0.6. Expression of recombinant proteins was induced by the addition of 0.5 mM IPTG, and cultures were grown for an additional 2 h. Outer membrane fractions were prepared as described previously (35). Samples from induced cultures (total bacteria or outer membranes) were resolved by SDS-PAGE on 12% polyacrylamide gels and visualized by Coomassie blue staining or transferred onto nitrocellulose membranes for Western blot analysis. Blots were probed with rabbit anti-YapV antiserum (1:1,000), followed by anti-rabbit horseradish peroxidase (HRP) secondary antibodies (Cappel; MP Biomedicals, Aurora, OH), prior to development with ECL substrate (Pierce), using several phosphate-buffered saline (PBS) washing cycles between each step. Expression levels of the three Yap proteins were compared by analyzing Western blots of 3-fold serial culture dilutions of *E. coli* strain SE5000 carrying plasmid pCS320, pCS319, or p1741, using a mixture of adsorbed anti-YapV and anti-YapK antibodies (1:500) and an anti-rabbit polyclonal antibody conjugated to an IRDye 800CW fluorophore (1:5,000; Li-Cor Biosciences Inc., Lincoln, NE). All images were taken by using an Odyssey Fc imaging system and its imager software (Li-Cor) for densitometric analysis. For fluorescence microscopy, cells of *E. coli* strain BL21(DE3) carrying plasmid pCS320, pCS319, p1741, or pMAL-p2X were grown and induced as described above for YapV and YapJ. For YapK, bacteria were grown for 1 h after IPTG induction. The bacterial cells were deposited onto slides and dried for 20 min. The slides were washed once with PBS, and the bacteria were fixed with 4% paraformaldehyde in PBS (pH 7.4) and then labeled with adsorbed anti-YapV and/or anti-YapK antiserum (1:500), followed by anti-rabbit Alexa Fluor 480 (1,1000; Invitrogen, Lifetechnologies, Grand Island, NY). Images were captured with a Coolsnap digital camera (Photometrics, Tucson, AZ) mounted onto a Nikon Eclipse E600 microscope with Coolsnap version 1.2.0 software.

Adhesion of bacteria to cells. Human cell lines used in this study included the type II alveolar epithelial cell line A549 (ATCC CCL-185) and the epithelial-like cell line WI26 VA4 (ATCC CCL-95.1) from embryonic lung tissue, a simian virus 40 (SV40)-transformed derivative of WI-26 that was later recognized as a diploid fibroblast cell line. Adhesion assays were done as described previously, by determining CFU counts of bound bacteria (4). Binding of bacteria to cells was also examined after Giemsa staining by using bright-field microscopy. For the *Y. pestis* binding studies, the bacterial strains were grown overnight at 26°C in RPMI buffered to pH 7.2 with 0.1 M MOPS (3-morpholinopropanesulfonic acid). Adherence percentages were calculated as the number of cell-associated bacteria divided by the total number of inoculated bacteria × 100.

Binding to extracellular matrix molecules. To analyze the ability of YapV, YapK, and YapJ to interact with extracellular matrix (ECM) proteins, 96-well Immuno Maxisorb plates (Nunc; Thermo Fisher Scientific, Rochester, NY) were coated with 10 μg/ml BSA (control); collagen type I, II, III, IV, or VI; laminin; or fibronectin in PBS at 4°C overnight and then washed with PBS and blocked with PBS plus 3% BSA (Sigma) for 1 h. All of the ECM proteins were obtained from Sigma-Aldrich Corp. (St. Louis, MO), with the exception of rat collagen type I (Corning, NY), human fibronectin (BD Biosciences, San Jose, CA), and human laminin (Trevigen, Gaithersburg, MD). Binding of His-tagged YapV, YapK, and YapJ to ECM proteins was assayed as described previously (36). Briefly, 96-well plates coated with ECM proteins were incubated with the purified YapV, YapK, or YapJ protein for 2 h. After washing, the corresponding anti-YapV or anti-YapK antiserum (1:2,000), or both antibodies together for YapJ, was added, followed by wash cycles and incubation with goat anti-rabbit HRP-conjugated antibody (1:2,000). After several wash cycles, bound antibodies were detected by using the 1-Step Turbo TMB ELISA substrate (Thermo Fisher Scientific) followed by 2 M sulfuric acid and measuring the absorbance at 450 nm. Binding of bacteria to the ECM proteins on plates was studied by using YapV-, YapK-, or YapJ-expressing *E. coli* BL21(DE3). Induced cultures of YapV-, YapK-, or YapJ-expressing bacteria were washed with PBS, and equal amounts (10^7 CFU) were added to wells coated with ECM protein. *E. coli* harboring vector pMal-p2x was used as a control. After incubation for 2 h, unbound bacteria were removed, plates were washed once with PBS, and adherent bacteria were fixed with 4% paraformaldehyde for 20 min. Plates were washed with PBS again and stained with crystal violet for 20 min. After washing, the dye was solubilized with 80% ethanol plus 20% acetone, and the absorbance was measured at 570 nm.

Statistical analysis. Student's *t* test (two tailed) was used to calculate statistical significance for the binding assays.

RESULTS

Autotransporter protein paralogs in *Yersinia* spp. Based on the deciphered *Y. pestis* KIM genome (32), 10 different ORFs for predicted autotransporter proteins, designated YapA, YapC, YapE to YapH, and YapK to YapN, were originally described. Several of these proteins were investigated in more detail in the same strain (14) and in strain CO92 (16, 17, 24, 26, 37). An originally unrecognized ORF for a predicted autotransporter protein present in

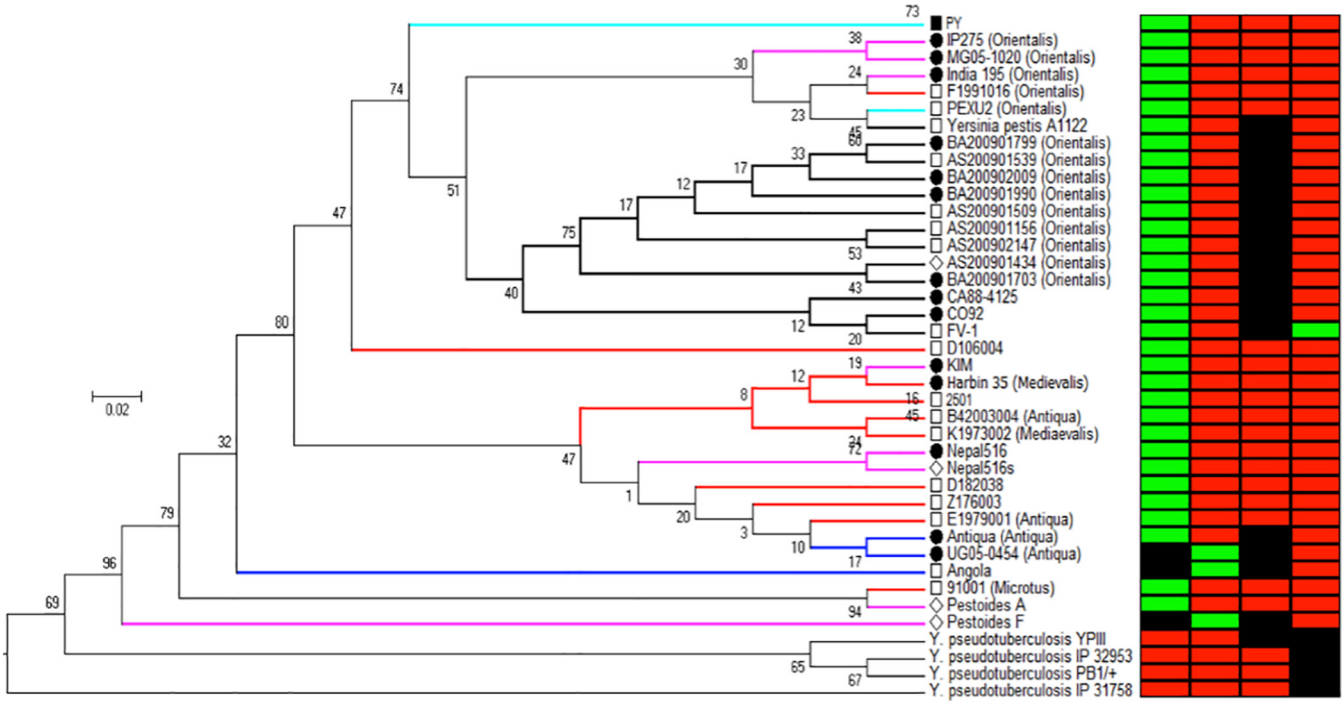

FIG 1 Coevolution of *Yersinia pestis* and *Y. pseudotuberculosis* YapX, YapK, YapV, and YapJ proteins. A *Yersinia* phylogenomic tree was constructed by using 35 highly conserved orthologous housekeeping genes. The scale indicates the number of substitutions per nucleotide, and numbers on the tree correspond to evolutionary distances. The branches of the tree are color-coded based on the known geographic origin of each *Y. pestis* isolate as follows: cyan, South America; pink, Asia (except China); red, China; blue, Africa; black, North America. The symbols at the ends of the tree branches indicate host origins as follows: solid circles, human; empty squares, rodents; empty diamonds, other mammals; black squares, mixture of human and rodent. Colored rectangles on the right represent genes with a complete ORF (red), pseudogenes (green), or absent genes (black).

KIM5 but absent in strain CO92 was later designated *yapV* (y3429) (27). YapV shares 86% amino acid identity with YapK (y0567). Comparisons of *Y. pestis* and *Y. pseudotuberculosis* genomes (38–42) revealed that most of the genomes carried a *yapV* gene as well as up to three paralogous genes or pseudogenes, designated *yapK*, *yapJ*, and *yapX* (see Fig. S2 and Table S2 in the supplemental material). Pairwise alignments of the four paralogous KIM proteins, using a patched ORF for the frameshifted gene (*yapX*), showed 79 to 86% amino acid identity, with 96 to 98% identity for the carboxy-terminal halves (~660 amino acids). These autotransporter proteins determined a phylogenetic group that was clearly separated from the other *Y. pestis* or *Y. pseudotuberculosis* Yap proteins (see Fig. S3 in the supplemental material). A phylogenetic tree based on *Yersinia* species housekeeping genes illustrated the sudden appearance of these paralogous genes/pseudogenes only in *Y. pseudotuberculosis*, considered ancestral to *Y. pestis* (Fig. 1; see also Table S2 in the supplemental material). All *Y. pestis* strains lacked or had a mutated *yapX* gene, highlighting a reductive evolutionary process that is typical for this species (39). Interestingly, all 13 North American strains of *Y. pestis* were biotype Orientalis strains and had lost *yapV*, even though the biotype Orientalis isolates from Asia and South America still carried *yapV* (43, 44). The three African isolates also lacked *yapV*. With the exception of strain FV-1, all *Y. pestis* strains carried the *yapJ* gene, in contrast to all the *Y. pseudotuberculosis* strains, which did not carry this gene. Taken together, specific reductive evolution patterns appeared to link geographic and phylogenic groups (Fig. 1; see also Table S2 in the supplemental material).

***Y. pestis* KIM5 recombinant YapV and YapK proteins.** Consistent with data from previous transcription studies of *yap* genes in *Y. pestis* grown *in vitro* (14, 26), we failed to detect YapV or YapK proteins in *Y. pestis* KIM5 and KIM6 by Western blot analysis under various *in vitro* growth conditions, including the use of BHI and heart infusion broths, at 26°C or 37°C (data not shown). Recent studies with a recombinant YapV protein showed that this protein is an autotransporter protein by localizing in the outer membrane and exposing part of itself on the bacterial surface (27). Earlier studies with the orthologous YapK and YapJ autotransporter proteins of strain CO92 used murine models of plague that highlighted their additive effect on increasing bacterial dissemination (26, 37). However, the mechanism responsible for the latter phenotype was not investigated further. Some earlier work on autotransporter proteins of *Y. pestis* had suggested that YapK might have adhesive properties based on a weak hemagglutinating reaction (14), and recent data highlighted the interaction of YapV with a host protein, albeit unexpectedly a cytoplasmic protein, neuronal Wiskott-Aldrich syndrome protein (N-WASP) (27). Since *Y. pestis* enters the respiratory tract to cause primary pneumonic plague, the *yapV* and *yapK* genes were cloned to study the adhesive properties of their products toward relevant cells and molecules encountered by *Y. pestis* when it invades lung tissue. Recombinant proteins of ~130 kDa were expressed by *E. coli*, in agreement with the calculated molecular masses of both proteins (Fig. 2A). YapV and YapK were shown by fluorescence microscopy to expose their passenger domain on the bacterial surface (Fig. 3). Some of the bacteria expressing a YapV construct that has

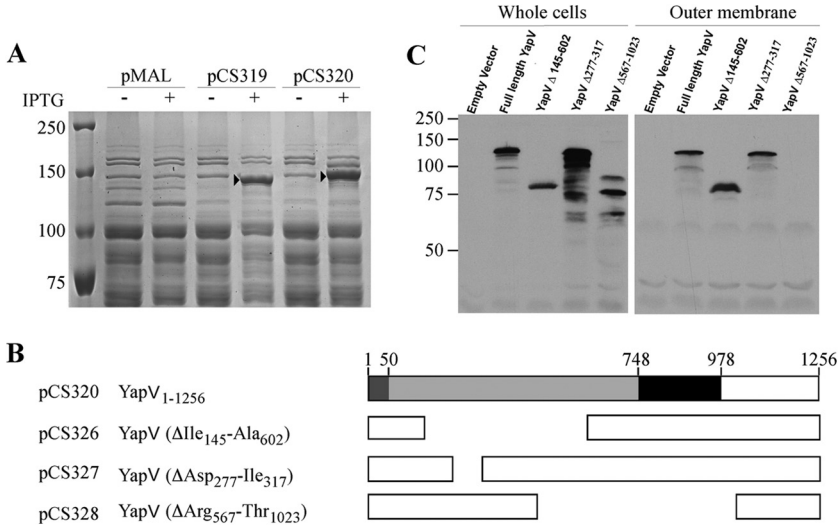

FIG 2 Characterization of YapK and YapV. (A) Expression of YapK and YapV in *Escherichia coli*. Cells of *E. coli* SE5000 transformed with pMal-p2X (empty vector), pCS319 (containing the *yapK* gene [y0567]), or pCS320 (containing the *yapV* gene [y3429]) were grown at 37°C, and protein expression was induced with IPTG. Whole cells were solubilized and analyzed by SDS-PAGE and Coomassie blue staining. Solid arrowheads indicate monomeric forms of YapK and YapV. The positions of molecular mass markers (in kDa) are indicated on the left. (B) DNA segments of the *yapV* gene included in each construct. The full-length *yapV* gene can be divided into DNA segments predicted to encode a typically long signal sequence (gray), a surface-exposed passenger domain (light gray), a linker domain (black), and the outer membrane-embedded β-barrel domain (white). The numbers correspond to the amino acid residues that flank the evaluated YapV regions. (C) Western blot analysis of whole cells and outer membrane fractions of *E. coli* SE5000 expressing full-length or truncated forms (in-frame deletions) of YapV detected with anti-YapV antiserum.

a portion of its C-terminal β-barrel domain deleted, YapV(Δ567–1023), were labeled in a punctate way (Fig. 3), despite the fact that it had been removed from the outer membrane fraction prepared with Sarkosyl (Fig. 2B and C). It is likely that the latter detergent removed YapV(Δ567–1023) from the outer membrane because the protein's insertion into the outer membrane was unstable but still partially detectable by fluorescence microscopy. In contrast, two constructs lacking only portions of the YapV passenger domain were clearly detected in the bacterial outer membrane fractions (Fig. 2B and C). Taken together, these results confirmed that the cloned *yapV* and *yapK* gene constructs were expressed in *E. coli* and, in agreement with data from previous studies, demonstrated attributes of autotransporter proteins (14, 26, 27, 37).

YapV- and YapK-expressing bacteria bind to human respiratory tract epithelial cells. Although many autotransporter genes of *Y. pestis* CO92 were transcribed only at very low levels in murine models of plague (26), some were shown to be significantly more expressed, including *yapK* and *yapJ*, with both of their products improving bacterial dissemination in murine models of plague (15, 16, 26, 37). The virulence properties that were responsible for this phenotype remained unknown. Since many autotransporter proteins have adhesive functions, we investigated whether YapV and YapK could mediate adhesion of bacteria to respiratory tract epithelial cells. The binding of *E. coli* SE5000 carrying pCS320 (YapV) or pCS319 (YapK) was studied with the type II alveolar epithelial cell line A549 and the lung epithelial-like cell line WI26 VA4. *E. coli* cells expressing recombinant YapV or YapK bound well to A549 cells, whereas *E. coli* cells carrying the empty vector did not bind significantly, as visualized by microscopy (Fig. 4A to C). Quantitative analysis of adherence of bacteria to A549 cells by colony counts determined that YapV- and YapK-expressing *E. coli* cells bound 7 and 8 times better, respectively, than did the *E. coli* control strain (Table 2). Comparable results were observed for WI26 VA4 cells. None of the *E. coli* strains expressing any of the three in-frame deletion constructs in YapV bound to A549 cells, indicating the importance of the full-length protein as a requirement for efficient binding (data not shown). When *Y. pestis* was grown *in vitro* at 37°C, YapK and YapJ could not be detected by Western blot analysis, even though *yapK* and *yapJ* had higher levels of transcripts at this temperature than at 26°C (26), leaving it possible that some proteins are expressed at levels below the level of detection. Since recombinant YapV and YapK were shown to have adhesive properties, we investigated whether *Y. pestis yapV* and *yapK* mutants would show reduced adhesive properties. Previous work done by using *Y. pestis* strain KIM6 demonstrated that the removal of the surface structures Psa, F1, and Pla (pPCP1$^-$ strain) improved the detection of other surface proteins with adhesive properties (4) and that the absence of the type III secretion system in KIM6 prevented eukaryotic cell death (12). Thus, to optimize the binding assays, the *yapV* and *yapK* mutations were engineered in the KIM6 pPCP1$^-$ *caf psa* strain, and the adhesive properties of the mutants were studied with A549 cells. The lack of both the *yapV* and *yapK* genes caused a significant decrease in the adherence of *Y. pestis* to A549 cells (Fig. 4D). The binding of the *yapV* single mutant to A549 cells was diminished significantly compared with that of the nonmutated strain. The *yapK* mutant also showed reduced, albeit not statistically significant, adhesion, suggesting a lower binding affinity or expression level of YapK than of YapV. When complemented in *trans* with *yapV*-containing plasmid pCS320, the *Y. pestis yapV yapK* double mutant regained the adhesive properties of the *yapK* mutant strain. These results indicated that YapV, and possibly YapK, might participate in primary pneumonic plague for the initiation of contact of bacteria with respiratory tract epithelial cells.

Binding of YapV-, YapK-, and YapJ-expressing bacteria or passenger domains to ECM proteins. Since *Y. pestis* expresses

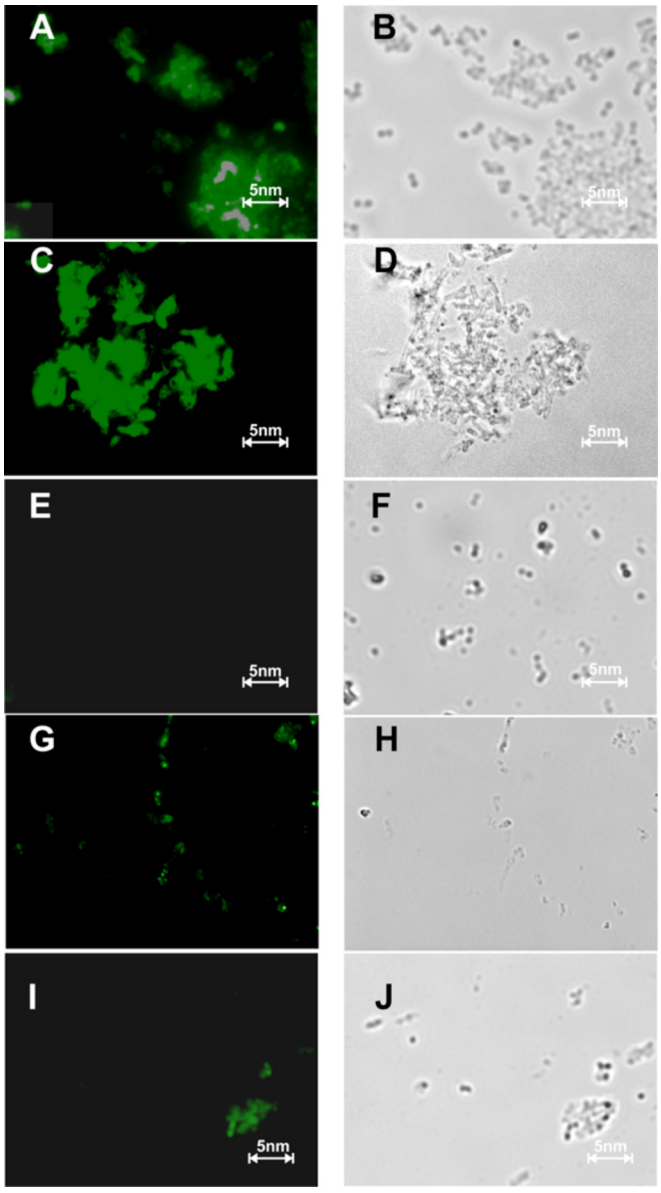

FIG 3 Visualization of Yap proteins on bacterial surfaces. (A and B) YapV; (C and D) YapK; (E and F) negative control; (G and H) YapVΔArg$_{517}$-Thr$_{1023}$; (I and J) YapJ expressed from *E. coli* BL21(DE3) transformed with pCS320, pCS319, pMal-p2x, pCS328, or p1741. Phase-contrast microscopy (right) and fluorescence microscopy (left) were used to detect bacteria labeled with anti-YapV (for YapV proteins), anti-YapK (for YapK), or anti-YapV and anti-YapK sera (for YapJ or the negative empty vector control), followed by Alexa Fluor 488-conjugated anti-rabbit IgG.

many virulence factors that allow it to remain and spread extracellularly (4, 45), and having shown that both YapV and YapK mediate binding of bacteria to cells, we further explored their adhesive properties toward immobilized ECM proteins. We complemented these studies by including binding assays with YapJ. Although *yapJ* was originally described as a pseudogene in *Y. pestis* KIM (32) (also designated KIM-10 [46]), it was later recognized as a complete ORF in KIM D27 (33), which we further confirmed with strain KIM5 (KIM5 and KIM D27 are both *pgm* deletion mutants derived from wild-type isolate KIM-10) (46). An engineered *yapJ* plasmid directed the expression of YapJ on bacterial surfaces, as visualized by fluorescence microscopy using passenger domain antibodies (Fig. 3E). Purified His-tagged passenger domains from YapV, YapK, and YapJ showed comparable binding preferences, interacting significantly better with type I, II, and III collagens; fibronectin; and laminin than with BSA and type IV or type VI collagens (Fig. 5A). The His-tagged Psa protein with anti-Yap antibodies or the antibodies without Yap proteins served as negative controls to confirm the binding specificities of the three investigated Yap proteins. Whole-cell lysates of recombinant *E. coli* cells expressing the Yap proteins revealed different levels of expression, as indicated by Western blot densitometry, with YapK and YapJ being detected at 30% and 15% of the levels of YapV, respectively (data not shown). Accordingly, YapV showed the highest level of bacterial adhesion (Fig. 5B). Although bacterial adhesion profiles for the various ECM proteins corresponded mostly to the binding of the corresponding His-tagged proteins, there was a discrepancy for YapV binding to type IV collagen and laminin, suggesting that the folding of the isolated His-tagged passenger domain did not match the exact conformation of the biologically more relevant bacterium-associated YapV protein. Since the ECM proteins were not all sourced from humans or the same animal species, host effects were evaluated by comparing the adhesions of the three Yap proteins to the same ECM proteins of human or animal origin (Fig. 6). Several host-specific binding properties were detected. The YapV- or YapJ-expressing bacteria bound significantly better to rat than to human collagen type I ($P < 0.05$), whereas the YapK-expressing bacteria bound better to human variants of collagen type I and type III than to rat collagen type I ($P < 0.001$) and bovine collagen type III ($P < 0.01$), respectively. In contrast, both the YapV- and YapK-expressing bacteria bound better to bovine than to human fibronectin ($P < 0.001$), whereas YapV-expressing bacteria bound better to human than to murine laminin ($P < 0.01$). All three Yap proteins demonstrated additional host-specific binding trends, as illustrated in Fig. 6. Moreover, for the human ECM proteins, collagen type I and type III acted as the best receptors for all Yap proteins, whereas the Yap proteins bound least to fibronectin. Taken together, these experiments indicated that YapV, YapK, and YapJ harbor interacting surfaces capable of recognizing a variety of ECM molecules and that these adhesive interactions occur in a host-specific manner.

DISCUSSION

This study examined the function of the three paralogous YapV, YapK, and YapJ proteins from *Y. pestis*. Most *Y. pestis* strains, including KIM, carry the genes for all three proteins; however, a few strains carry only the *yapK* and *yapJ* genes, such as strain CO92, or only one of these genes, with the other remaining as a pseudogene. A fourth paralogous gene, designated *yapX*, found in *Y. pseudotuberculosis* strains, is either present as a pseudogene or absent in all *Y. pestis* strains. No other *Yersinia* species carry DNA for these genes. YapK and YapJ of strain CO92 (26) and YapV of strain KIM5 were previously expressed in *E. coli* as recombinant proteins that were detected on the bacterial surface under *in vitro* growth conditions. We prepared constructs for the three corresponding autotransporter proteins from strain KIM and controlled for surface exposure of their passenger domains to evaluate their adhesive properties toward relevant cells and receptors predicted to be encountered during *Y. pestis* infection. *E. coli* expressing recombinant YapV or YapK was found to bind significantly to

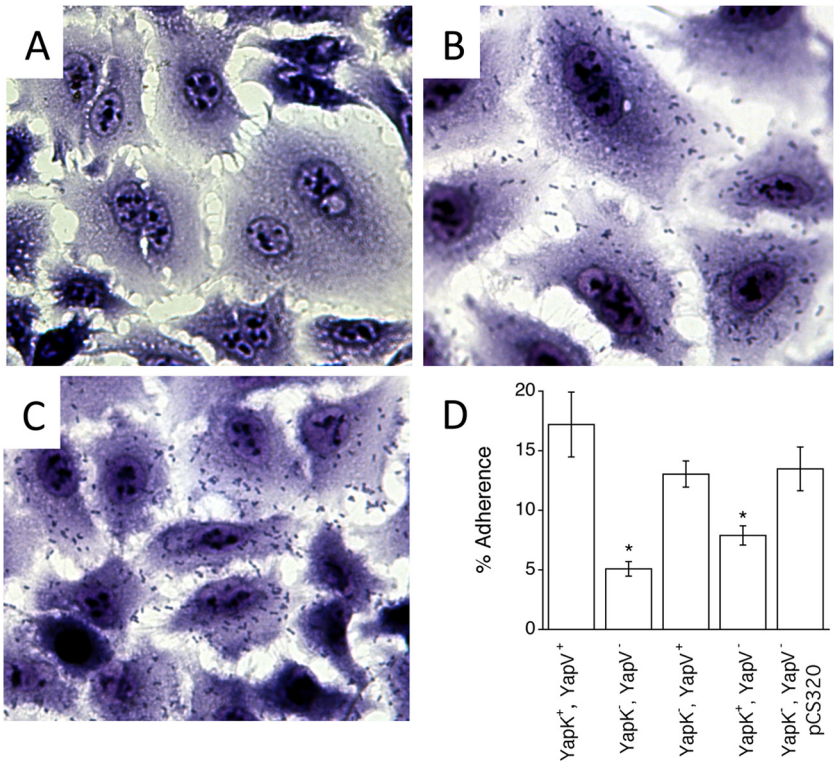

FIG 4 Yap protein-mediated binding of bacteria to A549 human type II alveolar epithelial cells. (A to C) Light microscopy of Giemsa-stained A549 cells and *E. coli* SE5000 transformed with the empty vector pMal-p2X (A), YapK-expressing plasmid pCS319 (B), or YapV-expressing plasmid pCS320 (C). Bacteria were grown at 37°C, and protein expression was induced by IPTG. Incubation of A549 cells with bacteria was done at a multiplicity of infection of 50. (D) Adherence of *Y. pestis* mutants to A549 cells. Cells of *Y. pestis* strains DSY50 ($YapK^+$ $YapV^+$), DSY52 ($YapK^-$ $YapV^-$), DSY51 ($YapK^-$ $YapV^+$), DSY53 ($YapK^+$ $YapV^-$), and DSY52 transformed with pCS320 were incubated at a multiplicity of infection of 10 for binding to A549 cells grown in 24-well plates. Percentages of cell-associated bacteria were determined by CFU counts. Data represent the means ± standard errors from three independent experiments done in duplicate. *, $P < 0.05$ compared to DSY50 binding.

alveolar A549 epithelial and WI26 VA4 epithelial-like cells. The *Y. pestis yapV yapK* double mutant bound significantly less to A549 cells than did the parental nonmutated strain, and the phenotype was significantly complemented with a plasmid expressing YapV. Interestingly, reduced binding was detected despite the presence of Ail in the mutants, which probably explains some of the background adhesion of the double *yap* mutant. Binding differences of the mutants also attested to the expression of the proteins, despite the proteins being undetectable by Western blotting. This interpretation is consistent with the previously reported detection of autotransporter transcripts from *in vitro*-grown *Y. pestis* CO92, with *yapK* and *yapJ* being among the three *yap* genes that were significantly more expressed than the other ones (26). Since *Y. pestis* is an invasive pathogen that uses several antiphagocytic mechanisms to remain extracellular and uses its Pla protease to disseminate through intercellular compartments, where it encounters ECM proteins, we investigated whether the paralogous Yap proteins of *Y. pestis* KIM interact with these host proteins. We demonstrated that not only YapV but also YapK and YapJ acted as adhesins recognizing specific ECM proteins. Most interestingly, the adhesive interactions demonstrated host-specific affinities. YapV was previously reported to bind to N-WASP (27); however, the biological relevance of this property remains unclear considering the expected separate compartmentalization of YapV and N-WASP. Even though *Y. pestis* can be detected in macrophages, particularly early during infection (47, 48), *Y. pestis* is not known to reach the host cytosol, where N-WASP is located. In addition, whether the passenger domain of an autotransporter protein can be translocated or injected into the host cytosol remains to be demonstrated. In contrast, the adhesive properties of the three Yap proteins described here provide a reasonable explanation for the reduced dissemination of the corresponding *Y. pestis* mutants in murine models of plague (37). It remains to be examined whether these paralogous adhesins and the previously described *Y. pestis* adhesins are each temporally and spatially regulated in a particular sequential manner so as to modulate the attachment and spreading properties of this pathogen in different hosts and host tissues (49–51).

Comparative genomics has led to the consideration of *Y. pestis*

TABLE 2 Binding of recombinant *E. coli* strains expressing YapK or YapV to human respiratory tract cells

Strain	Mean % adherence to cell line ± SD	
	A549	WI26 VA4
SE5000 empty vector	1.34 ± 0.10	1.38 ± 0.06
SE5000 $YapK^+$	10.86 ± 0.85[a]	7.99 ± 1.20[a]
SE5000 $YapV^+$	9.29 ± 1.76[a]	10.17 ± 0.68[a]

[a] Statistically significant difference from the *E. coli* SE5000 empty vector control ($P < 0.05$).

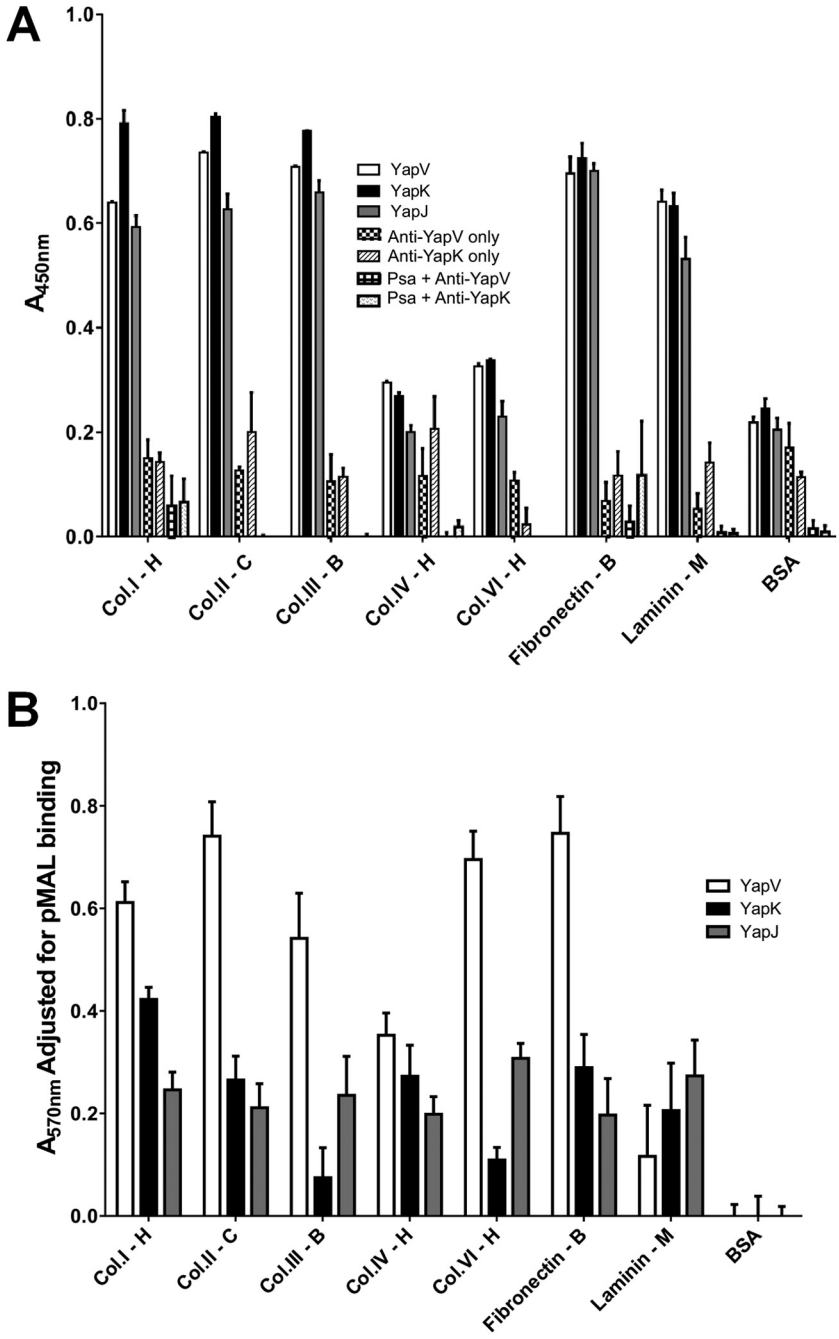

FIG 5 Binding of YapV, YapK, and YapJ to ECM proteins. Plastic wells were coated with human collagen type I, chicken collagen type II, bovine collagen type III, human collagen type IV, human collagen type VI, murine laminin, bovine fibronectin, and BSA. (A) Purified His-tagged passenger domains of YapV, YapK, and YapJ were added to wells coated with ECM proteins and detected with polyclonal anti-YapV and/or anti-YapK serum, HRP-conjugated secondary antibody, and a chromogenic substrate. Negative controls included anti-YapV or anti-YapK antibody used without any Yap proteins and the two antibodies used with a His-tagged PsaA protein. (B) Binding of YapV-, YapK-, or YapJ-expressing *E. coli* SE5000 cells to wells coated with ECM proteins. Bound bacteria were stained with crystal violet, and the absorbance was measured at 570 nm. Data represent the means ± standard errors from ≥3 independent experiments.

as an evolutionary descendant of an ancestral *Y. pseudotuberculosis* strain. Reductive evolution as well as the acquisition of two plasmids, each with a major virulence factor (Pla and F1), have played an integral part in the conversion to a more lethal pathogen with different transmission mechanisms, a narrow host range, and immune evasion factors that bypass innate immune defenses (38, 42, 52). Massive evolutionary gene loss and pseudogenization have been linked to the generation of specialized life-styles in other pathogens, such as *Shigella*, *Salmonella enterica* serovar Typhi, and *Mycobacterium leprae* (53–55). Evolving to a new life cycle could explain how many *Y. pestis* genes, such as the *inv* and *yad* genes, became pseudogenes by neutral genetic drift (55). Alternatively, the *rscA* biofilm repressor gene of *Y. pseudotuberculosis* mutated in *Y. pestis* is a typical example of selection by adaptive pseudogeni-

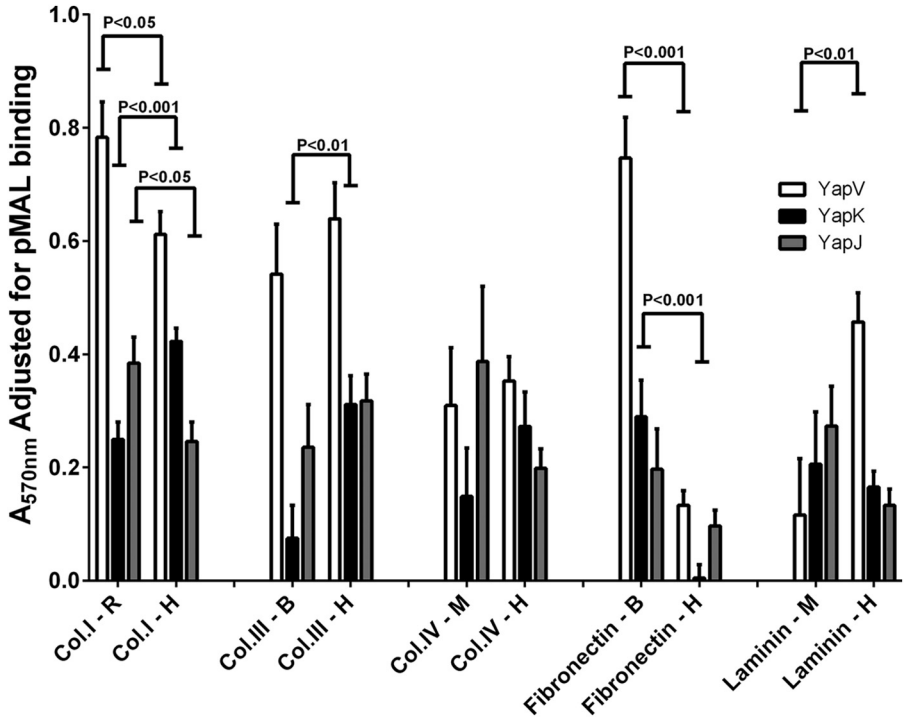

FIG 6 Comparisons of binding of YapV-, YapK-, and YapJ-expressing *E. coli* SE5000 cells to plastic wells coated with rat (R), human (H), bovine (B), or mouse (M) ECM proteins. The assays were done as described in the legend of Fig. 5B.

zation, as biofilm formation in fleas benefits bacterial transmission (56, 57). The pseudogenization or absence of *yapX* in full *Y. pestis* genomes confirms the pattern of reductive loss in this bacterium compared to *Y. pseudotuberculosis*. However, additional evolutionary processes must have directed the presence or absence of the four paralogous autotransporter genes in the 40 *Yersinia* genomes examined. From an evolutionary standpoint, the earliest presence of these genes was detected in *Y. pseudotuberculosis*. It is likely that *yapV* was acquired by horizontal gene transfer (HGT), considering that phage DNA is present downstream of this gene. The origins of *yapK* and *yapX* might be the same, or these genes might have evolved from a duplication(s) of *yapV* or from an ancestral gene of *yapE* (see Fig. S3 in the supplemental material). Alternatively, a *Yersinia frederiksenii* gene with a similar carboxy-terminal third (ATCC 33641; GenBank accession number ZP_04633941) might have served as an ancestral gene for gene duplication, and genomic rearrangements and drift would have resulted in the creation of *yapK* and *yapJ*. The strain-specific pallet of *yap* genes might illustrate the definitions of inparalogs (i.e., a lineage-specific duplication of *yapX* and *yapJ*) (see Fig. S2 in the supplemental material), outparalogs (i.e., a duplication preceding speciation for *yapK* and *yapV*), or pseudoparalogs (i.e., HGT acquisition for *yapJ*) (58). The reductive loss of similar genes suggests either redundant functions or the removal of genes that no longer serve *Yersinia pestis* maintenance in its hosts or environments (39). The acquisition of a new gene, *yapJ* (Fig. 1), and the maintenance of at least one of the four paralogous *yap* genes in any *Y. pestis* genome suggest a beneficial role for such genes for the perpetuation of *Y. pestis*.

Most intriguing is the significant relationship between the profile of the four paralogous genes/pseudogenes in *Y. pestis* strains and the geographic location of their isolation. Since the reservoirs of host species vary geographically, this suggests an evolutionary adaption of *Y. pestis* to a specific local host(s). YapV and its paralogous proteins could play a role in the maintenance of a reservoir, thus benefiting the life cycle of the pathogen. Whether the panoply of paralogous *yap* genes in the different *Y. pestis* strains represents effective functional adaption or only random gene losses geographically grouped because of time-dependent evolutionary steps remains to be investigated. Finally, the observed association between geographic origin and strain signatures for the *yapK*, *yapV*, *yapX*, and *yapJ* genes or pseudogenes could be useful to track strain origins and thus serve diagnostic and epidemiological purposes.

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