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Effect of the O-Antigen Length of Lipopolysaccharide on the Functions of Type III Secretion Systems in *Salmonella enterica*▽

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The virulence of *Salmonella enterica* critically depends on the functions of two type III secretion systems (T3SS), with the *Salmonella* pathogenicity island 1 (SPI1)-encoded T3SS required for host cell invasion and the SPI2-T3SS enabling *Salmonella* to proliferate within host cells. A further T3SS is required for the assembly of the flagella. Most serovars of *Salmonella* also possess a lipopolysaccharide with a complex O-antigen (OAg) structure. The number of OAg units attached to the core polysaccharide varies between 16 and more than 100 repeats, with a trimodal distribution. This work investigated the correlation of the OAg length with the functions of the SPI1-T3SS and the SPI2-T3SS. We observed that the number of repeats of OAg units had no effect on bacterial motility. The interaction of *Salmonella* with epithelial cells was altered if the OAg structure was changed by mutations in regulators of OAg. Strains defective in synthesis of very long or long and very long OAg species showed increased translocation of a SPI1-T3SS effector protein and increased invasion. Invasion of a strain entirely lacking OAg was increased, but this mutant strain also showed increased adhesion. In contrast, translocation of a SPI2-T3SS effector protein and intracellular replication were not affected by modification of the OAg length. Mutant strains lacking the entire OAg or long and very long OAg were highly susceptible to complement killing. These observations indicate that the architecture of the outer membrane of *Salmonella* is balanced to permit sufficient T3SS function but also to confer optimal protection against antimicrobial defense mechanisms.

Salmonella enterica is a remarkable pathogen with strategies for adaptation to different lifestyles in the environment as well as within various host organisms. The requirements for life within the host can vary dramatically, for example, after transition from extracellular life within the intestine to an intracellular life within a special organelle formed inside infected host cells (reviewed in reference 23). The presence of an outer membrane is an important structural feature that enables commensal as well as pathogenic bacteria to adapt to the intestine and to resist bile salts and various molecules of the host innate immune system (44). Of specific importance is the lipopolysaccharide (LPS), the major constituent of the outer leaflet of the outer membrane. LPS is composed of (i) the lipid A portion, consisting of acyl chains linked to phosphorylated *N*-acetylglucosamine; (ii) the inner and outer core moieties, consisting of rather conserved sugars; and (iii) a highly variable O antigen (OAg). The LPS of *S. enterica* serovar Typhimurium and several other serovars has special characteristics, with extreme heterogeneity in the length of the OAg repeats. LPS species with a short OAg (S-OAg) consisting of about 16 repeats of OAg units can be found. In addition, LPS species with long and very long OAg (L-OAg and VL-OAg, respectively) are present, containing about 35 and more than 100 repeats, respectively, of the OAg units. The synthesis of L- and VL-OAg in *S. enterica* serovar Typhimurium is regulated, and Wzz_{ST} and Wzz_{fepE}, respectively, have been characterized as the corresponding regulators (5, 40; reviewed in reference 41).

In contrast to the commensal intestinal flora, *S. enterica* has the ability to breach intestinal barriers, to invade host cells, and to enter into an intracellular lifestyle. These hallmarks of the pathogenesis of *Salmonella* critically depend on the functions of two type III secretion systems (T3SS) (31, 46). T3SS are complex molecular machines that mediate the contact-dependent translocation of sets of effector proteins (reviewed in reference 18). Translocation requires the insertion of a membrane pore, termed a translocon, in the target membrane and the assembly of a needle-like oligomeric structure that links the translocon to the T3SS apparatus in the bacterial envelope (reviewed in reference 13). The T3SS encoded by *Salmonella* pathogenicity island 1 (SPI1) is active in extracellular bacteria and mediates the internalization of *Salmonella* into nonphagocytic cells by injection of effector proteins that manipulate the host cell actin cytoskeleton (46). The second T3SS is encoded by SPI2 and mediates the translocation of a second set of effector proteins by bacteria residing inside the *Salmonella*-containing vacuole, a specialized compartment that allows the intracellular proliferation of bacteria (31). Although both the SPI1-T3SS and the SPI2-T3SS are likely to have structural similarities, the numbers of active T3SS and their subcellular localization appear to be different (11, 30, 48).

The flagellar assembly system is considered the third T3SS in *S. enterica* (34). Flagellum-mediated motility can be considered a basic function of a bacterial cell. However, a contribution of motility to the interaction with host cells during pathogenesis has been demonstrated (50, 51).

Previous work indicates that the function of the T3SS required for invasion of *Shigella flexneri* is dependent on the modification of the structure of the LPS (52). We assume that similar functional constraints also apply to the function of the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Comment and/or reference
Salmonella NCTC 12023 strains		
NCTC 12023	Wild type	Lab collection
MvP643	Δ*sseB*::FRT	Red deletion; this study
P2D6	*ssaV*::mTn*5*	49
P7D8	*invC*::mTn*5*	15
MvP706	Δwzz_{ST}::FRT	Red deletion; this study
MvP708	Δwzz_{fepE}::FRT	Red deletion; this study
MvP724	Δwzz_{ST}::FRT Δwzz_{fepE}::FRT	Red deletion; this study
MvP1036	Δ*waaL*::FRT	Red deletion (53)
MvP613	$sseJ_{200}$::*luc aph*	20
MvP661	$sseJ_{200}$::*luc* FRT	This study
MvP694	*ssaV*::mTn*5* $sseJ_{200}$::*luc* FRT	20
MvP716	Δwzz_{ST}::FRT $sseJ_{200}$::*luc aph*	P22 of MvP613; this study
MvP718	Δwzz_{fepE}::FRT $sseJ_{200}$::*luc aph*	P22 of MvP613; this study
MvP725	Δwzz_{ST}::FRT Δwzz_{fepE}::FRT $sseJ_{200}$::*luc aph*	P22 of MvP613; this study
MvP1288	Δ*waaL*::*aph* $sseJ_{200}$::*luc* FRT	Red deletion; this study
MvP1156	*sipA*::$sipA_{M45}$	19
MvP1290	Δ*invC*::FRT *sipA*::$sipA_{M45}$	P22 of MvP1156; this study
MvP1291	Δwzz_{ST}::FRT *sipA*::$sipA_{M45}$	P22 of MvP1156; this study
MvP1292	Δwzz_{fepE}::FRT *sipA*::$sipA_{M45}$	P22 of MvP1156, this study
MvP1293	Δwzz_{ST}::FRT Δwzz_{fepE}::FRT *sipA*::$sipA_{M45}$	P22 of MvP1156; this study
MvP1294	Δ*waaL*::*aph sipA*::$sipA_{M45}$	Red deletion; this study
MvP1281	*sipA*::$sipA_{M45}$::*tem1*	P22 of M1104; this study
MvP1282	*invC*::FRT *sipA*::$sipA_{M45}$::*tem1*	P22 of M1104; this study
MvP1283	Δwzz_{ST}::FRT *sipA*::$sipA_{M45}$::*tem1*	P22 of M1104; this study
MvP1284	Δwzz_{fepE}::FRT *sipA*::$sipA_{M45}$::*tem1*	P22 of M1104; this study
MvP1285	Δwzz_{ST}::FRT Δwzz_{fepE}::FRT *sipA*::$sipA_{M45}$::*tem1*	P22 of M1104; this study
MvP1286	Δ*waaL*::*aph sipA*::$sipA_{M45}$::*tem1*	Red deletion; this study
Salmonella SL1344 strains		
M913	Δ*fliGHI*::FRT	51
M1104	*sipA*::$sipA_{M45}$::*tem1*	47
Plasmids		
pWSK29	Low-copy-number vector	Lab stock
pBAD myc hisB	Vector for arabinose-inducible expression	Invitrogen
p3313	*waaL* in pWSK29	53
p3388	wzz_{fepE} in pWSK29	This study
p3389	wzz_{ST} in pWSK29	This study
p3390	wzz_{ST} wzz_{fepE} in pWSK29	This study
p3456	wzz_{ST} wzz_{fepE} in pBAD myc hisB	This study

Salmonella T3SS for invasion and intracellular proliferation. The reduction of LPS length by glucosylation, as identified in *S. flexneri*, is not found in *S. enterica*. We questioned if alternative mechanisms exist that allow the function of LPS in mediating protection against antimicrobial function of the host as well as the proper functions of the SPI1-T3SS, the SPI2-T3SS, and the flagellar assembly system.

We investigated the effects of reduced numbers of repeats of OAg on the invasion and intracellular replication of *Salmonella*. Our data demonstrate that OAg length is critical for the function of the SPI1-T3SS and for bacterial invasion. In contrast, no influence of OAg length was observed on the functions of the SPI2-T3SS and the flagellar assembly system.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Routinely, 3-ml bacterial cultures were grown aerobically in glass test tubes at 37°C in a roller drum (TC-7; New Brunswick) with agitation at 30 rpm. Antibiotics were used at the following concentrations: carbenicillin or kanamycin, 50 μg ml^{-1}; and tetracycline, 20 μg ml^{-1}.

Generation of mutant strains and plasmids for complementation. Bacterial strains used in this study are described in Table 1. The one-step inactivation method (14) was used for the generation of strains deleted for wzz_{ST}, wzz_{fepE}, or wzz_{ST} and wzz_{fepE} in the background of wild-type (WT) *Salmonella* and for the deletion of *waaL* in the background of strain MvP1156 (*sipA*::$sipA_{M45}$), MvP1281 (*sipA*::$sipA_{M45}$::*tem1*), or MvP661 ($sseJ_{200}$::*luc* FRT). After mutagenesis, the *aph* resistance cassettes were subsequently deleted by FLP-mediated recombination. Primers used for deletion and controls are listed in Table 2.

For analyses of secretion and translocation, the SPI1 effector SipA-M45 from *Salmonella* 12023-derived strain MvP1156 and $SipA_{M45}$-TEM1 from SL1344-derived strain M1104 (47) or the SPI2 effector $SseJ_{200}$-Luc (20) was transferred into WT *Salmonella* 12023 and *invC*, *ssaV*, wzz_{ST}, wzz_{fepE}, and wzz_{ST} wzz_{fepE} mutants, using P22 transduction according to standard methods (35).

Plasmids carrying WT alleles of the deleted genes under the control of their own promoters were generated by PCR amplification of wzz_{ST} or wzz_{fepE}, using primers listed in Table 2. The PCR products were purified using a nucleotide removal kit (Qiagen); digested by BamHI/XbaI or EcoRI/BamHI, for wzz_{fepE} or wzz_{ST}, respectively; and cloned into pWSK29 for the generation of p3388 and p3389, for expression of wzz_{fepE} and wzz_{ST}, respectively. For complementation of the wzz_{ST} wzz_{fepE} double mutant, wzz_{fepE} was excised from p3388 by digestion with BamHI/NotI, gel purified using a gel extraction kit (Qiagen), and subcloned into BamHI/NotI-digested p3389.

For inducible expression of wzz_{ST} and wzz_{fepE}, these genes were amplified by PCR using primers listed in Table 2. A nucleotide removal kit (Qiagen) was used for purification of the PCR product; the purified wzz_{ST} fragment was digested with EcoRI and XbaI and cloned into EcoRI/XbaI-digested vector pBAD myc hisB. Subsequently, the wzz_{fepE} fragment was digested by PstI and EcoRI and

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)[a]
Deletion primers	
WzzB-Del-For	TGTCTCCAGCTTCATCCTTTTTTTAGTTAGGGTATCTATGgtgtaggctggagctgcttc
WzzB-Del-Rev	CCTGTCGTAGCCGACCACCATCCGGCAAAGAAGCTTACAAcatatgaatatcctccttag
Wzz-FepE-Del-For	TGTTCTTTCATTGGATAAAGTTTTCAGGTCATACGGCATGgtggtaggctggagctgcttc
Wzz-FepE-Del-Rev	AAGCCGGATATCGCTATCCGGCTTTTCGGGTAAATCAGACCatatgaatatctccttag
RfaL-Del-For	CTCATCCCAAACCTATTGTGGAGAAAAGATGCTAACCACAgtggtaggctggagctgcttc
RfaL-Del-Rev	ATACCGTAATAATAAGTATCAGCGCGTTTTTTTATCTATTActatgaatatcctccttag
Cloning primers	
WzzB-For2-EcoRI	gat*gaattc*ttattgccgacgctatcctc
WzzB-Rev-BamHI	atc*ggatcc*gtctgatttgatgtaccacc
FepE-For-EcoRI	tcc*gaat*tcaggtcatacggcatgccatc
FepE-Rev-XbaI	gga*tctaga*tcagactaaccgttcatctatc
WzzB-For-EcoRI	*tgcgaattcttagttagggtatctatgacagtg*
WzzB-Rev-XbaI	*gcatctagattacaaggcttttggcttatag*
FepE-For-PstI	tcc*ctgcag*caggtcatacggcatgccatc
FepE-Rev-EcoRI	gga*gaattc*tcagactaaccgttcatctatc
Control primers	
WzzB-DelCheck-For	aaaagtgtataccgcgatc
WzzB-DelCheck-Rev	agtgatgtagtggcattgag
Wzz-DelCheck-Rev2	ccaatgaagcgtctgatttg
Wzz-FepE-DelCheck-For	aaactatcgggcccatcatc
Wzz-FepE-DelCheck-Rev	tgttaagcgatctcaaccgc
RfaL-DelCheck-For	gctggctggcgcaaaatttg
RfaL-DelCheck-Rev	tattgtgccatctcaggttg

[a] Sequences complementary to chromosomal targets are in capital letters, and introduced restriction sites are in italics.

subcloned into the pBAD myc hisB-wzz_{ST} plasmid. The resulting plasmid, p3456, carries wzz_{fepE} upstream of wzz_{ST}, with both under the control of the P_{BAD} promoter.

Analysis of serum sensitivity and killing by polymyxin B. Sensitivity to polymyxin B was tested as described before, with slight modifications (15). Bacterial overnight cultures were diluted 1:31 in fresh LB with appropriate antibiotics and were grown at 160 rpm and 37°C for 3.5 h to late logarithmic phase (SPI1-inducing conditions). Cultures were washed twice with tryptone saline (0.5% Bacto tryptone [Difco], 0.9% NaCl) and diluted to 1×10^4 bacteria per ml in tryptone saline with or without various concentrations of polymyxin B (Sigma). The assay mixtures were incubated for 1 h at 37°C in a thermomixer (Eppendorf, Hamburg, Germany) at 550 rpm. Following the incubation period, the samples were serially diluted in tryptone saline and plated on Mueller-Hinton (MH) plates to determine numbers of viable bacteria. The survival rates of bacteria exposed to polymyxin B were compared to those of controls without polymyxin B in order to normalize all samples, and the percent survival of WT bacteria was calculated.

To determine the MIC of polymyxin B, Etest strips (bioMérieux, Deutschland GmbH, Nürtingen, Germany) were used. Overnight cultures of various strains in BBL MH II broth (Becton Dickinson GmbH, Heidelberg, Germany) were adjusted to a 0.5 McFarland standard in fresh medium. Bacteria were spread on dry MH plates by use of a sterile cotton swab according to the manufacturer's recommendation. The Etest strips were applied in the middle of the agar surface, and the plates were inoculated overnight at 37°C.

For analyses of the sensitivities of various *Salmonella* strains to serum complement, bacteria were grown under SPI1-inducing conditions as described above, washed twice in phosphate-buffered saline containing 5 mM $MgCl_2$ (PBSM), and diluted in PBSM to 1×10^8 CFU ml^{-1}. Normal human sera (NHS) of four healthy volunteers were pooled and diluted to 50% in PBSM. Heat-inactivated serum (HIS) was prepared by incubation for 30 min at 56°C and dilution to 50% in PBSM. To test serum sensitivity, 50 μl of the bacterial suspension was mixed with an equal volume of NHS or HIS and incubated for 60 min at 37°C as described by Bliska and Falkow (9). Serial dilutions of the samples were prepared in PBSM and plated onto MH plates to determine the number of viable bacteria. The percent survival of the WT strain was calculated as follows: $100 \times$ [mean (CFU ml^{-1} in NHS/CFU ml^{-1} in HIS) of mutant]/[mean (CFU ml^{-1} in NHS/CFU ml^{-1} in HIS) of WT].

Cell culture. The murine monocyte cell line RAW264.7 and the nonpolarized epithelial cell line HeLa were cultured in Dulbecco's modified Eagle's medium (DMEM) containing a high glucose concentration (4.5 g/liter), L-glutamine, and sodium pyruvate (PAA, Pasching, Austria) and supplemented with 10% fetal calf serum (Sigma-Aldrich, Seelze, Germany) as well as 2 mM Glutamax (Invitrogen, Karlsruhe, Germany). The canine kidney epithelial cell line MDCK was cultured in minimum essential medium (MEM; PAA) supplemented with penicillin-streptomycin (PAA) as described previously (19). All cell lines were maintained at 37°C in an atmosphere containing 5% CO_2 and 90% humidity.

Analysis of LPS structure. Bacteria grown on LB agar were inoculated into 3 ml of LB medium and grown for 16 h at 37°C. Subsequently, bacteria were subcultured (1:31 from LB overnight culture) for 3.5 h at 37°C. One hundred fifty microliters of bacterial culture was harvested by centrifugation for 3 min at $18,000 \times g$ and resuspended in lysis buffer (2% sodium dodecyl sulfate [SDS], 4% β-mercaptoethanol, 10% glycerol, 1 M Tris-HCl, pH 6.8, and bromophenol blue) according to the optical density at 600 nm (OD_{600}) (50 μl of lysis buffer per 1.6 OD_{600} units). Lysates were boiled at 100°C for 10 min, and subsequently, 2 U Turbo DNase was added to the chilled samples for 15 min at room temperature (RT). For protein digestion, samples were treated with 50 μg proteinase K (MBI-Fermentas) at 60°C for 1 h. LPS preparations were separated in 15% polyacrylamide gels, using a Laemmli buffer system at 25 mA (2 gels) for 1.5 h according to standard protocols (4), and were visualized by silver staining using the procedure described by Hitchcock and Brown (24).

Analysis of motility. The swimming and swarming behaviors of WT *Salmonella* and various mutants were analyzed using semisolid plates as described elsewhere (29). Briefly, the surfaces of swim plates containing 0.25% Difco Bacto agar and 0.5% glucose in LB were carefully pierced in the center with 0.2 μl of bacterial liquid overnight cultures. Swarm plates containing 0.5% Difco Bacto agar and 0.5% glucose in LB were inoculated with 0.5 μl of bacterial overnight cultures dropped on the surface of the plate. After incubation at 37°C for 5 to 6 h (swimming) or 12 to 13 h (swarming), the plates were photographed with a Canon EOS 450D camera.

Analyses of adhesion to and invasion of epithelial cells. MDCK cells were seeded in 24-well plates (Greiner Bio-One) at a density of 1×10^5 cells/well and allowed to polarize for 5 to 6 days, reaching a density of about 5×10^5 cells per well. Three hours prior to infection, culture MEM was replaced by fresh MEM without penicillin-streptomycin. HeLa cells were seeded in 24-well plates at a density of 5×10^5 cells per well 1 day before infection and allowed to duplicate. For infection under SPI1-inducing conditions, bacteria were subcultured 1:31 from an overnight culture in LB with appropriate antibiotics and incubated for 3.5 h at 37°C. The bacterial cultures were diluted in PBS to an OD_{600} of 0.2 (equivalent to 4×10^8 bacteria ml^{-1}). A master mix of the inoculum of approximately 2.5×10^6 bacteria per well (multiplicity of infection [MOI], 5) for MDCK infection or 1×10^6 bacteria per well (MOI, 10) for HeLa cell infection was prepared in MEM or DMEM, and 300 μl was added to each well. For the adhesion assay, the inoculum and the cells were prechilled on ice for 15 min in order to inhibit invasion. The infection procedure was performed on ice, with further incubation at 4°C for 30 min. Subsequently, the infected monolayer was washed twice with prechilled PBS and lysed with 500 μl per well of 0.5% sodium deoxycholate (for MDCK cells) or 0.1% Triton X-100 (for HeLa cells) for 15 min at 4°C. For the invasion assay, infection was performed for 30 min at 37°C, and subsequently, plates were washed twice with PBS. In order to allow bacterial invasion, cells were incubated for an additional 1 h at 37°C in MEM or DMEM containing 100 μg ml^{-1} gentamicin to kill extracellular bacteria. The cells were lysed with 500 μl per well of 0.5% sodium deoxycholate (for MDCK cells) or 0.1% Triton X-100 (for HeLa cells) for 5 min at 37°C. Serial dilutions of the inoculum and the lysates were plated onto MH plates to determine the number of intracellular bacteria. The percentages of adherent (number at 30 min versus number in inoculum $\times$ 100) and invading (number at 1 h versus number in inoculum $\times$ 100) bacteria were calculated.

For arabinose induction of strains containing p3456 prior to infection, overnight cultures were diluted 1:50 in fresh medium and incubated at 37°C for 1.5 h (OD_{600} of approximately 0.6 to 0.8) in a shaking air incubator at 150 rpm. The

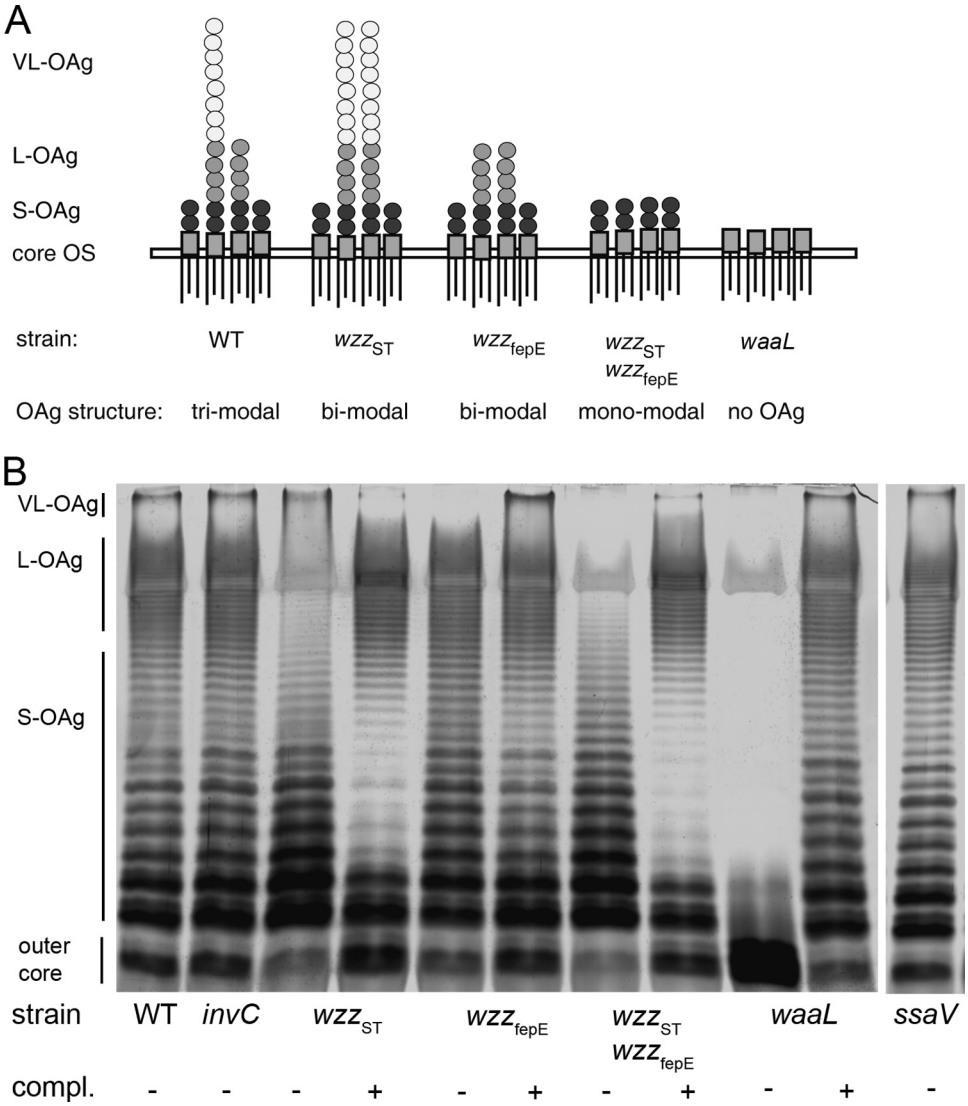

FIG. 1. Characteristics of LPS of *S. enterica* serovar Typhimurium strains used in this study. (A) Schematic representation of OAg in *S. enterica* serovar Typhimurium. WT *S. enterica* serovar Typhimurium processes LPS species with trimodal OAg lengths, i.e., VL-, L-, and S-OAg. A new set of isogenic mutant strains was generated, as well as low-copy-number plasmids for complementation of the mutations. The mutant strains deficient in the synthesis of the entire OAg (*waaL*) possesses only the core oligosaccharide (core OS) of LPS. Mutant strains defective in the regulators of L-OAg (wzz_{ST}) or VL-OAg (wzz_{fepE}) and a wzz_{ST} wzz_{fepE} double mutant strain contain LPS species with bimodal or monomodal OAg lengths, as indicated. (B) WT *Salmonella* and various mutant strains were grown in LB broth, and bacterial cells were processed for LPS analyses by SDS-polyacrylamide gel electrophoresis and silver staining. The mutant strains were complemented by plasmids harboring the deleted gene and the native promoters. The presence and absence of plasmids for complementation (compl.) are indicated by + and −, respectively.

cells were collected by centrifugation in a microcentrifuge for 10 min at 5,000 rpm and diluted 1:3 in LB containing 0.2% arabinose (P_{BAD} induction) or 0.2% glucose (P_{BAD} repression) as described by Khlebnikov et al. (28). Bacteria were grown for an additional 3.5 h at 37°C in order to obtain SPI1-inducing conditions.

Phagocytosis and intracellular replication in macrophages. RAW264.7 macrophages were seeded at a density of 2×10^5 cells per well into 24-well plates 24 h prior to infection and then allowed to duplicate. Bacteria were grown overnight in LB with appropriate antibiotics and stored at 4°C until use. Immediately prior to infection, the OD_{600} of the bacterial culture was measured and the bacteria were diluted in PBS to an OD_{600} of 0.2 (equivalent to 4×10^8 bacteria ml^{-1}). A master mix of the inoculum of approximately 4×10^5 bacteria per well (MOI = 1) was prepared in DMEM, and 300 μl was added to RAW264.7 cells. Infection was synchronized by centrifugation at 500 × *g* for 5 min, and macrophages were allowed to internalize bacteria for 25 min at 37°C. The cells were washed twice with PBS, and 500 μl/well DMEM containing 100 μg ml^{-1} gentamicin was added for 1 h to kill remaining extracellular bacteria. For the rest of the experiment, 500 μl/well DMEM containing gentamicin at 15 μg ml^{-1} was applied to the infected cells. At 2 h and 16 h postinfection, the macrophages were washed twice with PBS and lysed for 10 min at RT with 500 μl per well of 0.1% Triton X-100. Serial dilutions of the inoculum and the lysates were plated onto MH plates to enumerate the intracellular CFU. The percentage of phagocytosis (value at 2 h versus value for inoculum × 100) and *x*-fold intracellular replication (value at 16 h versus value at 2 h) were calculated.

Quantification of translocation of T3SS effector proteins. Analyses of the translocation of SPI1-encoded proteins were conducted as described by Schlumberger et al. (48). Briefly, 1 day prior to infection, 6×10^3 HeLa cells were seeded in 96-well half-area μclear plates (Greiner Bio-One), and if indicated, 50 μM cytochalasin D (Sigma-Aldrich) was added 30 min prior to infection to inhibit actin polymerization and *Salmonella* invasion. Cells were washed twice and infected with SPI1-induced *Salmonella* strains expressing a chromosomal $SipA_{M45}$-TEM1 fusion at an MOI of 100 for 30 min at 37°C (17). Cells were washed twice at room temperature with Hanks balanced salt solution containing

10% fetal calf serum and 100 μg ml^{-1} gentamicin. The infected cells were loaded with 1 μg ml^{-1} CCF2-AM (Invitrogen) according to the manufacturer's recommendation and incubated for another 3 h at RT. The fluorescence signals were detected via a Victor3 plate reader (PerkinElmer), with excitation at 405 nm and emission at 460 nm (blue fluorescence) as well as at 535 nm (green fluorescence). Background signals were subtracted, and the translocation of $SipA_{M45}$-TEM1 was quantified by calculating the blue/green ratio of the background-corrected data.

Analyses of SPI2 protein translocation in RAW264.7 cells infected with *Salmonella* harboring an SseJ-Luc fusion protein were performed as described before (20). Briefly, 8×10^5 RAW264.7 macrophages seeded in 24-well plates were infected for 30 min with *Salmonella* strains expressing the SseJ-Luc fusion at an MOI of 10. Infected cells were washed twice with PBS, and gentamicin at 100 μg ml^{-1} was added for 1 h and replaced by gentamicin at 15 μg ml^{-1} for the remaining experiment. At 15 h postinfection, macrophages were washed twice with PBS and lysed with 100 μl of eukaryotic lysis buffer (Roche). Ten microliters of the lysate was used to perform serial dilution series to determine the number of viable intracellular bacteria. The residual lysate was centrifuged, and aliquots of the supernatant were applied to 96-well microtiter plates (Microfluor; Dynatech). Luciferase activities were quantified using a TopCount instrument (PerkinElmer) and expressed in relative light units. The amount of translocated SseJ-Luc was calculated as the number of relative light units per intracellular bacterium.

Preparation and analyses of protein expression and secretion. For analyses of in vitro expression and secretion of either SPI1- or SPI2-encoded proteins in the background of the various OAg mutants, samples were prepared as follows. For induction of SPI1-encoded proteins, bacteria expressing a chromosomal *sipA*::M45 fusion were cultured overnight in LB with appropriate antibiotics, diluted 1:31 in fresh medium, and subcultured for another 3.5 h at 37°C. Samples of 2.5 ml of the bacterial cultures were harvested by centrifugation. Induction of SPI2 proteins was done according to the method of Coombes et al. (12). Briefly, *Salmonella* cells grown overnight in LB were washed twice in low-phosphate, low-magnesium medium (LPM) at pH 5.8 and diluted 1:50 in fresh LPM, pH 5.8. Cultures were grown for 7 h with shaking at 180 rpm, and 1.8 ml of culture was harvested by centrifugation.

The culture supernatants were filtered through a 0.2-μm filter (0.2-μm Acrodisc syringe filter with PES membrane; Pall Life Sciences), and 100 ng purified p39 (a gift from Gerhard Groer, Institute for Microbiology, Erlangen, Germany) was added as a precipitation control. Proteins were recovered by precipitation with trichloroacetic acid (TCA) (final concentration, 10% [wt/vol]) overnight at 4°C and centrifugation for 45 min at 18,000 × *g*. The pellet was washed twice with 1 ml cold acetone and recovered by centrifugation at 18,000 × *g* for 30 min. The air-dried final protein pellet of the supernatant as well as the bacterial cell pellet was solubilized with a volume of SDS sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 2% β-mercaptoethanol, 12.5% glycerol, and 0.01% bromophenol blue) adjusted according to the OD_{600} of the original culture.

Samples were separated by SDS-polyacrylamide gel electrophoresis on a Tris-Tricine system (45). For Western blot analysis, proteins were transferred onto 0.2-μm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) in Towbin buffer, using discontinuous semidry immunoblotting, as previously described (7).

The SPI1 protein SipA-M45 was detected via a monoclonal antibody against the M45 tag, and the SPI2 protein SseB was detected via antisera against recombinant SseB. Anti-DnaK antibody was used to control for equal loading of bacterial lysates as well as for release of cytosolic protein into the culture supernatant. Anti-p39 (a gift from Gerhard Groer) was used to detect purified p39 added to culture supernatants as a precipitation control.

IR800 and IR680 (gifts from Reinhard Voll) were used as secondary antibodies, and an Odyssey infrared imaging system (Li-Cor) was employed to visualize the infrared signals. The intensities of the protein bands were quantified by the Odyssey software, and the values were expressed as ratios of protein to loading control or protein to precipitation control and plotted on a graph.

Statistical analyses. Prism 4.0 software was used to calculate statistical significance. Student's *t* test was used for comparison of two groups, while the one-sample *t* test was used for comparison of the mean of a sample to a known value (see Fig. 6 and Fig. 7A and B).

RESULTS

Generation of mutant strains with defined defects in OAg length. The biosynthesis of the LPS of *S. enterica* serovar Typhimurium has been studied in detail, and gene functions for the addition of the OAg and for the regulation of the OAg chain length are known from previous studies (8, 33, 38, 39). To generate a set of isogenic mutant strains with defined defects in the OAg, wzz_{fepE} and wzz_{ST} were deleted, as regulators of VL-OAg and L-OAg, respectively. Furthermore, a double mutant in wzz_{fepE} and wzz_{ST} was generated that expressed only S-OAg. Finally, mutant strains were generated in *waaL* (previous designation, *rfaL*), which is required for the addition of the OAg chain to the outer core. The strains were grown in liquid media and processed for analyses of the LPS (Fig. 1). LPS profiles indicated that all mutant strains had the expected phenotypes with respect to OAg length. The *waaL* mutant

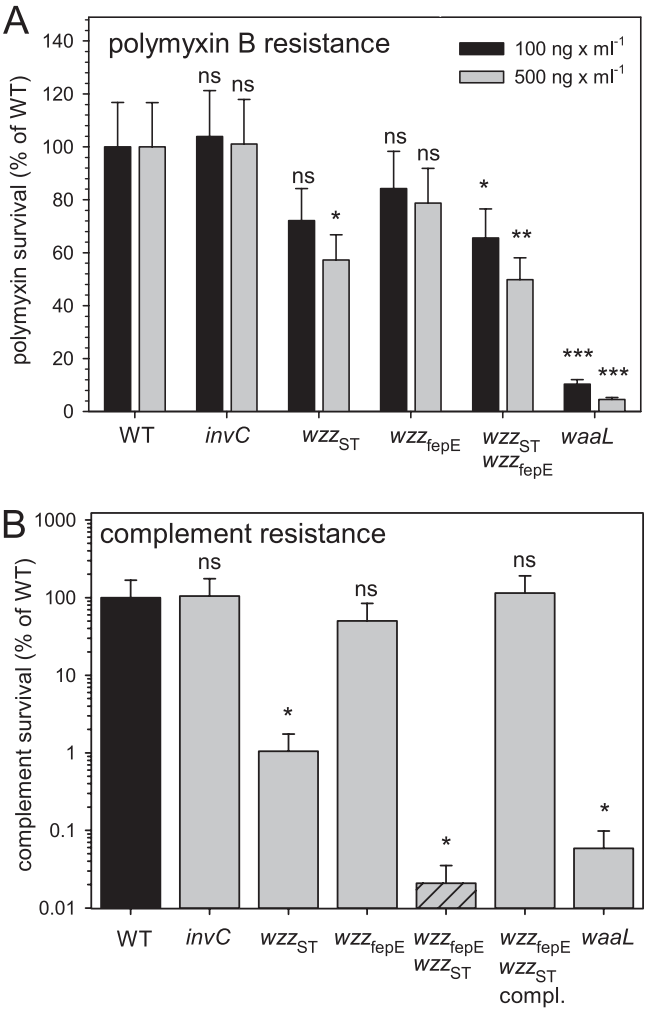

FIG. 2. Effect of OAg structure on resistance to polymyxin or complement. WT *S. enterica* serovar Typhimurium and various mutant strains with defects in OAg synthesis were incubated with buffer containing 100 or 500 ng ml^{-1} polymyxin B (A) or with 50% HIS or NHS (B). (A) After incubation for 60 min, serial dilutions of the suspensions were plated onto LB plates for the determination of the number of surviving bacteria. (B) For complement survival, the ratio of survival in NHS and HIS was calculated. Means and standard deviations for three assays are shown and are expressed as percentages of the survival of the WT strain. For statistical analysis, Student's *t* test was applied. The mutant strains were compared to the WT strain, and significance is expressed as follows: ns, not significant ($P \geq 0.05$); *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.01$.

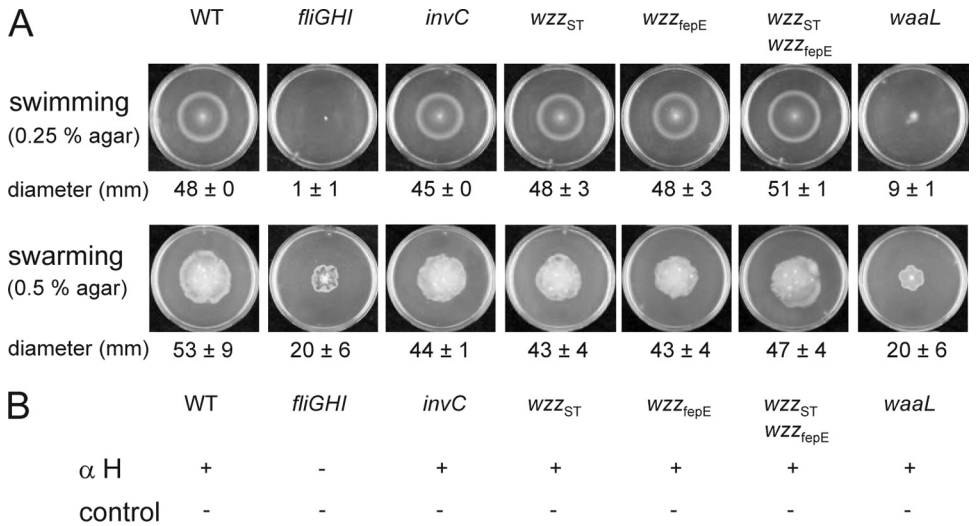

FIG. 3. Effects of variations in OAg length on flagellar assembly and motility of *Salmonella*. (A) WT *Salmonella* and various mutant strains were used to inoculate the center of agar plates containing swimming agar or swarming agar, containing 0.25 or 0.5% agar, respectively. The plates were incubated for 5 to 6 h (swimming) or 12 to 13 h (swarming), and the diameters of the bacterial growth zones were quantified. The mean diameter of the growth zone, in mm, and standard deviations are shown for three independent tests. (B) To test for the presence of the flagella, the various strains were also tested by slide agglutination with anti-H1-H1,2 antiserum against a flagellar protein. To control for the effect of OAg defects on bacterial aggregation, additional tests with anti-Hg,Hm antiserum against a flagellar protein of *S. enterica* serovar Enteritidis were performed. The presence and absence of agglutination are indicated by + and −, respectively.

strain entirely lacked OAg, the wzz_{fepE} wzz_{ST} double mutant expressed only S-OAg, and the wzz_{ST} and wzz_{fepE} strains were devoid of L-OAg and VL-OAg species, respectively. To restore their functions, the WT alleles of wzz_{ST} and/or wzz_{fepE} were cloned into low-copy-number plasmids under the control of their native promoters. Analysis of the mutant strains harboring the complementation plasmids indicated fully restored OAg length of the LPS (Fig. 1). We also analyzed growth rates of the various strains and did not observe differences in growth in rich medium (LB broth) or in minimal medium (PCN at pH 7.4 or 5.8) (data not shown).

Role of OAg length in *Salmonella* resistance to complement and polymyxin. For further functional characterization of the set of mutant strains, we determined the sensitivities of the various strains to polymyxin B, as a representative antimicrobial peptide, and to human serum complement (Fig. 2). The routinely applied Etest for polymyxin B indicated that the MIC for the WT strains, all mutant strains, and complemented mutant strains was 500 ng ml^{-1} polymyxin B (data not shown). As a more sensitive approach, the sensitivity toward polymyxin B was investigated in liquid medium (Fig. 2A). The strains lacking L-OAg, VL-OAg, or both L-OAg and VL-OAg exhibited a small increase in their sensitivity to polymyxin. In contrast, the *waaL* strain lacking the entire OAg showed a highly increased sensitivity, with about 5% survival after exposure to 500 ng ml^{-1} polymyxin B. The contribution of OAg length to complement resistance was more dramatic (Fig. 2B). A strain lacking VL-OAg was not affected in survival in serum. In accordance with a previous study (39), we observed that the mutant lacking L-OAg was about 100-fold more sensitive to complement-mediated lysis. Interestingly, we observed that the wzz_{ST} wzz_{fepE} mutant strain as well as the *waaL* mutant lacking the entire OAg showed similar highly increased (about 1,000-fold) sensitivities to complement killing.

Collectively, these data indicate that OAg is an important factor for the protection of *Salmonella* against polymyxin B and serum complement. With respect to the different modal lengths, L-OAg and VL-OAg have only minor contributions to resistance against polymyxin but an important function for resistance of *Salmonella* against serum complement.

Effect of altered OAg length on bacterial motility. We investigated if variations of the OAg length affect the function of the flagellar assembly system and compared the motilities of WT *Salmonella* and various mutant strains (Fig. 3). A mutation resulting in the loss of the entire OAg led to a highly reduced motility that was comparable to that for a flagellar defect caused by a deletion of the main flagellar subunit. As a simple test for the assembly of flagella, we performed slide agglutination with Hi-H1,2 antisera against flagellar protein. Agglutination was observed for the WT and all mutant strains with defects in OAg or T3SS. No agglutination was detected for a mutant strain defective in *fliGHI* (Fig. 3).

Host cell invasion by *Salmonella* is modulated by OAg length. During the interaction of *Salmonella* with the intestinal mucosa, adhesion to epithelial cells and host cell invasion are important virulence functions. We investigated the effect of OAg on this interaction (Fig. 4). Since our previous work indicated a profound difference in the invasion of nonpolarized and polarized epithelial cells (19), cell culture models of HeLa cells (nonpolarized) and MDCK cells (polarized) were used. The ability to adhere to HeLa cells or MDCK cells was not affected by the lack of L-OAg or VL-OAg and was slightly increased for a strain lacking both L-OAg and VL-OAg. The mutant strain lacking the entire OAg showed highly increased (about fivefold) rates of adhesion. The SPI1-mediated invasion was quantified by a gentamicin protection assay. A strain defective in the SPI1-T3SS was dramatically reduced in triggering its entry into HeLa cells as well as into MDCK cells. The wzz_{ST}

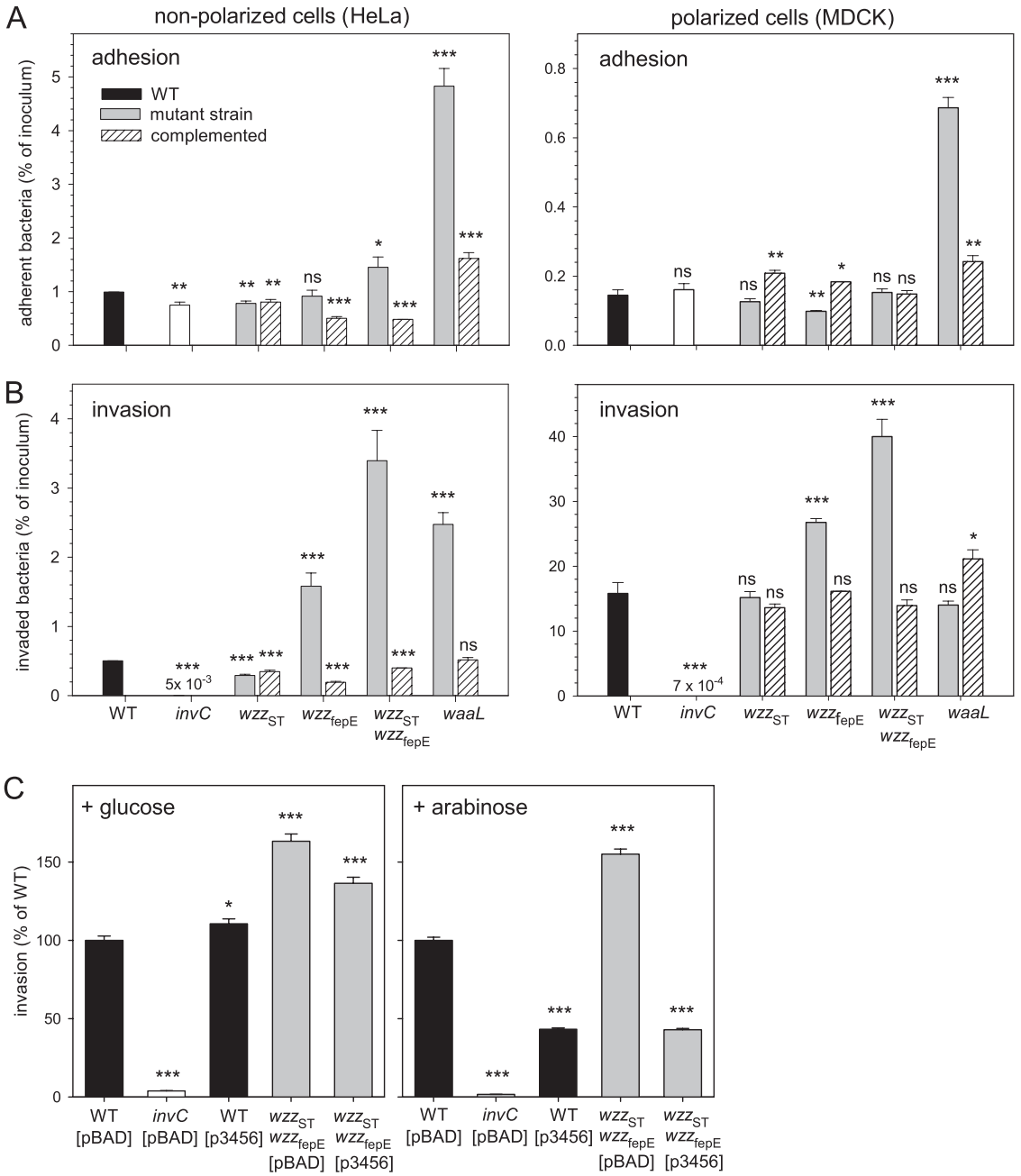

FIG. 4. Effect of altered OAg length on adherence to and invasion of epithelial cells. WT *Salmonella enterica* serovar Typhimurium (black bars), various mutant strains with a defect of the SPI1-T3SS (*invC*; white bars) or genes for OAg biosynthesis (gray bars), and complemented mutant strains (hatched bars) were used to infect epithelial cells. Infections were performed with nonpolarized cells (HeLa) (left) or polarized cells (MDCK) (right). (A) For quantification of adhesion, bacteria were allowed to adhere for 30 min at 4°C, and nonadherent bacteria were removed by washing. Subsequently, the cells were lysed and serial dilutions of the suspensions were plated onto LB plates for the quantification of adherent bacteria. (B) For quantification of invasion, bacteria were grown to late log phase and added to the cells at an MOI of 10 or 5 for HeLa or MDCK cells, respectively, followed by an incubation of 30 min at 37°C to allow invasion. Noninternalized bacteria were removed by washing, and the remaining extracellular bacteria were killed by addition of medium containing 100 μg ml^{-1} gentamicin for 1 h. Subsequently, the cells were washed and lysed to release internalized bacteria, and serial dilutions of the suspensions were plated onto agar plates for the quantification of intracellular bacteria. The numbers of adherent or invading bacteria are expressed as percentages of the inoculum, and means and standard deviations for triplicate experiments are displayed. (C) Overexpression of V-OAg and VL-OAg negatively affects invasion of epithelial cells. HeLa cells were infected with WT *S. enterica* serovar Typhimurium, the *invC* strain, or the wzz_{ST} wzz_{fepE} mutant strain harboring plasmid pBAD as a control or plasmid p3456 for the overexpression of wzz_{ST} and wzz_{fepE}. The bacteria were cultured in LB containing 0.2% glucose, as a noninducing control, or 0.2% arabinose, to induce overexpression of wzz_{ST} wzz_{fepE}, as indicated. Statistical analysis was performed as described in the legend for Fig. 2.

strain showed slightly reduced invasion, while the wzz_{fepE} strain had threefold higher rates of invasion. The most dramatic increase in invasiveness was observed for the wzz_{ST} wzz_{fepE} strain. This strain invaded HeLa and MDCK cells at 6.8-fold and 2.7-fold higher rates, respectively, than that of WT *Salmonella*. The presence of complementing plasmids again reduced the rate of invasion to the level of the WT strain. The *waaL* strain was also fivefold more invasive in HeLa cells, and this increase is similar to the increase in adhesion of this strain. Although increased adhesion to MDCK cells was observed for the *waaL* strain, the invasion of the polarized cells was similar to that of the WT strain.

Since invasion was increased in the absence of L-OAg and VL-OAg, we next investigated if invasion would be affected by overexpression of these LPS species. To test this hypothesis, a plasmid for the arabinose-inducible expression of wzz_{ST} and wzz_{fepE} was generated and introduced into the WT and wzz_{ST} wzz_{fepE} double mutant strains. We observed that invasion of the mutant strain was higher than that of the WT strain after growth under noninducing conditions. In contrast, induction of wzz_{ST} wzz_{fepE} expression by growth in arabinose-containing medium resulted in 3.6-fold decreased invasion. This result further demonstrates the correlation between OAg chain length and host cell invasion by *Salmonella*.

Our data show that adhesion to host cells is largely independent of the length of the OAg and that only a complete lack of the OAg increases binding to epithelial cells. The invasion of host cells is strongly affected by OAg length. The presence of L-OAg and VL-OAg appears to interfere with efficient entry into epithelial cells, but this effect is distinct from an altered adhesion to host cell surfaces. We did not observe major differences in the effect of OAg length during interaction with nonpolarized and polarized cells, but the effect on invasiveness was more pronounced for nonpolarized cells.

Alteration of OAg length does not affect intracellular replication of *Salmonella*. We analyzed the effect of alterations of OAg length on phagocytic uptake and intracellular replication of *Salmonella* in the macrophage-like cell line RAW264.7 (Fig. 5). The phagocytic uptake of *ssaV*, wzz_{ST}, and wzz_{fepE} strains was not significantly different from that of WT *Salmonella*. The uptake of the wzz_{ST} wzz_{fepE} double mutant was slightly higher, and the *waaL* strain was phagocytosed at about threefold higher rates (Fig. 5A).

The intracellular fate of *Salmonella* is critically dependent on the function of the SPI2-encoded T3SS and its effector proteins. Thus, we used a strain defective in the SPI2-T3SS as a control with reduced intracellular proliferation. As expected, the SPI2 strain showed highly reduced intracellular proliferation. In contrast, the intracellular replication of all mutant strains with altered OAg lengths was similar to that of the WT strain (Fig. 5B). These data indicate that the OAg length is not critical for the intracellular fate of *Salmonella* in macrophages.

OAg length affects translocation by the SPI1-T3SS but not by the SPI2-T3SS. The observation that OAg length influences host cell invasion but not intracellular proliferation prompted us to investigate the functions of SPI1-T3SS and SPI2-T3SS in strains expressing various OAg lengths. To analyze if the number of OAg repeats affects the function of the SPI1-T3SS or the SPI2-T3SS, we quantified the secretion and translocation of selected effector proteins of either T3SS.

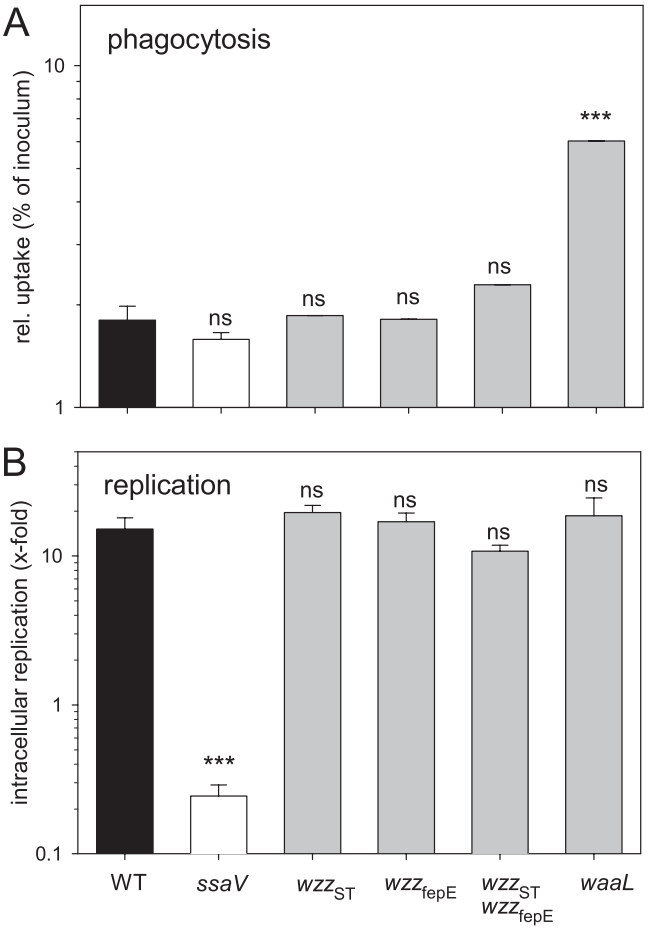

FIG. 5. Effect of altered OAg length on phagocytosis and intracellular replication of *S. enterica* serovar Typhimurium. RAW macrophages were infected at an MOI of 1 with WT *S. enterica* serovar Typhimurium, a strain deficient in the SPI2-T3SS (*ssaV*), or various mutant strains deficient in OAg biosynthesis. (A) For quantification of phagocytosis, the CFU of intracellular bacteria was determined 2 h after infection, and relative uptake was expressed as a percentage of the inoculum. (B) For quantification of intracellular replication, infected cells were lysed 2 h and 16 h after infection, and the CFU of intracellular bacteria was quantified. The *x*-fold change in intracellular replication is the ratio of CFU recovered at 16 h versus that recovered 2 h after infection. Means and standard deviations for triplicate assays are shown. Statistical analysis was performed as described in the legend for Fig. 2.

For the quantification of secretion by the SPI1-T3SS, we monitored SipA, a substrate protein with direct effector function. The synthesis of SipA under SPI1-inducing conditions was similar for the WT and for strains with altered OAg lengths (Fig. 6A). The secretion of SipA into the culture supernatant was similar for all mutant strains except for the wzz_{ST} strain, which exhibited a slight secretion defect of SipA (Fig. 6B). Only the *invC* strain, as expected, lacked secretion of SipA. To quantify translocation by the SPI1-T3SS, a fusion protein consisting of SipA and the TEM β-lactamase as a reporter was used. As previously described, the translocation of this hybrid protein can be followed by the conversion of a TEM substrate by a fluorescence resonance energy transfer-based assay (47). We observed that SipA translocation, corre-

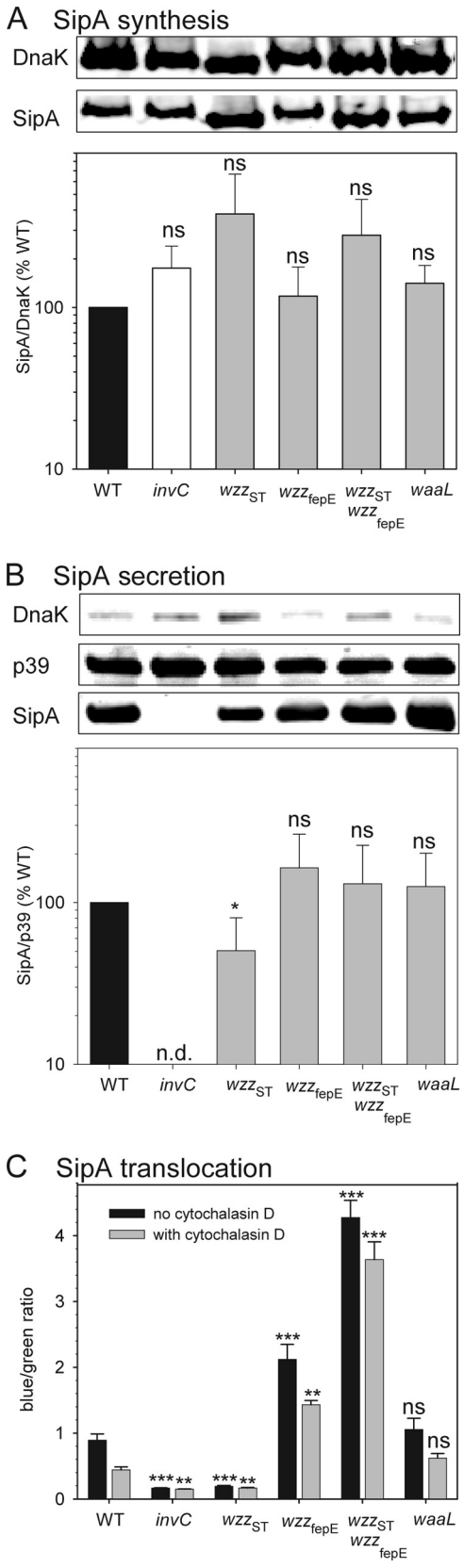

FIG. 6. Effect of altered OAg length on function of SPI1-T3SS. SipA was analyzed as a representative SPI1-T3SS substrate protein. (A) The synthesis of SipA by various strains was analyzed. Cultures were grown in LB broth, and cell lysates were subjected to Western blot analyses for SipA and for DnaK, as a constitutively expressed, nonsecreted protein. The histogram shows the quantification of the band intensities of DnaK and SipA. (B) To quantify secretion by the SPI1-T3SS, various strains were grown in LB broth, and protein in the culture supernatant was recovered by TCA precipitation and subjected to Western blot analysis. As a control for the recovery of protein, equal amounts of recombinant p39 were added to the supernatants prior to precipitation. The histogram shows the quantification of the band intensities of p39 and SipA. (C) Translocation of the fusion protein SipA-TEM into HeLa cells was quantified by a fluorescence resonance energy transfer-based assay. Infection of HeLa cells was performed with various strains, as indicated, at an MOI of 100, without cytochalasin D or in the presence of 50 μM cytochalasin D to inhibit internalization. Statistical analysis was performed as described in the legend for Fig. 2.

sponding to TEM activity, was very low for the *invC* and wzz_{ST} strains (Fig. 6C). Translocation by the *waaL* strain was similar to that by the WT, the wzz_{fepE} strain showed a >2-fold higher translocation rate, and about 4.5-fold higher reporter activities were detected for the wzz_{ST} wzz_{fepE} double mutant strain. The very low level of translocation of the SipA-TEM reporter by the wzz_{ST} strain was in contrast to the minor effect of this mutation on invasion (Fig. 4B) but correlates with the reduced SipA secretion in vitro (Fig. 6B). To test if the presence of the SipA-TEM reporter protein affects invasion, we compared the invasion by wzz_{ST} strains with and without the *sipA*::*tem* fusion (data not shown). Our data indicate that the presence of the reporter fusion results in reduced invasion. This interference was observed only for the wzz_{ST} strain, not for other mutant strains harboring the reporter construct.

For analysis of secretion by the SPI2-T3SS, we monitored SseB, a component of the translocon. The synthesis of SseB was affected neither by any of the alterations in the OAg nor by a defect in *ssaV*, encoding an essential subunit of the SPI2-T3SS (Fig. 7A). No secretion of SseB into the culture supernatant was observed for the *ssaV* strain (Fig. 7B). The secretion of SseB by the wzz_{ST}, wzz_{fepE}, and wzz_{ST} wzz_{fepE} strains was comparable to that of the WT strain, while secretion by the *waaL* strain was about fourfold lower (Fig. 7B). None of the strains released detectable amounts of the cytoplasmic protein DnaK, indicating that none of the OAg defects disturbed the integrity of the cell envelope. To quantify translocation by the SPI2-T3SS, we used a luciferase fusion to SseJ, an effector protein that is translocated in large amounts by intracellular *Salmonella* (22, 42). The use of a chromosomal fusion between SseJ and luciferase has been described previously (20). In order to compensate for any differences in the degree of intracellular replication of the various strains, the number of intracellular bacteria was determined and the luciferase activity was normalized to the number of intracellular bacteria. In accordance with our previous observations, we found that a mutation in *ssaV* resulted in a severe reduction of luciferase activity. The luciferase activities determined for lysates of cells infected with strains with altered OAg lengths were similar to that for WT-infected host cells. These results show that the function of the SPI2-T3SS of intracellular *Salmonella* is not affected by the length of the OAg.

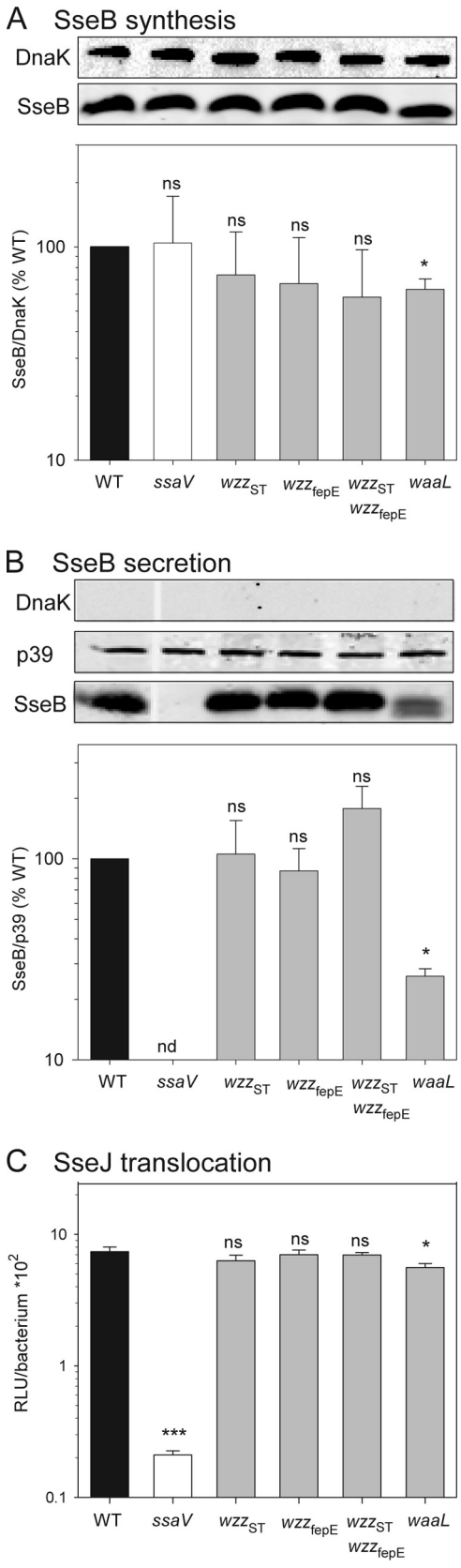

FIG. 7. Effect of altered OAg length on function of SPI2-T3SS. The secretion of SseB and translocation of SseJ, representative SPI2-T3SS substrate proteins, were analyzed. (A) Synthesis of SseB. Various strains were grown in LPM, and cell lysates were subjected to Western blot analyses for SseB and for DnaK, as a constitutively expressed, nonsecreted protein. The histogram shows the quantification of the band intensities of DnaK and SseB. (B) To quantify secretion by the SPI2-T3SS, various strains were grown in LPM, and protein in the culture supernatant was recovered by TCA precipitation and subjected to Western blot analysis. As a control for the recovery of protein, equal amounts of recombinant p39 were added to the supernatants prior to precipitation. The histogram shows the quantification of the band intensities of p39 and SseB. nd, not detectable. (C) Translocation of the fusion protein SseJ-Luc was quantified by a luciferase assay. RAW macrophages were infected with WT *Salmonella* or various mutant strains, each harboring a chromosomal *sseJ*::*luc* fusion. At 15 h after infection, host cells were lysed and intracellular bacteria recovered and processed for quantification of luciferase activity. In parallel, the number of intracellular bacteria was determined by plating of lysates onto agar plates. The relative translocation of the SseJ-Luc reporter fusion was expressed as luciferase activity (relative light units [RLU]) per intracellular bacterial cell, and means and standard deviations for triplicate samples were calculated. Statistical analysis was performed as described in the legend for Fig. 2.

DISCUSSION

The LPS constitutes a ubiquitous component of the cell envelopes of the large group of gram-negative bacteria. Despite its wide distribution, the LPS is highly variable in structure, and the most variability can be observed in the chemical structure and length of the OAg of the LPS. In this study, we systematically investigated the correlation between the OAg length of the LPS of *Salmonella enterica* serovar Typhimurium and the functions of the three T3SS of this pathogen.

The effect of OAg defects on complement resistance was in line with previous studies (21, 27). Early studies did not indicate a role of the OAg in resistance to antimicrobial peptides, and this resistance is thought to be independent of OAg but dependent on the lipid A portion of LPS. The functions of the T3SS were affected to different degrees by variations of the OAg length. Our data from Etest assays confirmed these results, but analyses in liquid culture suggested increase polymyxin B-mediated killing of wzz_{fep} wzz_{ST} and *waaL* strains. Similar observations have been made for *Erwinia carotovora* strains with defects in OAg (6). No change in flagellum-mediated motility was observed for mutants with truncated OAg, and only the *waaL* strain lacking the entire OAg was highly reduced in motility. This correlates with previous reports on the effect of a *waaL* mutation on the motility of *Pseudomonas aeruginosa* (1) or *Erwinia amylovora* (6). The SPI2-T3SS is essential for intracellular replication of *Salmonella* in cultured RAW264.7 macrophages. Intracellular replication was not affected by removal of L-OAg and VL-OAg or by the entire lack of the OAg. This finding is in accord with the intracellular function of the SPI2-T3SS, since translocation of a representative SPI2-T3SS effector was not affected by any of the modifications of the OAg length investigated here. If *Salmonella* is residing in the *Salmonella*-containing vacuole and closely surrounded by a vacuolar membrane, the presence of OAg would not affect the function of the SPI2-T3SS needle or the insertion of the translocon due to steric hindrance.

In contrast, we found that the OAg length affected extracellular *Salmonella* in a rather different manner. The adhesion to

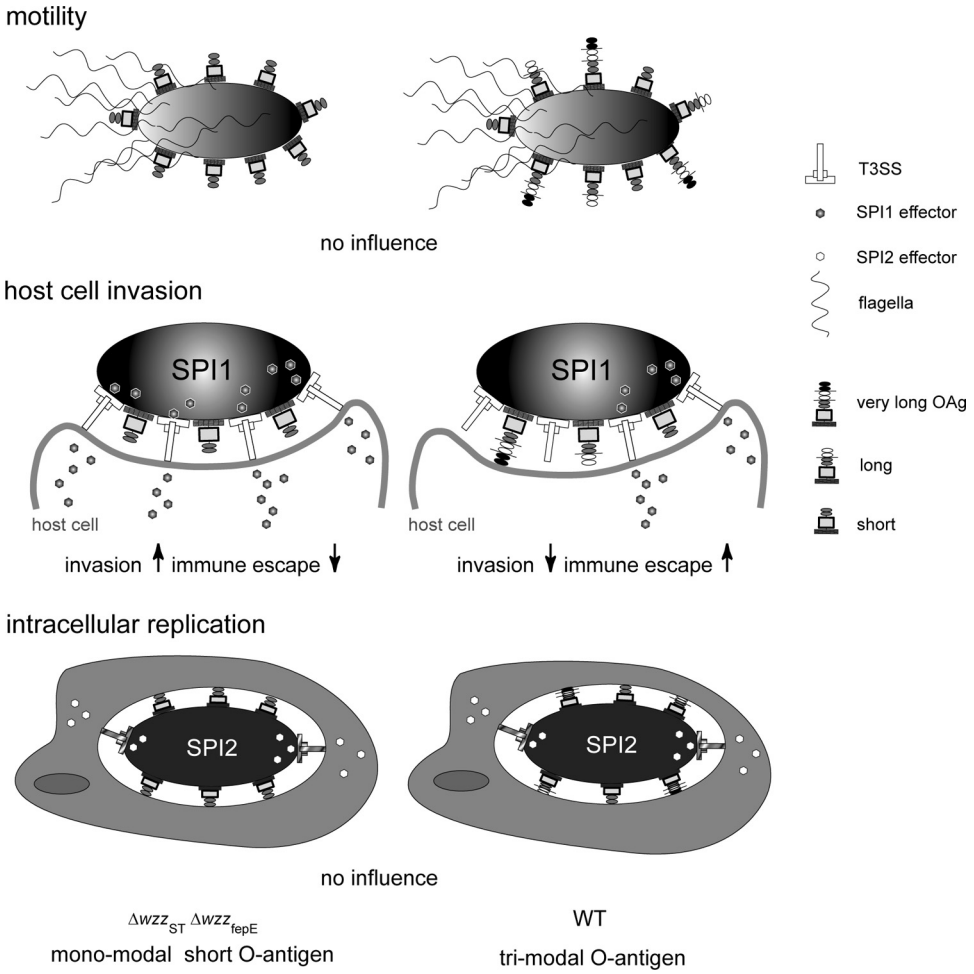

FIG. 8. Model for effects of LPS OAg length on functions of T3SS. The efficiency of invasion of host cells is impaired by the presence of LPS species with L- and VL-OAg. However, the presence of these LPS species provides protection against antimicrobial factors of the host. In contrast, the OAg length does not interfere with the function of the SPI2-T3SS during the intracellular life of *Salmonella* and has no effect on flagellum-mediated motility.

and invasion of epithelial cells were strongly influenced by truncations of the OAg. This finding may indicate that WT *Salmonella* synthesizes OAg that negatively affects invasion. However, the protection mediated by the long and very long forms of OAg is likely more important for adaptation of intestinal colonization, or invasion is already very efficient, so that a compromised invasion may be tolerated.

Currently, alternative roles of *Salmonella* invasion are being discussed. Ackermann et al. (2) proposed that invasion by a proportion of the intestinal *Salmonella* organisms is important to trigger an inflammatory response that affects the competing microbial flora. This effect requires invasion by only a certain subpopulation of *Salmonella* and would be compatible with a suboptimal invasiveness. The work of Gerlach et al. (19) revealed that the interaction between *Salmonella* and polarized epithelial cells is more complex than initially thought. The presence of the apical brush borders appears to be an efficient barrier, and *Salmonella* requires the function of the SPI4-encoded large adhesin SiiE to efficiently adhere to and subsequently invade polarized cells. The divergent roles of OAg species of *Salmonella enterica* serovar Typhimurium in motility, host cell invasion, and intracellular replication are also summarized in Fig. 8.

In contrast to observations by Murray et al. (38), we could not detect an influence of OAg length on uptake by macrophages. In our setting, only mutant strains lacking the OAg were taken up in significantly larger amounts by phagocytic cells. An experimental difference may be the competition invasion/uptake assay performed by Murray et al., in contrast to the conventional phagocytosis assay performed in our study. We also failed to observe the reduction of OAg length in intracellular *Salmonella* (data not shown) that has been reported previously (32) and is thought to be dependent on the PmrA/PmrB and RcsC/YojN/RcsB regulators (16). It is likely that modifications resulting in OAg truncation occur late in the intracellular life cycle (>16 h postinfection). The function of the SPI2-T3SS in host cell modification is more important in the initial intracellular phase (2 to 6 h postinfection). Any interference with SPI2-T3SS function by OAg would be critical in this phase.

In contrast to *Salmonella*, *Shigella* possesses only two modal forms of the OAg. The synthesis of S-OAg chains with a modal

length of 11 to 17 repeat units is mediated by Wzz_{SF}. This function is required for sufficient expression of the cell surface protein IcsA, which mediates F-actin comet tail formation and cell-to-cell spread (43). *Shigella* OAg is also thought to partially mask IcsA (37). The VL-OAg, containing more than 90 repeat units, is determined by Wzz_{pHS2} (Cld_{pHS-2}), which is encoded on the pHS-2 plasmid. This OAg type is required for serum resistance and enhances the Sereny reaction in mice (26, 36). Previous work (52) demonstrated that a modification of the OAg in *Shigella flexneri* is required for the optimal balance between invasion and resistance against antimicrobial factors. This modification was mediated by glucosylation of OAg. Mutant strains in *gtr* deficient in LPS glucosylation are highly attenuated in virulence in a rabbit ligated ileal loop model. It was also found that *gtr* mutants show an about 10-fold reduced invasion compared to the WT strain with glucosylated OAg, and ultrastructural analyses indicated that the needle structures assembled by the *Shigella mxi*/*spa* T3SS are less accessible at the cell surface of a *gtr* strain. Glucosylation of OAg has been reported for *S. enterica* serovar Typhimurium as well (10), but the resulting O12-2 OAg variant appears to be important mainly for immune evasion, and the effect on OAg length has not been investigated.

Our data show that the functional constraints on OAg are similar in *Shigella* and *Salmonella*, but both species evolved different mechanisms to modify the OAg to balance between resistance against antimicrobial effectors and host cell invasion. In both organisms, the resistance against antimicrobial effectors appears most important, and a reduction of the efficiency of invasion is tolerated.

It was recently shown that changes in the OAg structure of *P. aeruginosa* also affect the secretion of T3SS effectors, such as *exoS*, *exoT*, or *pcrV*, the cytotoxic effect mediated by *Pseudomonas*, and the severity of disease in an animal model (3). In contrast, we did not detect altered expression levels or amounts of secreted effector proteins as a consequence of OAg defects, but we saw altered characteristics of adhesion, invasion, and effector proteins in the case of OAg-deficient *Salmonella*.

At present, it is not known if the different LPS species are homogenously distributed in the outer membranes of *S. enterica* and related species or if membrane microdomains exist that are enriched for S-OAg, L-OAg, or VL-OAg. Such a distribution could minimize the interference of the OAg with the functions of T3SS that may be located in regions with smaller amounts of L-OAg and VL-OAg. Unfortunately, the inability to directly visualize LPS by electron microscopy hampers the analysis of the distribution of LPS species within the outer membrane.

In conclusion, our study demonstrates that the presence of three length variants of OAg of LPS allows *S. enterica* serovar Typhimurium to balance between defensive and offensive virulence functions required for adaptation to various niches within the eukaryotic host. A major challenge for future research will be the visualization of the bacterial cell surface with approaches that maintain the integrity of the carbohydrates.

ACKNOWLEDGMENTS

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