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Role of *Salmonella* Pathogenicity Island 1 Protein IacP in *Salmonella enterica* Serovar Typhimurium Pathogenesis▽

Jin Seok Kim,[1]§ Jeong Seon Eom,[1]§ Jung Im Jang,[1] Hyeon Guk Kim,[1]† Doo Won Seo,[1]‡ Iel-Soo Bang,[2] Seong Ho Bang,[3] In Soo Lee,[4] and Yong Keun Park[1]*

School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, South Korea[1]; Department of Microbiology and Immunology, Chosun University School of Dentistry, Gwang ju 501-759, South Korea[2]; Department of Biological Science, Hanseo University, Chungcheongnam-do 356-706, South Korea[3]; and Department of Biotechnology, Hannam University, DaeJeon 300-791, South Korea[4]

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Gram-negative bacteria, including *Salmonella enterica* serovar Typhimurium, exploit type III secretion systems (T3SSs) through which virulence proteins are delivered into the host cytosol to reinforce invasive and replicative niches in their host. Although many secreted effector proteins and membrane-bound structural proteins in the T3SS have been characterized, the functions of many cytoplasmic proteins still remain unknown. In this study, we found that IacP, encoded by *Salmonella* pathogenicity island 1, was important for nonphagocytic cell invasion and bacterial virulence. When the *iacP* gene was deleted from several *Salmonella* serovar Typhimurium strains, the invasion into INT-407 epithelial cells was significantly decreased compared to that of their parental strains, and retarded rearrangements of actin fibers were observed for the *iacP* mutant-infected cells. Although IacP had no effect on the secretion of type III translocon proteins, the levels of secretion of the effector proteins SopB, SopA, and SopD into the culture medium were decreased in the *iacP* mutant. In a mouse infection model, mice infected with the *iacP* mutant exhibited alleviated pathological signs in the intestine and survived longer than did wild-type-infected mice. Taken together, IacP plays a key role in *Salmonella* virulence by regulating the translocation of T3SS effector proteins.

The injection of bacterial proteins by the type III secretion system (T3SS) into the host cytoplasm has been broadly applied to study pathogen-host interactions ranging from the invasion of plant and animal pathogens to a symbiont interaction of *Rhizobium* (22, 42). The T3SS is composed of more than 20 different structural proteins that form needle-like appendages through which effector proteins are delivered directly into host cells to manipulate various host cell signaling events. Moreover, cytoplasmic chaperones are involved in the stability and efficient translocation of effector proteins (14). *Salmonella enterica* serovar Typhimurium, a facultative intracellular pathogen, has evolved two distinct T3SSs encoded by *Salmonella* pathogenicity island 1 (SPI-1), responsible for the invasion of nonphagocytic cells, and by SPI-2, required for intracellular survival and replication inside the *Salmonella*-containing vacuole (SCV). The expressions of the two T3SSs are inversely regulated during the pathogenic process. Although the expression of the SPI-1 T3SS at systemic sites has remained controversial, some effector proteins of SPI-1 (e.g., SipA and SopB) are persistently expressed and secreted under favorable conditions for SPI-2 expression during the biogenesis and maturation of the SCV (17).

After the SPI-1 T3SS is activated upon host cell contact, the translocators SipB and SipC appear to be inserted into the host cell membrane, where they form a translocation pore, which is connected to the needle complex. A variety of effector proteins encoded within and outside SPI-1 can be translocated into a host cytoplasm and cooperatively induce membrane ruffling (11) and macropinocytosis (16). Among SPI-1 effector proteins, SopE, SopE2, and SopB trigger the actin rearrangement in host cells by activating small GTPases, including Rac1, Cdc42, and RhoG, directly or indirectly (39). A *Salmonella* serovar Typhimurium mutant carrying null mutations in these effector proteins failed to invade epithelial cells. After bacterial invasion, an activated membrane was subsequently recovered by SptP, another effector protein possessing GTPase-activating protein activity (13).

The *iacP* gene, which is located downstream of *sicA-sipBCDA* in the SPI-1 locus, was initially identified as a putative acyl carrier protein (ACP) by sequence similarity (26). ACP is an abundant small acidic and highly conserved protein that is essential for various biosynthetic pathways (5). In the process of fatty acid (FA) biosynthesis in *Escherichia coli*, ACP sequentially delivers the acyl intermediates for FA elongation as a cofactor of FA synthase (20). For the enzymatic activity of ACP, a prosthetic group 4′-phosphopantetheine (4′-PP) that was covalently incorporated into apo-ACP serves as the binding site of acyl groups. It was reported previously that the substitution of serine 36 in *Escherichia coli* ACP eliminated the attachment site of the 4′-PP and inhibited FA incorporation (27).

In addition to lipid biosynthesis, acyl-ACP is required for various bacterial virulence processes: the synthesis of the lipid

* Corresponding author. Mailing address: School of Life Sciences and Biotechnology, Korea University, Seoul 136-701, South Korea. Phone: 82 2 3290 3422. Fax: 82 2 3290 3922. E-mail: ykpark@korea.ac.kr.

§ J.S.K. and J.S.E. contributed equally to this work.

† Present address: National Center for Lot Release, Korea Food & Drug Administration, Seoul 122-704, South Korea.

‡ Present address: Biopharmaceuticals and Herbal Medicine Evaluation Department, Korea Food & Drug Administration, Seoul 122-704, South Korea.

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A moiety of lipopolysaccharide (LPS) (43) and the *N*-acylhomoserine lactones as signal molecules in quorum sensing (52) and the posttranslational modification of bacterial toxins such as *E. coli* hemolysin (HlyA) (24). The activation of HlyA requires posttranslational acylation at two internal lysine residues by ACP and the acyl transferase HlyC. The conformation of acylated HlyA is matured into a molten globular form comprised of disordered regions, which is necessary for the hemolytic effects of a toxin to occur (21).

As a *Salmonella* serovar Typhimurium mutant that lacks an entire SPI-1 locus was found to grow as well as the wild type, it is predicted that IacP would be responsible for the modification of other proteins in the T3SS (26). However, it is not known which proteins are targeted by IacP or how the invasion process during SPI-1 activation is affected in the *iacP* mutant. In this study, we report that IacP promotes SopB, SopA, and SopD secretion during cell entry, thus contributing to the virulence of *Salmonella* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *Salmonella* serovar Typhimurium strains used in this study are listed in Table 1. Unless otherwise noted, *Salmonella* serovar Typhimurium bacteria were incubated at 37°C in Luria-Bertani (LB) medium with 0.3 M NaCl for SPI-1 activation. When necessary, L-arabinose was added to induce the expression of plasmid-borne genes, and the following antibiotics were added to the cultures: ampicillin (Ap) (100 μg/ml), chloramphenicol (Cm) (30 μg/ml), kanamycin (Km) (50 μg/ml), and streptomycin (Sm) (50 μg/ml).

Construction of *Salmonella* serovar Typhimurium strains. The disruption or epitope tagging of specific genes was conducted by using the red recombinase system (9, 51) with the appropriate primers listed in Table 2. Briefly, Cm^r cassettes of pKD3 and pSU314 flanked by an Flp recombination target (FRT) site were amplified with the following primers: *iacP* mut-L and *iacP* mut-R for YKJ034, tag*iacP* HA-L and tag*iacP* HA-R for YKJ074, *sopE* mut-L and *sopE* mut-R for YKJ204, and *sptP* HA-L and *sptP* HA-R for YKJ119. Km^r cassettes of pKD4 and pSUB11 were amplified with the following primers: *sopB* mut-L and *sopB* mut-R for YKJ029 and tag*sopB* 3FL-L and tag*sopB* 3FL-R for YKJ042. Purified PCR products were electroporated into *Salmonella* serovar Typhimurium bacteria possessing red recombinase (pKD46), and transformants were incubated at 37°C for 1 h and then plated onto LB plates containing the appropriate antibiotics. Insertions of the antibiotic resistance gene were verified by PCR and DNA sequencing. If necessary, the resistance gene cassette was excised by using pCP20 carrying the Flp recombinase to generate YKJ032, YKJ033, YKJ035, and YKJ036. YKJ052, YKJ205, YKJ156, YKJ099, YKJ098, and YKJ123 were generated by the P22-mediated transduction of M587, YKJ204, YKJ029, YKJ042, and YKJ119 into UK1, YKJ032, and YKJ035.

Construction of recombinant plasmids. All plasmids and primers used in this study are listed in Tables 1 and 2. The promoter region of the *sicA-sipBCDA-iacP* operon was amplified by PCR from the UK1 chromosome and cloned into plasmid pMW118 to generate pYKJ029. For native expression, the *iacP*, *sipC*, *sipD*, and *sipA* genes were amplified and subcloned into pYKJ029, generating pYKJ033, pYKJ290, pYKJ291, and pYKJ038, respectively. To generate pYKJ032, an approximately 1-kb upstream region of the *iacP* gene was amplified by PCR and then cloned into plasmid pMW118. To construct pYKJ065, the *sopE* gene and upstream promoter region were amplified from the SL1344 chromosome and cloned into pMW118. This strategy was applied to the construction of pYKJ305 (*sopB*) except that the UK1 chromosome was used as a PCR template. For the arabinose-inducible expression of *iacP* (pYKJ034) and *sopA* (pYKJ304), amplified genes were placed under the control of the pBAD promoter of the pBAD24 plasmid. Site-directed substitutions of serine at position 38 (pYKJ035) and glutamate at position 51 (pYKJ036) with alanine in IacP were generated by using the QuikChange mutagenesis kit (Agilent Technologies) with pYKJ033 as a PCR template. Plasmid constructs were verified by DNA sequencing analysis.

Preparation of membrane and secreted proteins of *Salmonella* serovar Typhimurium. For the activation of the T3SS-related genes, *Salmonella* serovar Typhimurium bacteria were grown under the growth conditions described above for 3 h. Membrane proteins of *Salmonella* serovar Typhimurium were prepared as previously described (29). Briefly, isolated whole membranes were placed onto a six-step sucrose gradient, centrifuged at 100,000 × *g* for 36 h, and then divided into 14 fractions. Fractions of the inner and outer membrane proteins were collected and precipitated with 10% (vol/vol) trichloroacetic acid (TCA). All steps were performed at 4°C. For the preparation of secreted proteins, the culture supernatant was passed through a 0.45-μm filter and then recovered by precipitation with 10% (vol/vol) TCA and acetone as described elsewhere previously (7). Equal amounts of sample proteins were subjected to SDS-PAGE and visualized by Western blotting.

TABLE 1. *Salmonella* strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
UK1	Wild type	38
SL1344	Wild type	54
LT2	Wild type	45
ATCC 14028s	Wild type	ATCC
CAS152	CS401 Δ*sspB* (*sipB*); Sm^r	50
YKJ013	UK1 Δ*sipB*	29
YKJ032	SL1344 Δ*iacP*	This study
YKJ033	LT2 Δ*iacP*	This study
YKJ034	UK1 *iacP*::Cm; Cm^r	This study
YKJ035	UK1 Δ*iacP*	This study
YKJ036	ATCC 14028s Δ*iacP*	This study
YKJ074	UK1 *iacP*::HA; Cm^r	This study
YKJ204	SL1344 *sopE*::Cm; Cm^r	This study
YKJ205	SL1344 Δ*iacP sopE*::Cm; Cm^r	This study
YKJ029	UK1 *sopB*::Km; Km^r	This study
YKJ099	UK1 Δ*iacP sopB*::Km; Km^r	This study
YKJ042	UK1 *sopB*::3×FLAG; Km^r	This study
YKJ098	UK1 Δ*iacP sopB*::3×FLAG; Km^r	This study
YKJ119	UK1 *sptP*::HA; Cm^r	This study
YKJ123	UK1 Δ*iacP sptP*::HA; Cm^r	This study
Plasmids		
pMW118	Low-copy-no. plasmid; Ap^r	Nippon Gene
pBAD24	Expression plasmid containing the arabinose-inducible promoter P_{BAD}; Ap^r	18
pYKJ029	pMW118 Psip; Ap^r	This study
pYKJ032	pMW118 Pint-*iacP*; Ap^r	This study
pYKJ033	pMW118 Psip-*iacP*::HA (p*iacP*); Ap^r	This study
pYKJ034	pBAD24 P_{BAD}-*iacP*::HA; Ap^r	This study
pYKJ035	pMW118 Psip-*iacP*(*S38A*)::HA; Ap^r	This study
pYKJ036	pMW118 Psip-*iacP*(*E51A*)::HA; Ap^r	This study
pYKJ290	pMW118 Psip-*sipC*::FLAG; Ap^r	This study
pYKJ291	pMW118 Psip-*sipD*::FLAG; Ap^r	This study
pYKJ038	pMW118 Psip-*sipA*::FLAG; Ap^r	This study
pYKJ304	pBAD24 P_{BAD}-*sopA*::FLAG; Ap^r	This study
pYKJ305	pMW118 P*sopD*::FLAG; Ap^r	This study
pYKJ065	pMW118 P*sopE*::HA; Ap^r	This study

Isolation and silver staining of *Salmonella* serovar Typhimurium LPS. LPS of *Salmonella* serovar Typhimurium cells grown under SPI-1-inducing conditions for 3 h was prepared by using an LPS extraction kit (Intron Biotechnology, South Korea) according to the manufacturer's protocols. Extracted LPS was routinely analyzed on 14% SDS-PAGE gels and subsequently visualized by LPS-modified silver staining as described previously (49).

Invasion assays. A gentamicin protection assay was conducted as described elsewhere previously (37). Briefly, the human embryonic intestinal epithelial cell line INT-407 (ATCC CCL-6) was seeded onto a 24-well plate and then infected with *Salmonella* serovar Typhimurium strains (multiplicity of infection [MOI] of 10) for 15 min at 37°C. After washing three times with fresh medium, cells were incubated in medium containing gentamicin (100 μg/ml) for 1 h to remove the extracellular bacteria. Cells were treated in 1% Triton X-100 for 15 min, after which intracellular bacteria were enumerated by serial dilution.

Translocation assay. The protein translocation into cultured INT-407 cells was determined as previously described, with slight modifications (6). Briefly, 4×10^6 INT-407 cells seeded into two 100-mm-diameter culture dishes were infected with *Salmonella* serovar Typhimurium (MOI of 10) for 15 min. The cells were

TABLE 2. Primer sequences used in this study

Primers	Sequence[a]	Description
Chromosome		
iacP mut-L	5′-AGTCAAAAAAGTGATCACCTCTTGTATTGCCGTTGATGTTGTGTAGGCTGGAGCTGCTTC	YKJ034
iacP mut-R	5′-ACACGGCATATATCCGCAAAGGTCATCATATCAGGAAGATCATATGAATATCCTCCTTAG	YKJ034
tag*iacP* HA-L	5′-TATATGCCGTGTTGTTAAAAAAAGTCTTGAGTCCAGGGTGTATCCGTATGATGTTCCTGA	YKJ074
tag*iacP* HA-R	5′-TTACTTTCCTCTTGAATTATATCTTTTATAAGATTGCTTCCATATGAATATCCTCCTTAG	YKJ074
sopE mut-L	5′-TCCAAAAACAGGAAACCACACTACTAAAGAAAAATCAACCGTGTAGGCTGGAGCTGCTTC	YKJ204
sopE mut-R	5′-TGTTTTGTATATATTTATTAGCAATGTTTTCTAGTATCAGCATATGAATATCCTCCTTAG	YKJ204
sopB mut-L	5′-CATTTTTTTAAAGTTCCTGGTGCATAAAAGTCACATCCTTGTGTAGGCTGGAGCTGCTTC	YKJ029
sopB mut-R	5′-GTTTTATCTTTACCGTCCTCATGCACACTCACCGTGGACACATATGAATATCCTCCTTAG	YKJ029
tag*sopB*3FL-L	5′-TTGGCAGTCAGTAAAAGGCATTTCTTCATTAATCACATCTGACTACAAAGACCATGACGG	YKJ042
tag*sopB*3FL-R	5′-ATCCTTGCTATTGACTTCCTTCATTTCCAGCATAGCTTACCATATGAATATCCTCCTTAG	YKJ042
sptP HA-L	5′-AAAAGCAATGCAAGCCCAGTTGCTTATGACGACGGCAAGCTATCCGTATGATGTTCCTGA	YKJ119
sptP HA-R	5′-AGAGAAAAACAAAAGACTTTCTATCGCGGCAAACAAATAACATATGAATATCCTCCTTAG	YKJ119
Plasmids		
Psip-L	5′-CCGGCATGCAGCATGGCTGACTACAGTTT	pYKJ029
Psip-R	5′-GTCGACTACTTACTCCTGTTATCTGTCACC	pYKJ029
com*iacP*-L	5′-CCGGTCGACATGAATATGGATATTGAAGCAA	pYKJ033
com*iacP* HA-R	5′-CCGGAGCTCTTAAAGAGCGTAATCTGGAACATCGTATGGGTACACCCTGGACTCAAGACTTTTT	pYKJ033
iacP pro-L	5′-CCGGCATGCGCGAGTCACACCATTCGACTA	pYKJ032
iacP S38A-L	5′-GAGGATCTTTACGCTGACGCCTTGGATTTAATTGATATTG	pYKJ035
iacP S38A-R	5′-CAATATCAATTAAATCCAAGGCGTCAGCGTAAAGATCCTC	pYKJ035
iacP E51A-L	5′-GTATTTGGTCTTAGTGAGGCCTTTGACATTAGTTGCAATG	pYKJ036
iacP E51A-R	5′-CATTGCAACTAATGTCAAAGGCCTCACTAAGACCAAATAC	pYKJ036
B*iacP*-L	5′-CCGGAATTCACCATGAATATGGATATTGAAGCAA	pYKJ034
B*iacP*-R	5′-CCGGCATGCTTAAAGAGCGTAATCTGGAACATCGTATGGGTACACCCTGGACTCAAGACTTTTT	pYKJ034
sopE-L	5′-CCGGCATGCCAATGCCAGAACGGCAAG	pYKJ065
sopE HA-R	5′-CCGGAGCTCTTAAAGAGCGTAATCTGGAACATCGTATGGGTAGGGAGTGTTTTGTATATATTTA TTAGC	pYKJ065
BsopA-L	5′-CCGGAATTCACCATGAAGATATCATCAGGCGCAA	pYKJ304
BsopA FL-R	5′-CCGGCATGCCTACTTATCGTCGTCATCCTTGTAATCCGCCCAGGCCAGTGGCAGGAT	pYKJ304
sopD-L	5′-CCGGCATGCTTATAGTCACCACAAAGGATTACCAAC	pYKJ305
sopD FL-R	5′-CCGGAGCTCCTACTTATCGTCGTCATCCTTGTAATCTGTCAGTACTATATTACGACTGCA	pYKJ305
sipC FL-L	5′-CCGGTCGACATGTTAATTAGTAATGTGGGAA	pYKJ290
sipC FL-R	5′-CCGGAGCTCTTACTTATCGTCGTCATCCTTGTAATCAGCGCGAATATTGCCTGCGA	pYKJ290
sipD FL-L	5′-CCGGTCGACATGCTTAATATTCAAAATTATTCCGCTT	pYKJ291
sipD FL-R	5′-CCGGAGCTCTTACTTATCGTCGTCATCCTTGTAATCTCCTTGCAGGAAGCTTTT	pYKJ291
sipA FL-L	5′-CCGGTCGACATGGTTACAAGTGTAAGGACTCAGC	pYKJ038
sipA FL-R	5′-CCGGAGCTCTTACTTATCGTCGTCATCCTTGTAATCACGCTGCATGTGCAAGCCA	pYKJ038

[a] For chromosome primers, FRT sequences are underlined. For plasmid primers, underlined sequences represent restriction enzyme recognition sites.

then further incubated in Dulbecco's modified Eagle's medium (DMEM) containing gentamicin (100 μg/ml) for 1 h, followed by three washes with phosphate-buffered saline (PBS). Next, 75 μl of PBS and 1% Triton X-100 (translocated protein) were added to one culture dish, while 75 μl of PBS and 1% SDS (internalized bacterial protein) were added to another culture dish. Following incubation at room temperature (RT) for 20 min, the cells were collected by using a cell scraper and centrifuged at 15,000 × *g* for 10 min to remove the cellular debris. The lysates were then loaded onto a 12% SDS-PAGE gel, and the translocated protein was detected by Western blotting.

Subcellular fraction and total proteins in infected INT-407 cells. INT-407 cells were seeded into two 100-mm-diameter culture dishes (2×10^6 cells/dish) and then infected with bacteria at an MOI of 1:10 for 15 min. Next, the cells were washed with fresh DMEM and further incubated with DMEM supplemented with gentamicin (100 μg/ml) for 1 h. Following washes with PBS, membrane and nonmembrane fractions were prepared by using a ProteoExtract native membrane protein extraction kit (EMD) according to the manufacturer's instructions. To detect the intracellular level of phosphorylated Akt (phospho-Akt) in the infected cells, cells on the dishes were collected by using a scraper, lysed by brief sonication in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 0.25% deoxycholate, 1 mM EDTA) containing a protease inhibitor cocktail, and cleared by centrifugation. The total protein concentrations in the isolated fractions were determined and normalized via a Bradford protein assay (Bio-Rad).

Western blotting and antibodies. A nitrocellulose membrane was blocked with 5% (wt/vol) skim milk in TBS-T (20 mM Tris, 0.2 M NaCl [pH 7.5], 0.1% Tween 20) for 1 h and then incubated with one of the following primary antibodies at the proper dilution for 1 h: a monoclonal anti-hemagglutinin (HA) antibody (Cell Signaling), a polyclonal anti-SipB antibody (28), a polyclonal anti-OmpW antiserum (a gift from H. Y. Kang, Pusan University, South Korea), a monoclonal anti-DnaK antibody (Assay Designs), a polyclonal anti-LPS antibody (BD), a monoclonal anti-FLAG antibody (Sigma-Aldrich), a monoclonal anti-β-tubulin antibody (Sigma-Aldrich), a monoclonal anti-caveolin-1 antibody (BD), a monoclonal anti-phospho-Akt antibody (Cell Signaling), and a polyclonal anti-Akt antibody (Sigma-Aldrich). After washing three times, the second antibody of goat anti-mouse IgG horseradish peroxidase (HRP) conjugate or goat anti-rabbit IgG HRP conjugate (Bio-Rad) was added, and the membranes were incubated for 1 h. The blots were then developed by using a BM chemiluminescence blotting substrate (POD) (Roche).

Immunofluorescence study. A study of the invasion of *Salmonella* serovar Typhimurium bacteria into epithelial INT-407 cells was performed as described above, with some modifications. Briefly, *Salmonella* serovar Typhimurium bacteria were incubated with Hoechst 33342 dye (Invitrogen) for 20 min prior to infection. Monolayers of INT-407 cells (1×10^4 cells/well) grown on glass coverslips in 24-well plates were infected with *Salmonella* serovar Typhimurium at an MOI of 1:10 for 15 min. After further incubation with medium containing gentamicin, the samples on the coverslips were fixed with 3.7% paraformaldehyde in PBS for 20 min, permeabilized with 0.1% (vol/vol) Triton X-100 in PBS for 10 min, and then blocked in 3% (wt/vol) bovine serum albumin in PBS for 1 h. To visualize phosphorylated Akt, anti-phospho-Akt (Ser473) and subsequent Alexa Fluor 488-conjugated goat anti-rabbit antibodies (Invitrogen) were used for 1 h of incubation at RT. Following mounting, the specimens were analyzed with a confocal laser scanning microscope (LSM700; Carl Zeiss). To examine

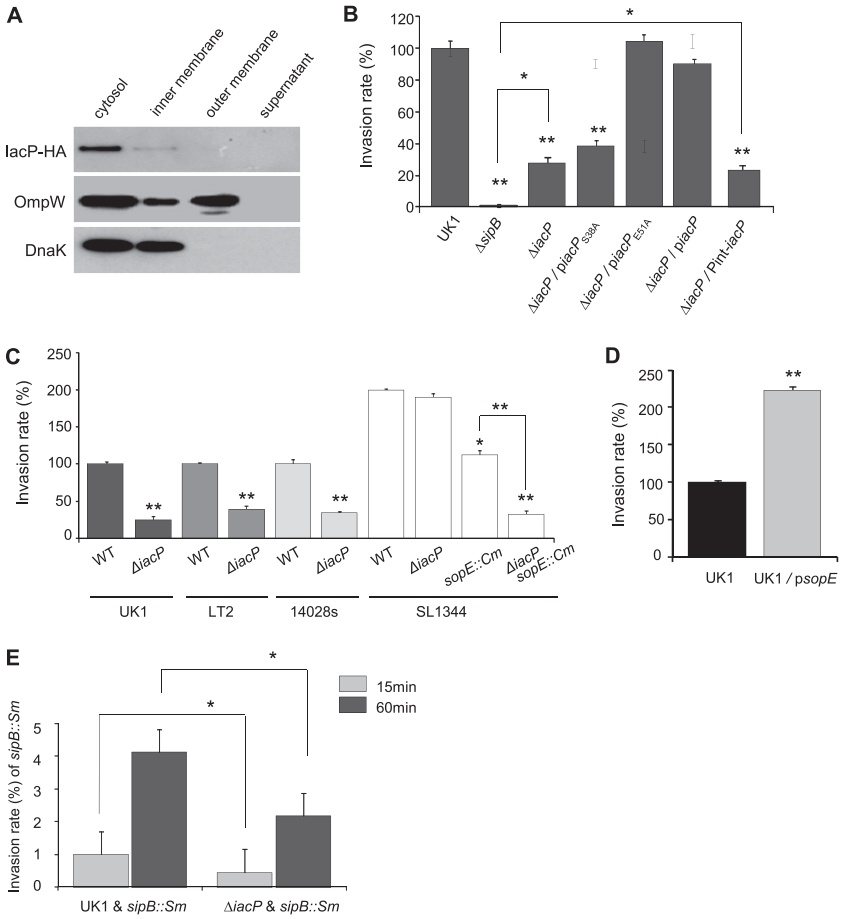

FIG. 1. IacP contributes to *Salmonella* serovar Typhimurium invasion into nonphagocytic cells. (A) *Salmonella* serovar Typhimurium bacteria grown under SPI-1-inducing conditions were fractionated on a sucrose gradient. HA-tagged IacP was detected in the cytoplasmic fraction. As a control for the membrane and cytoplasmic proteins, the blot was reprobed with antibodies against OmpW and DnaK. (B to E) To determine the invasion rate, bacterial strains were allowed to infect an INT-407 cell for 15 min or 60 min, after which a gentamicin protection assay was conducted. The intracellular bacteria were enumerated by the plating of serial dilutions, and the percent bacterial invasion rate was calculated in comparison to that of the wild type (WT), which was set as 100% (in the case of strain SL1344, the invasion rate of UK1 was set as 100%). All experiments were performed on separate days at least three times. Bars correspond to the means ± standard deviations (SD). *, $P < 0.01$; **, $P < 0.001$ (compared with invasion by the respective wild-type or control strain, determined by a Student's *t* test).

actin fibers of the infected cells, they were fixed with paraformaldehyde without gentamicin incubation, as significant differences in accumulations of actin fibers were previously observed immediately after bacterial entry (25). To label F-actin, samples was incubated for 1 h with tetramethyl rhodamine isocyanate (TRITC)-conjugated phalloidin (Sigma-Aldrich), mounted using Fluoromount-G (SouthernBiotech) antifade medium, and then observed by using a fluorescence microscope (Axio Imager A1; Carl Zeiss) coupled with a charge-coupled-device (CCD) camera (AxioCam MRc5; Carl Zeiss).

***In vivo* virulence assays.** For the histochemistry of the small intestine and determinations of the bacterial burden in the spleen, 5-week-old female C3H/HeN mice (Labanimal, South Korea) were orally infected with 1×10^8 CFU of *Salmonella* serovar Typhimurium. Seven days later, the mice were sacrificed, and tissue samples from the intestine were embedded in paraffin. Subsequent hematoxylin and eosin (H/E) staining was performed as described previously (32). For the pathological scoring of infections, H/E-stained samples were examined and scored as described previously (8). The following features of the surface epithelium were evaluated: a sum of no pathological change (score of 0), regenerative change (mild, score of 1; moderate, score of 2; severe, score of 3), desquamation (patchy, score of 1; diffuse, score of 2), and polymorphonuclear leukocytes (PMNs) in the epithelium (score of 1). For the lumen, the sum of empty cells (score of 0), necrotic epithelial cells (scant, score of 1; moderate, score of 2; dense, score of 3), and PMNs (scant, score of 2; moderate, score of 3; dense, score of 4) were scored; for the mucosa, a sum of no pathological change (score of 0), crypt abscesses (rare [<15%], score of 1; moderate [15% to 50%], score of 2; abundant [>50%], score of 3), the presence of mucinous plugs (score of 1), and the presence of granulation tissue (score of 1) were evaluated; and for the submucosa, a sum of no pathological change (score of 0), mononuclear cell infiltrates (1 small aggregate, score of 0; more than 1 aggregate, score of 1; large aggregates plus increased numbers of single cells, score of 2), PMN infiltrate (none, score of 0; single, score of 1; aggregates, score of 2), and edema (mild, score of 0; moderate, score of 1; severe, score of 2) were evaluated. Spleens were removed from three mice in each group and homogenized in PBS. CFU were enumerated by plating serial dilutions of homogenized samples. For the survival assay, 5-week-old female C3H/HeN mice were infected with 1×10^7 CFU *Salmonella* serovar Typhimurium or PBS and then monitored daily. The survival rate was evaluated as the percentage of mice surviving after oral challenge.

Statistical analysis. A Student's *t* test (SPSS 18.0) was used to identify significant differences in the bacterial invasion rates, and a Kaplan-Meier survival curve was plotted by using GraphPad Prism 5.

RESULTS

IacP is required for the invasion of nonphagocytic cells. To investigate the expression and subcellular localization of IacP in *Salmonella* serovar Typhimurium, we chromosomally tagged

IacP at the C terminus with HA using the red recombinase system. *Salmonella* serovar Typhimurium strains carrying an HA-tagged IacP protein were cultured for 3 h under SPI-1-inducing conditions and then subcellularly fractionated by six-step sucrose density gradient centrifugation into the cytoplasm, inner/outer membrane, and supernatant fractions. Figure 1A shows that IacP-HA (~10 kDa) localized primarily within the cytoplasm.

As the SPI-1 T3SS is essential for nonphagocytic cell invasion, we examined the effect of IacP during the invasion of INT-407 intestinal epithelial cells. INT-407 cells were infected by a *Salmonella* serovar Typhimurium wild-type strain, a *sipB* mutant, and an *iacP* mutant with or without various *iacP*-bearing plasmids grown under SPI-1-inducing conditions. As shown in Fig. 1B, the *iacP* mutant was less invasive than wild-type *Salmonella* serovar Typhimurium after 15 min of infection although not to the same extent as the *sipB* mutant. To complement the *iacP*-null mutant, we generated two plasmid constructs carrying the *iacP* gene under the control of the *sicA-sipBCDA* operon promoter (p*iacP*) or under the control of ~1,000 bp of the upstream sequence from the start codon of *iacP* (Pint-*iacP*). The *iacP* mutant possessing the p*iacP* plasmid could be internalized into INT-407 cells similarly to the wild type, whereas the Pint-*iacP* plasmid was unable to recover the invasion of the *iacP* mutant (Fig. 1B). These findings suggest that the *iacP* gene is not regulated independently but may be cotranscribed as a *sicA-sipBCDA-iacP* operon during bacterial entry.

To characterize the specific role of IacP required for bacterial invasion, we replaced the putative 4′-PP attachment site, the serine at position 38, with alanine ($IacP_{S38A}$) by site-directed mutagenesis. When INT-407 cells were infected with *Salmonella* serovar Typhimurium possessing substituted plasmids for 15 min, the $IacP_{S38A}$ plasmid failed to complement the invasion-defective phenotype of the *iacP* mutant, while no differences in invasion rates were observed between the wild type and the mutant harboring the $IacP_{E51A}$ plasmid used as a control (Fig. 1B). This result implies that the linkage between the 4′-PP and Ser38 of IacP is required for invasion into INT-407 cells.

To confirm the effect of *iacP*-mediated cell invasion in other *Salmonella* serovar Typhimurium strains, we introduced the *iacP* mutation into widely used laboratory strains such as ATCC 14028s, LT2, and SL1344. As shown in Fig. 1C, *iacP* mutants of ATCC 14028s and LT2 invaded INT-407 cells less than did their respective parental strains after 15 min of infection. However, the invasion defect could not be reproduced in SL1344 lacking the *iacP* gene (Fig. 1C), which is consistent with a previous report showing that the mutation of *iacP* did not alter the assembly of the T3SS apparatus and cell invasion of SL1344 (48).

As a mechanism for pathogenic adaptation, many lysogenic phages that participate in bacterial pathogenesis have been transferred between independent lineages of *Salmonella* (10). The *Salmonella* serovar Typhimurium effector SopE, which is encoded by a Fels-like prophage in SL1344 and translocated through the SPI-1 T3SS, activates cellular Rho GTPases such as Rac1 and Cdc42 to induce membrane ruffling (12). The *sopE* gene was not found in strain LT2, ATCC 14028s, or UK1 by PCR using specific primers (data not shown); however, the addition of the *sopE* plasmid into strain UK1 caused a 2-fold increase in bacterial entry (Fig. 1D). Thus, we reasoned that the IacP function might be masked by SopE in strain SL1344. As shown in Fig. 1C, the rate of internalization of the SL1344 *sopE* mutant into INT-407 cells was decreased to half that of wild-type SL1344 (19), and an additional *iacP* mutation resulted in a significant decrease in the invasion rate, which was identical to that of the *iacP* mutant of UK1. Together, these results suggested that *Salmonella* serovar Typhimurium IacP is required for early invasion into nonphagocytic cells and is not replaceable by other ACPs responsible for the biosynthesis of essential cellular lipids.

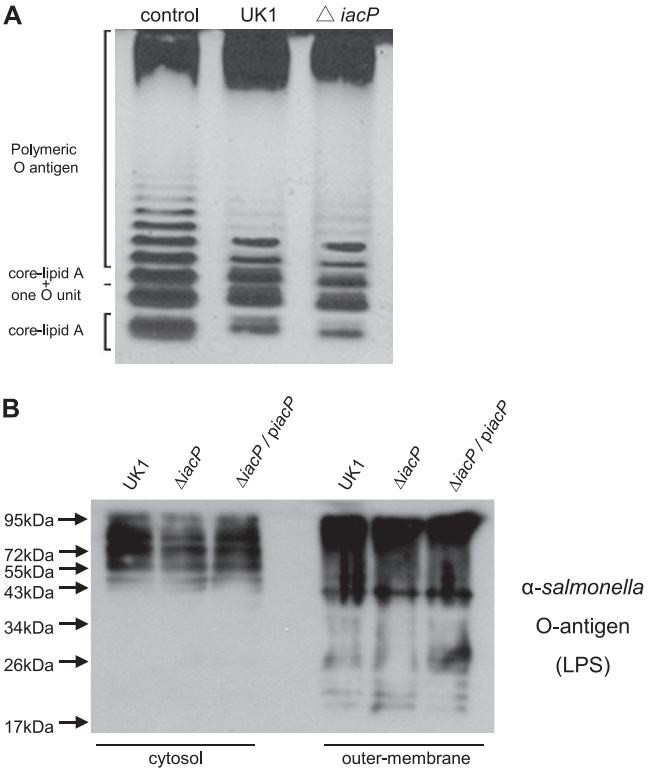

FIG. 2. Comparisons of the LPS profiles prepared from the wild type and the *iacP* mutant. (A) Silver-stained image of LPSs purified from the wild type and the *iacP* mutant grown under SPI-1-inducing conditions. Purified LPS from *Salmonella* serovar Typhimurium (Sigma-Aldrich) was used as a standard. (B) Cytosolic and outer membrane fractions isolated from the *Salmonella* serovar Typhimurium wild type, the *iacP* mutant, and the complement strain were immunoblotted with an anti-LPS antibody.

IacP is not necessary for LPS biosynthesis in *Salmonella* serovar Typhimurium. In many Gram-negative bacteria, LPS on the bacterial surface has been implicated as a virulence factor required for the adherence and invasion of epithelial cells (47). LPS is composed of three major parts: lipid A, the core oligosaccharide, and O antigen. As the acyl-ACP of *E. coli* is required for the biosynthesis of the lipid A component (43), we examined whether the lipid A portion was influenced by the *iacP* mutation. However, we found a typical LPS ladder pattern in the purified LPS and the isolated outer membrane fraction of the *iacP* mutant grown under SPI-1-inducing conditions (Fig. 2). These findings suggested that IacP is not required for

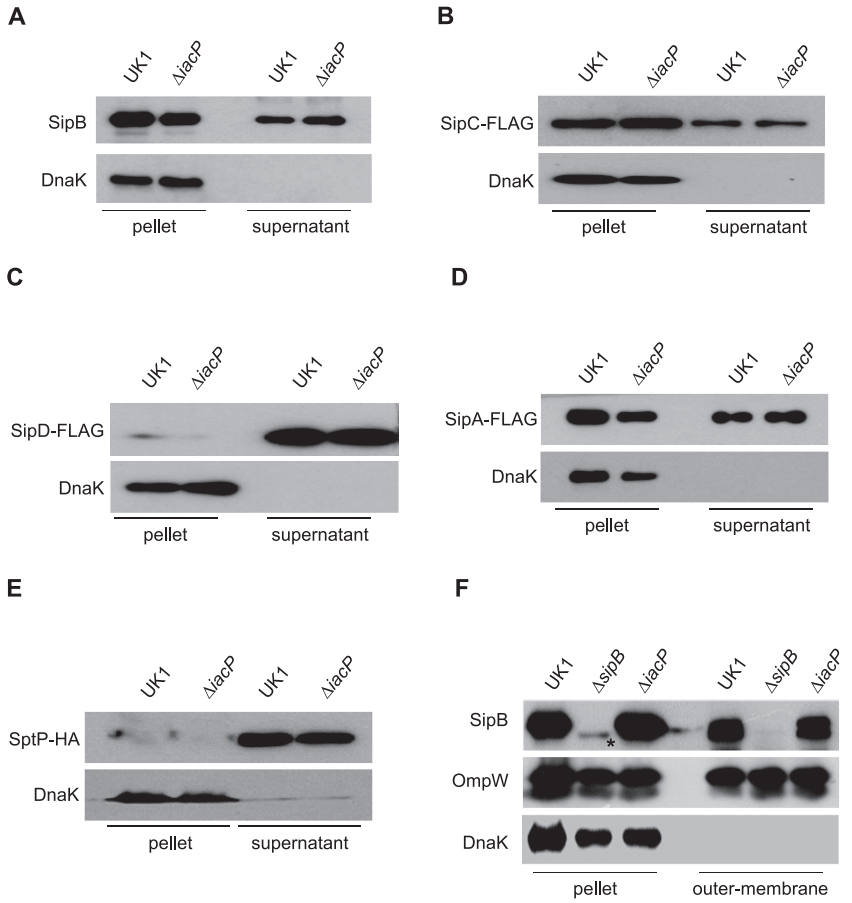

FIG. 3. IacP did not influence the secretion of SipB, SipC, SipD, SipA, and SptP. Wild-type *Salmonella* serovar Typhimurium and the *iacP* mutant were grown under SPI-1-inducing conditions for 3 h. (A to E) The levels of SipB, SipC-FLAG, SipD-FLAG, SipA-FLAG, and chromosomally HA-tagged SptP in the pellet and culture supernatant were determined by Western blotting using anti-SipB, anti-FLAG, and anti-HA antibodies. Anti-DnaK antibody was used as a control for cytoplasmic proteins. (F) *Salmonella* subcellular fractionation was undertaken to compare the outer membrane localizations of SipB in the wild type, the *iacP* mutant, and complement strains. As a control for membrane and cytoplasmic proteins, the blot was reprobed with antibodies to OmpW and DnaK. The asterisk denotes the nonspecific band.

the biosynthesis of LPS but rather is involved in the regulation of virulence factors necessary for SPI-1-mediated invasion.

IacP did not influence the secretion of SipB, SipC, SipD, SipA, and SptP. The SPI-1 effector proteins SipB, SipC, SipD, and SipA are encoded in the same operon of IacP and are required for bacterial internalization. To test the possibility that IacP was involved in the optimal secretion of these proteins under SPI-1-inducing conditions, we compared the secretion levels of SipB, SipC, SipD, and SipA. When measured by Western blotting using anti-SipB and anti-FLAG antibodies, SipB, SipC, SipD, and SipA were expressed and efficiently secreted into the culture supernatant in both the wild type and the *iacP* mutant (Fig. 3A to D). Similarly, the secretion of SptP, which is located downstream of the *iacP* gene, was not affected by the *iacP* mutation (Fig. 3E). These results indicate that IacP does not affect the expression and secretion of the SPI-1 translocon (SipB, SipC, and SipD) or other effector proteins (SipA and SptP).

The posttranslational acylation of some membrane-associated proteins is required for membrane targeting (46). Because SipB was observed on a bacterial surface for the attachment of host cells and may be inserted into the plasma membrane to form the translocation pore with SipC (33), we next examined whether the membrane localization of SipB was altered in the *iacP* mutant. As shown in Fig. 3F, the SipB localization on the membrane of the *iacP* mutant was equivalent to that of the wild type, indicating that the outer membrane localization of SipB did not appear to be modified by IacP.

Levels of secretion of SopB, SopA, and SopD were decreased in the *iacP* mutant. Actin cytoskeleton rearrangement in host cells is a characteristic of *Salmonella* serovar Typhimurium invasion and is accomplished by the translocation of several effector proteins through the SPI-1 T3SS. To determine if the decreased invasion of the *iacP* mutant was due to delayed actin remodeling, actin filaments were visualized by staining INT-407 cells with rhodamine-conjugated phalloidin immediately after *Salmonella* serovar Typhimurium infection for 15 min. As shown in Fig. 4A, the extended actin stress fibers at the cell periphery and the profound membrane protrusion were evidently shaped in INT-407 cells infected with the wild type. Actin fibers, however, were diffusely observed inside the cytoplasm, and the cellular morphology remained unchanged in many *iacP* mutant-infected cells. These findings indicate that IacP might be in-

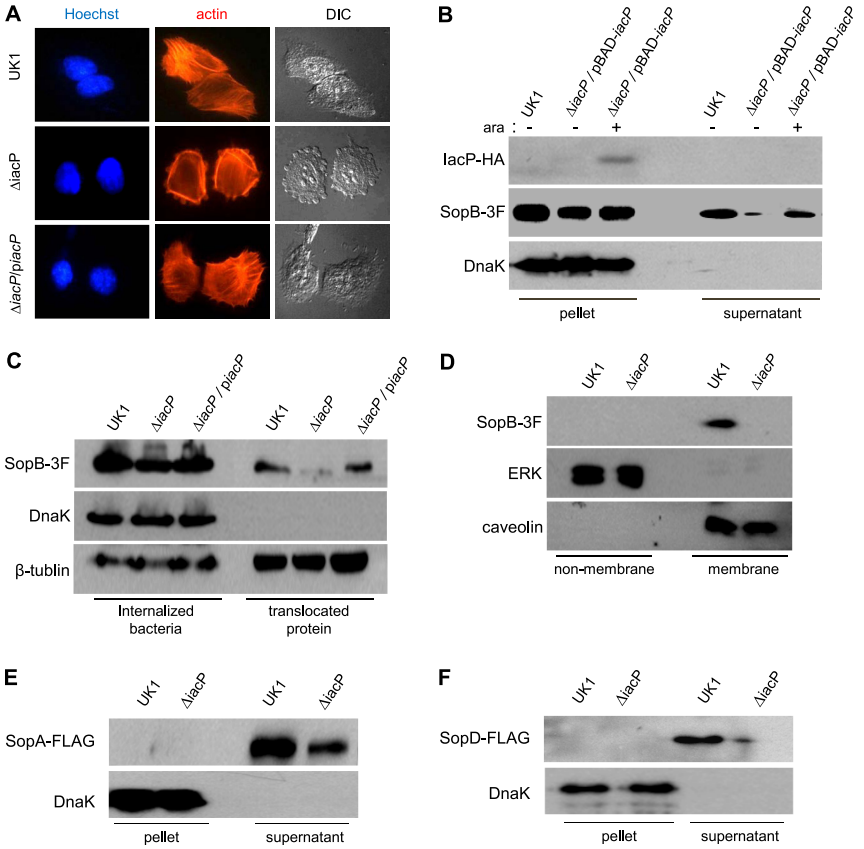

FIG. 4. IacP promotes the T3SS-mediated secretion and translocation of SopB. (A) After bacterial infection for 15 min, filamentous actins were stained with rhodamine phalloidin. The nucleus and intracellular *Salmonella* serovar Typhimurium bacteria were stained with Hoechst 33342 dye (original magnification, ×1,260). (B) The secretion of SopB tagged with 3×FLAG (SopB-3F) in cultured supernatants was examined by Western blotting. L-Arabinose (0.2 mM) was added to induce the expression of IacP. Anti-DnaK antibody was used as a control for cytoplasmic proteins. (C) INT-407 cells infected with *Salmonella* serovar Typhimurium were treated with Triton X-100 (internalized bacteria) or SDS (translocated protein). Anti-β-tubulin antibody was used as a loading control. (D) To determine the subcellular localization of SopB, the membranes of infected INT-407 cells were isolated. Extracellular signal-regulated kinase (ERK) and caveolin were used as nonmembrane and membrane controls, respectively. (E and F) The culture supernatants of *Salmonella* serovar Typhimurium possessing SopA-FLAG (E) and SopD-FLAG (F) plasmids were immunoblotted with anti-FLAG antibody. Anti-DnaK antibody was used as a control for cytoplasmic proteins.

volved in the modification of T3SS effectors required for actin cytoskeleton remodeling (36). Among these effector proteins, the secretion of five proteins (SipA, SipC, SopE, SopE2, and SptP) was not affected by IacP (Fig. 3 and data not shown for SopE2). To examine the effect of IacP on SopB secretion, we used a 3×FLAG epitope tag at the C terminus of SopB in the wild type and the *iacP* mutant. The level of secretion of SopB into the culture supernatant was dramatically reduced in the *iacP* mutant relative to that of the wild type or the *iacP* mutant possessing plasmid pBAD-*iacP*::HA, in which the transcription of the *iacP* gene was controlled by the arabinose-inducible promoter (Fig. 4B). This result was reproduced in *Salmonella* serovar Typhimurium without epitope tagging by using an anti-SopB antibody (data not shown). We then compared the translocations of SopB in INT-407 cells infected with the wild type and the *iacP* mutant. Consistent with the secretion profile shown in Fig. 4B, the *iacP* mutation resulted in a significant decrease (−3.9-fold) in SopB translocation into INT-407 cells (Fig. 4C). Moreover, it was reported previously that the inositol phosphatase activity of SopB is achieved in the host membrane of the bacterial entry site and nascent SCV (34). When *Salmonella*-infected INT-407 cells were fractionated into membranes and nonmembranes, SopB was not observed in the membrane fraction of the *iacP* mutant-infected cells (Fig. 4D). This might result from the decrease in SopB translocation from the *iacP* mutant.

As other effector proteins, SopA and SopD, like SopB, are synthesized and translocated during the late stage of infection and contribute to the invasion of host cells (2, 17, 44), we next examined the secretion of SopA or SopD in the *iacP* mutant. Although no SopA or SopD was detected in the bacterial cytosol for some unknown reason, the secretion of both proteins was obviously decreased in the *iacP* mutant (Fig. 4E and F). These results suggest that IacP promotes the invasion of *Salmonella* serovar Typhimurium by enhancing SopB, SopA, and SopD secretion and translocation to trigger actin cytoskeleton rearrangements in host cells.

IacP is important for *Salmonella* serovar Typhimurium virulence. Kuijl et al. recently showed that translocated SopB induces the phosphorylation of Akt, which subsequently activates Rab14 and AS160 phosphorylation to inhibit phagosomal

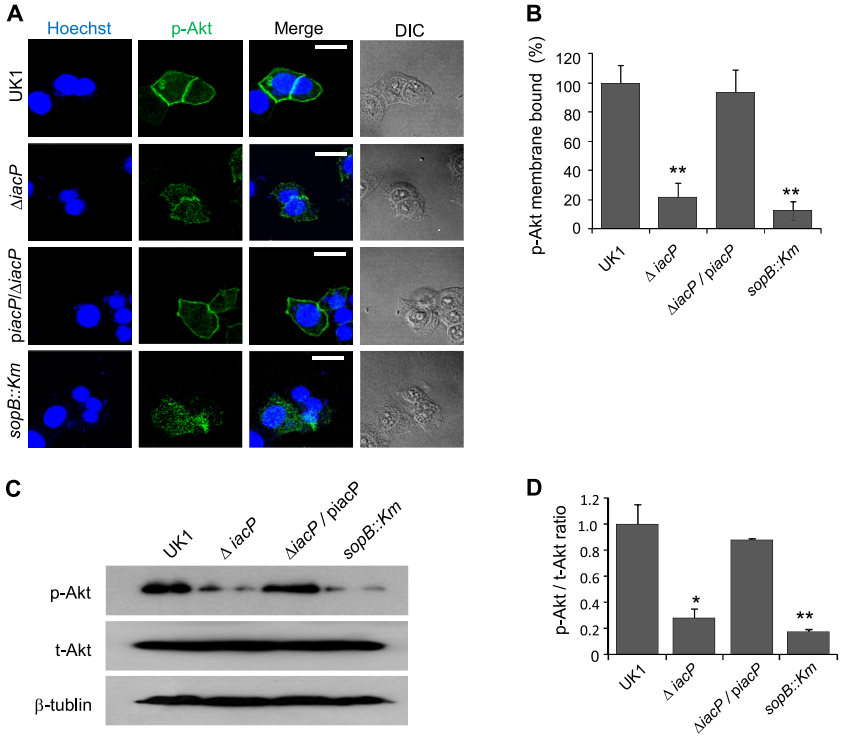

FIG. 5. Akt phosphorylation is decreased in INT-407 cells infected with the *iacP* mutant. (A) To visualize phosphorylated Akt at the plasma membrane, INT-407 cells infected with *Salmonella* serovar Typhimurium were stained with anti-phospho-Akt. Scale bar, 40 μm. (B) Images from three different confocal planes (at least 30 cells) per sample were analyzed to determine the quantification of phosphorylated Akt bound to the membrane. Bars correspond to means ± SD. (C) Total cellular proteins of INT-407 cells infected with the wild type or its isogenic mutants were subjected to Western blot analysis. β-Tubulin was used as a loading control. (D) Quantification of the amount of phosphorylated Akt and total Akt in bacterially infected cells in C. Each band was densitometrically normalized to β-tubulin, and the phospho-Akt/total Akt ratio is expressed as the mean ± SD. *, $P < 0.05$; **, $P < 0.001$ (statistically significant difference from the control group). p-Akt, phosphorylated Akt; t-Akt, total Akt.

maturation (31). To further examine the Akt phosphorylation of host cells infected with the *iacP* mutant, we stained the infected INT-407 cells with anti-phospho-Akt (p-Akt) antibody. As shown in Fig. 5A and B, p-Akt was accumulated around the plasma membrane of the host cells infected by the wild-type and the *iacP*-p*iacP* strains, whereas Akt activation was markedly decreased in *iacP* mutant-infected cells. Consistent with the confocal images, the amount of p-Akt was also decreased in the total extract from *iacP*-infected cells (Fig. 5C and D).

Furthermore, given the fact that the inositol phosphatase activity of SopB is required for SCV trafficking (1), we investigated whether the decreased translocation of SopB in the *iacP* mutant resulted in an attenuation of virulence in mice. C3H/HeN mice were orally infected with *Salmonella* serovar Typhimurium, and the pathological changes in the lining of the small intestine were investigated 7 days after infection by histochemistry. As shown in Fig. 6A and B, shortened and diffused desquamations of the epithelial brush border were observed for mice infected with the wild-type and *iacP*-p*iacP* strains. Inflamed small intestines were densely filled with infiltrated neutrophils and necrotic epithelial cells. However, the *iacP* mutant evoked significantly less damage in the small intestine of C3H/HeN mice, showing no difference compared with the PBS control group. These results suggest that the *iacP* mutant is less virulent than the wild type and is likely to be expeditiously cleared by the host defense system after oral ingestion. This was confirmed by the bacterial load from the spleen at 7 days after oral infection. As shown in Fig. 6C, the level of bacterial colonization in the spleen was significantly lower in mice administered the *iacP* mutant than in the wild-type-infected mice.

We next monitored the survival of C3H/HeN mice after the oral administration of *Salmonella* serovar Typhimurium. Two groups of mice challenged with the wild-type and *iacP*-p*iacP* strains succumbed to infection until 15 days postinfection. In addition, one mouse each from the group infected with the *iacP* mutant died on day 13 and day 15 after infection; however, the remaining three mice recovered from the bacterial infection and survived for the rest of the study (Fig. 6D). These results indicated that IacP is required for the infection and virulence of *Salmonella* serovar Typhimurium in mice.

DISCUSSION

During the invasion of a nonphagocytic cell by *Salmonella* serovar Typhimurium, the SPI-1 T3SS is activated upon host cell contact and is used to export an arsenal of effector proteins into the host cytoplasm. In this study, we first showed that the SPI-1 cytosolic protein IacP is important for bacterial virulence by regulating the secretion of T3SS effector proteins.

IacP is encoded from a *sicA-sipBCDA-iacP* operon and ex-

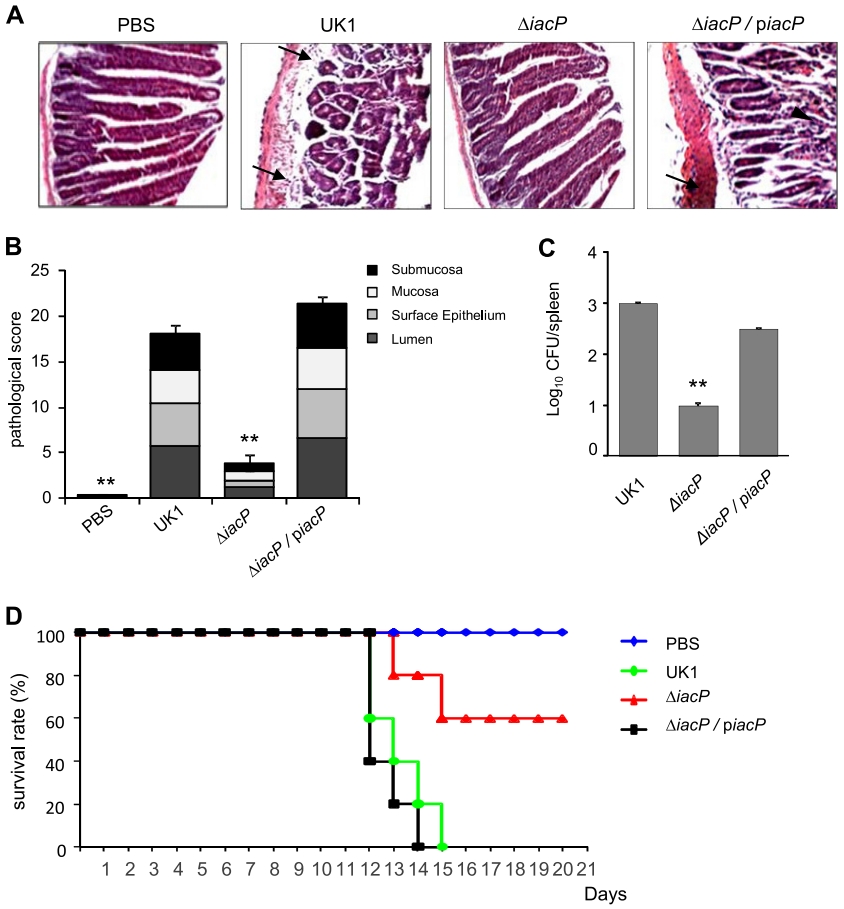

FIG. 6. IacP is required for full virulence of *Salmonella* serovar Typhimurium. (A) The intestinal linings of C3H/HeN mice orally infected with the wild type, the *iacP* mutant, and the complement strain were analyzed by histochemistry after 7 days of infection. The sections were counterstained with hematoxylin and eosin and mounted onto microscope slides (original magnification, ×200). The arrow indicates infiltrated PMNs, and the arrowhead indicates the diffused desquamation of epithelial cells. (B) The pathological scores are stacked averages from mice per group at 7 days after infection with *Salmonella* serovar Typhimurium. Bars correspond to means ± SD of the total score. (C) The bacterial load from mouse spleen was determined 7 days after oral infection with *Salmonella* serovar Typhimurium strains. Bars correspond to means ± SD. **, $P < 0.001$ compared with the mouse group infected with the wild-type strain. (D) The survival of each C3H/HeN mouse group following an oral injection of *Salmonella* serovar Typhimurium was recorded at daily intervals until the end of the study. The data are from a single representative experiment of three independent trials.

pressed in the bacterial cytosol under SPI-1-inducing conditions. While the growth of *Salmonella* serovar Typhimurium lacking the *iacP* gene could not be distinguished from that of the wild type under SPI-1-inducing conditions (data not shown), the *iacP* mutant invaded INT-407 epithelial cells less efficiently than did the parental strain, and its activity was not limited to a particular strain of *Salmonella* serovar Typhimurium. Moreover, the substitution of the putative attachment site of 4′-PP (Ser38) was sufficient to cause a loss of function of the *iacP* gene. These data indicated that IacP might play a role as a specific ACP by associating with the 4′-PP group during *Salmonella* entry and that IacP might be involved in the post-translational modification of invasion-related molecules that have a function at the bacterial or host membrane rather than fatty acid biosynthesis. LPS is necessary for bacterial adhesion and invasion in epithelial cells, and the lipid A component of LPS is synthesized in an ACP-dependent manner (43). However, we did not find significant differences in the biosyntheses and outer membrane localizations of the isolated LPSs from the wild type and the *iacP* mutant.

We next investigated the secretion pattern of effector proteins in the SPI-1 locus that are located upstream or downstream of flanking genes (SipB, SipC, SipD, SipA, and SptP). In the *iacP* mutant grown under SPI-1-inducing conditions, these proteins were equally expressed and secreted into the culture medium, indicating that these proteins were not targeted by IacP. In the present study, the invasion rate of the *iacP* mutant was significantly higher than that of the *sipB* mutant. In addition, we found that the membrane ruffling in INT-407 cells induced by the *iacP* mutant was insufficient to restore the internalization of a coinfected *sipB* mutant (Fig. 1E). Moreover, as shown in Fig. 1C, the deletion effect of *iacP* in the SL1344 strain was masked by SopE, which manipulated the actin rearrangement of host cells. Collectively, these data supported the notion that IacP might be involved in the modification of T3SS effectors required for actin cytoskeleton remod-

eling but not T3SS translocator proteins. Consistent with these results, we demonstrated that the rearrangement of actin fibers was delayed in INT-407 cells infected with the *iacP* mutant by staining the actin filaments. These findings could be ascribed to a decreased level of secretion of the SPI-1 effector protein SopB in the *iacP* mutant. SopB translocation and membrane localization in host cells infected with the *iacP* mutant were also dramatically reduced compared to wild-type-infected cells.

SopB activates the RhoG exchange factor SGEF (Src homology 3 domain-containing guanine nucleotide exchange factor) required for cytoskeletal rearrangement and enhanced bacterial entry (39). Upon the transient transfection of HeLa cells with a plasmid harboring *sopB*, SopB is associated with host cell membranes, and residues 117 to 167 of SopB are necessary for membrane targeting (35). We tested the hypothesis that IacP is involved in the modification of this domain. The expression and secretion of SopB lacking residues 117 to 167 in the wild-type strain were the same as those of full-length SopB, whereas the level of secretion of SopB without the membrane-targeting region was reduced in the *iacP* mutant (data not shown). In addition, ubiquitin-mediated localization, which directs the differential cellular function of SopB, is preceded by the plasma membrane accumulation of SopB, suggesting that the region at residues 117 to 167 of SopB is independent of the IacP modification and that the subcellular distribution of SopB in infected cells may be mediated by host factors (40).

This study found that IacP regulated the secretion of effector proteins encoded outside SPI-1, such as SopA or SopD, which are involved in bacterial adhesion, invasion, and virulence. This implies that IacP activity is not confined to the modification of some effectors and may be involved in the modification of some membrane-associated protein in the T3SS apparatus which controls the translocation of SPI-1 effectors. The hierarchical secretion of the T3SS between the membrane-bound translocon and secreted effectors has been well defined in previous studies of InvE of *Salmonella* serovar Typhimurium (30), the YopN/TyeA heterodimer of *Yersinia enterocolitica* (23), MxiC of *Shigella flexneri* (4), and SepL of enteropathogenic *Escherichia coli* (53). In addition, an alteration in the helical packing of the T3SS needle subunit upon host cell contact was reported previously to explain the signal responsible for the triggering of effector secretion (3, 41). If this signal is transmitted from the needle to the membrane-bound receiver protein in the T3SS base, IacP may be involved in the membrane localization of that protein, contributing to the establishment of a secretion hierarchy in the SPI-1 T3SS. However, further studies are needed to elucidate the IacP-dependent modification of membrane proteins, including InvE.

In INT-407 cells infected with the *iacP* mutant, the level of SopB-mediated phosphorylation of Akt was found to be significantly lower than that in wild-type-infected cells. These findings indicate that the amount of SopB translocated from the *iacP* mutant is was insufficient to activate the cytosolic Akt in host cells. The *sopB* mutation of *Salmonella* serovars Typhimurium and Dublin resulted in the attenuation of both gastroenteritis in calves (56) and inflammation in bovine ileal loops (15). Moreover, the *sopB* mutants augmented antigen uptake and presentation by dendritic cells (55). Therefore, we expected the *iacP* disruption to render *Salmonella* spp. aviru-lent. The pathological alterations observed in the intestine of C3H/HeN mice were prominent in a histochemical study of the *iacP* mutant-infected mouse group in which no flattened villi or signs of inflammation were observed. In our mouse survival assay, a group of C3H/HeN mice subjected to oral challenge with the *iacP* mutant survived longer than did wild-type-infected mice. Even though a small amount of SopB secretion was consistently observed for the *iacP* mutant, the level of attenuation achieved by the deletion of the *iacP* gene was greater than that of a single mutation in *sopB* (data not shown). This can be explained by the possibility that IacP might be involved in the translocation of various T3SS effector proteins, which is essential for the survival and replication of bacteria following host cell invasion. Taken together, our results indicate that IacP acts as a virulence factor by regulating the secretion and the translocation of type III effector proteins during host cell entry.

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