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Deletion of Amino Acids 29 to 81 in Adhesion Protein YadA of *Yersinia enterocolitica* Serotype O:8 Results in Selective Abrogation of Adherence to Neutrophils

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In order to analyze the multiple functions of the yersinia adhesin YadA in more detail, we constructed an N-terminally truncated YadA protein (deletion of amino acids [aa] 29 to 81) of *Yersinia enterocolitica* serotype O:8. The region aa 29 to 81 of YadA is located between the signal sequence and the amino-terminal hydrophobic domain (aa 80 to 101), which is involved in surface polymerization and collagen binding. The deletion of aa 29 to 81 (resulting in $YadA_{\Delta 29\text{-}81}$) had no effect on the well-known features of YadA such as autoagglutination, serum resistance, HEp-2 cell adherence, binding of collagen, and binding of the complement-inhibiting factor H. In contrast to this, mutant WA(pYVO8-$A_{\Delta 29\text{-}81}$), producing the truncated $YadA_{\Delta 29\text{-}81}$ had lost the ability to adhere to polymorphonuclear leukocytes and to induce an oxidative burst. This functional deficiency was comparable to that of a *yadA*-null mutant (K. Ruckdeschel, A. Roggenkamp, S. Schubert, and J. Heesemann, Infect. Immun. 64:724–733, 1996). Moreover, mutant WA(pYVO8-$A_{\Delta 29\text{-}81}$) turned out to be attenuated in virulence comparably to the *yadA*-null mutant, as demonstrated with orogastrically and intravenously infected mice. In summary, this study shows that specific functions of YadA (i) can be impaired by designed mutations and (ii) are important in distinct stages of the infection process.

The two human enteropathogenic species *Yersinia enterocolitica* and *Y. pseudotuberculosis* cause food-borne disease with a broad spectrum of intestinal and extraintestinal manifestations (9). The process of infection has been studied in mouse infection models (6). A few hours after peroral uptake, the yersiniae are detectable inside Peyer's patches in the gut. Here they are able to resist the nonspecific immune defense of the host and to multiply extracellularly (13, 26, 42). A 70-kb virulence plasmid (pYV) is essential for the pathogenicity of the *Yersinia* species. This plasmid encodes a number of secreted proteins called Yops (Yersinia outer proteins) (5, 47), which are thought to disturb the innate immune response in diverse ways. At least two of the Yops (YopE and YopH) are translocated into host cells after adherence of the bacterium (4, 33, 38, 46). The intracellular activity of YopE and YopH results in inhibition of phagocytosis, cytotoxicity, and probably dysregulation of signal transduction pathways (3, 36, 37). The V antigen interferes with the immune response by suppression of cytokines (30). This effect can be neutralized by anti-V antigen serum (25), indicating that V antigen is probably not translocated into host cells.

Cell adherence is mediated by the chromosomally encoded invasin Inv and the plasmid-encoded surface protein YadA (yersinia adhesin) (15, 20). The expression of *yadA* and *inv* is temperature regulated. *yadA* expression depends on the AraC-like activator VirF, encoded by pYV, which upregulates *yadA* at 37°C (24). In contrast, *inv* is maximally expressed at 27°C and downregulated at 37°C (21, 32). *yadA* genes of *Y. pseudotuberculosis* serotype III and *Y. enterocolitica* serotypes O8 and O3 have been sequenced, and a 41.3- to 44.4-kDa polypeptide has been predicted, whose size agrees well with those of the 45- to 50-kDa bands of YadA in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (44). YadA forms a fibrillar matrix covering the outer membrane of the pathogen (22). Multiple functions are ascribed to YadA in vitro, of which the most relevant are adhesion to epithelial cells (15) and to extracellular matrix (ECM) proteins such as collagen (10, 41), cellular fibronectin (40), and laminin (48) and interaction with intestinal brush border vesicles and mucus (31) and with guinea pig erythrocytes (22). YadA binds the serum complement-inhibiting factor H (8), a feature that may enable yersiniae to resist the bacteriolytic effect of human serum (2, 34). The phenomenon of autoagglutination depends on surface localization and correct polymerization of YadA (43, 48). Moreover, accumulating evidence suggests that YadA recognizes a specific receptor on polymorphonuclear leukocytes (PMN), leading to rapid interaction and extracellular localization of yersiniae on PMN (7, 39). The importance of YadA for the virulence of *Y. enterocolitica* has been demonstrated in the mouse infection model. A YadA-negative mutant of *Y. enterocolitica* serotype O:8 is avirulent in intragastrically infected mice and showed significant attenuation in intravenously challenged mice (35, 48).

To elucidate the contribution of YadA in the infection process in more detail, *yadA* mutants have been created by site-directed mutagenesis. Deletion of the hydrophobic domain of amino acids (aa) 80 to 101 (48) and substitution of the histidine residues by tyrosine at aa positions 156 and 159 (35) in the *yadA* gene of *Y. enterocolitica* serotype O:8 revealed that functions of YadA such as binding to ECM proteins and/or HEp-2 cells are important for virulence. Additionally, the hydrophobic domain of aa 80 to 101 is necessary for surface polymerization of YadA. In contrast to this, deletion of the hydrophobic domain in the carboxy-terminal part of YadA suggests that

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
Strains		
WA-314	*Y. enterocolitica* serotype O:8, clinical isolate, pYVO8$^+$	17
WA-C	pYVO8$^-$ derivative of WA-314	17
DH5α	*E. coli endA1 supE44 hsdR17* (r^-_K m^+_K) *thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*) *U169* (ϕ80*lacZ*ΔM15)	12
Sm10 λpir	*E. coli* K-12 *thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu-Km(λpir)	29
Plasmids		
pUC-A-1	pUC13 carrying *yadA* of pYVO8 as a 5-kb *Eco*RI-*Hin*dIII fragment	35
pUC-A-0	pUC-A-1, *yadA* gene inactivated by insertion of a Km-Genblock	35
pUC-$A_{\Delta 29\text{-}81}$	pUC-A-1, bp 437–595 in *yadA*, (corresponding to aa 29–81 of YadA) deleted	This study
pGP704	Suicide vector, R6K replicon	29
pRK290B	Derivative of pRK290, RK2 replicon, IncP	18
pHP45Ω	Origin of the Spc cassette	P. J. Sansonetti
pGP-$A_{\Delta 29\text{-}81}$	pGP704, carrying the *yadA*$_{\Delta 29\text{-}81}$ gene as an *Eco*RI-*Sph*I fragment	This study
pGPS-$A_{\Delta 29\text{-}81}$	pGP-$A_{\Delta 29\text{-}81}$, carrying a 1.8-kb Spc cassette in the *Eco*RI site	This study
pYVO8	Virulence plasmid of WA-314	17
pYVO8-A-0	pYVO8, *yadA* inactivated by a Km cassette	35
pYVO8-A-1	pYVO8-A-0 with integrated pGPS-A-1	35
pYVO8-$A_{\Delta 29\text{-}81}$	pYVO8-A-0 with integrated pGPS-$A_{\Delta 29\text{-}81}$	This study
pB8-5	pRK290B with the 5-kb *Bam*HI fragment of pYVO8 carrying *virF*	This study

this region is involved in the transport of YadA into the outer membrane (48).

In the present study we deleted aa 29 to 81 of the YadA protein of *Y. enterocolitica* serotype O:8. This part of YadA is located between the signal sequence (aa 1 to 25) and the amino-terminal hydrophobic domain (aa 80 to 101) and is presumably not involved in transport and correct surface conformation of YadA. As expected, the deletion of aa 29 to 81 did not affect the surface localization and polymerization of YadA but had a differential effect on the multiple functions of YadA. Autoagglutination, attachment to ECM proteins and HEp-2 cells, and binding of factor H remained unchanged, whereas the interaction of YadA with PMN was disturbed by this truncation. The altered interaction with PMN was accompanied by loss of virulence in the experimental mouse model.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains used in this study are listed in Table 1. *Escherichia coli* DH5α was used for the construction of YadA$_{\Delta 29\text{-}81}$. Bacteria were grown in Luria-Bertani medium: *Y. enterocolitica* at 27°C, *E. coli* at 37°C. For the induction of *yadA* expression, overnight cultures at 27°C were diluted 1:20 in Luria-Bertani medium and grown at 37°C for 2 h (for binding assays and chemiluminescence [CL] experiments) or 6 h (for preparation of outer membrane proteins). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin, 25 μg/ml; nalidixic acid, 60 μg/ml; spectinomycin, 50 μg/ml.

DNA manipulations. Plasmid DNA preparations were isolated with Qiagen kits (Qiagen, Hilden, Germany) as recommended by the manufacturer. Restriction enzyme digestions, recovery of DNA from low-melting-point agarose, DNA ligations, and transformations were performed as described by Ausubel et al. (1). Enzymes, deoxynucleoside triphosphates, and *Taq* polymerase were purchased from Pharmacia LKB (Uppsala, Sweden). Oligonucleotides were synthesized by Roth (Karlsruhe, Germany) in the TRITYL-OFF mode.

PCR was done with an annealing temperature of 55°C as described elsewhere (35). The nucleotide sequence of the mutant *yadA* gene was determined by the TaqDyeDideoxy terminator method using the 373A DNA sequencer (Applied Biosystems Gmbh, Darmstadt, Germany).

Construction of YadA$_{\Delta 29\text{-}81}$. Deletion of aa 29 to 81 from YadA of *Y. enterocolitica* serotype O:8 was achieved by removing the *Cla*I-*Pst*I fragment of the *yadA* gene (spanning bp 195 to 600; GenBank accession no. X13881) and by subsequently integrating an appropriate PCR fragment (bp 195 to 436) with *Cla*I and *Pst*I restriction sites at the ends, as outlined in Fig. 1. pUC-A-1 harboring the *yadA* gene and the flanking regions as a 5-kb *Eco*RI-*Hin*dIII fragment (35) was digested with *Cla*I (cutting at bp positions 177 and 194) and *Pst*I (cutting at bp positions 600 and 1476). The pUC-A-1 fragment missing bp 178 to 1476 of the *yadA* gene (pUC-A$_{\Delta bp\ 178\text{–}1476}$) and the *Pst*I fragment (bp 601 to 1476, an interior part of the *yadA* gene) were purified in low-melting-point agarose gel. A PCR

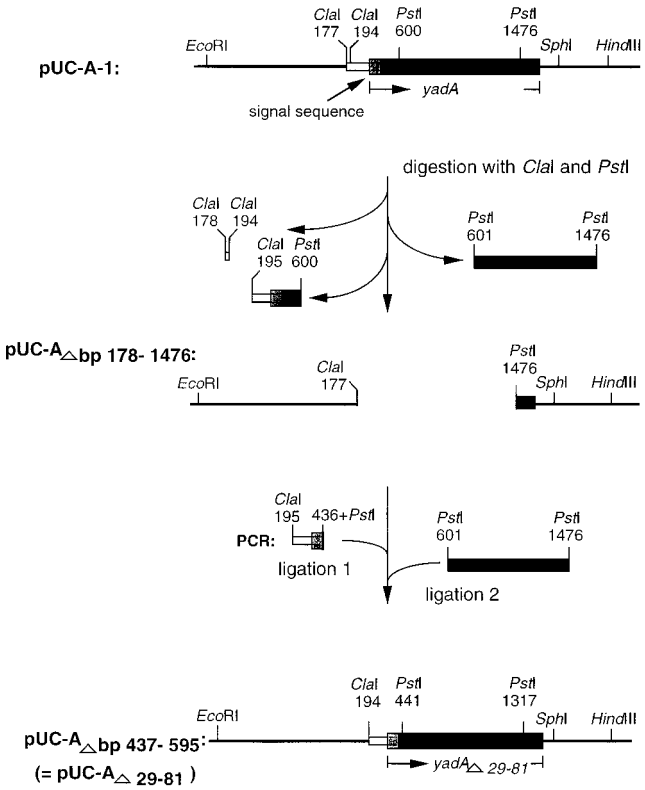

FIG. 1. Schematic drawing of the construction of pUC-A$_{\Delta 29\text{-}81}$. The restriction digest of plasmid pUC-A-1 with *Cla*I and *Pst*I resulted in four fragments. The *Cla*I-*Pst*I fragment pUC-A$_{\Delta bp\ 178\text{–}1476}$ and the *Pst*I-*Pst*I fragment (bp 601 to 1476) were purified in low-melting-point agarose. An appropriate PCR product (spanning positions 195 to 436) with *Cla*I and *Pst*I restriction sites at its ends was ligated first into pUC-A$_{\Delta bp\ 178\text{–}1476}$, followed by the *Pst*I-*Pst*I fragment (bp 601 to 1476). The resulting plasmid has a deletion in the *yadA* gene spanning bp 437 to 595 (aa 29 to 81) and was named pUC-A$_{\Delta 29\text{-}81}$. The *yadA* gene (start codon at position bp 352) and its signal sequence are indicated. The restriction sites for *Cla*I, *Eco*RI, *Pst*I, *Sph*I, and *Hin*dIII are marked with their cutting positions according to GenBank sequence X13881.

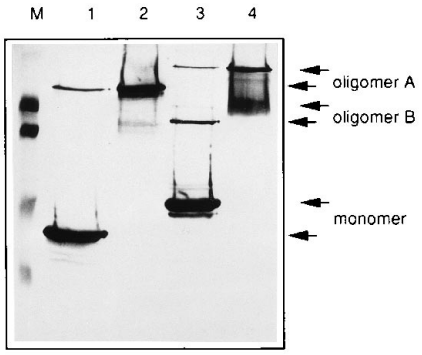

FIG. 2. Immunoblot for detection of YadA and YadA$_{\Delta 29\text{-}81}$. Outer membrane preparations of strain WA(pYV-A$_{\Delta 29\text{-}81}$) (lanes 1 and 2) and strain WA-314 (lanes 3 and 4) were resuspended in electrophoresis buffer. One-half of each sample was boiled for 5 min (lanes 1 and 3); the other half was incubated at 37°C for 2 h (lanes 2 and 4). The four samples were separated on SDS-PAGE and transferred to nitrocellulose sheets. The YadA-specific monoclonal antibody 8D1 was used for detection of YadA. After incubation at 37°C, besides oligomeric form A (~200 kDa), oligomeric form B (~116 kDa) of wild-type YadA (lane 4) and, in reduced amount, oligomeric form B of YadA$_{\Delta 29\text{-}81}$ (lane 2) are visible at the expected size. Lane M, marker proteins of 116, 96, 48, and 37 kDa.

product spanning bp positions 195 to 436 of the *yadA* gene (upper primer, TTTTTAAGATCGATTAGTGCTGT; lower primer with a *Pst*I restriction site [underlined] at the end, TCTCCTGCAGCGTCATTATTGGCAAATGCATA) was cut with *Cla*I and *Pst*I and ligated into pUC-A$_{\Delta bp\ 178\text{–}1476}$, resulting in pUC-A$_{\Delta bp\ 437\text{–}1476}$. In a final step, the interior *Pst*I fragment of the *yadA* gene (bp 601 to 1476) was ligated back into pUC-A$_{\Delta bp\ 437\text{–}1476}$, generating pUC-A$_{\Delta bp\ 437\text{–}595}$ or pUC-A$_{\Delta 29\text{-}81}$. The deletion of bp 437 to 595 in the *yadA*$_{\Delta 29\text{-}81}$ gene was verified by sequencing. This PCR-based mutation strategy also caused the deletion of bp 178 to 194, located between the two neighboring *Cla*I restriction sites (44). The deletion of the 17 bp occurred 150 bp upstream of the start codon of the *yadA* gene. The function of this noncoded region is unknown. The promoter region (the −10 region is located 253 bp upstream of the *yadA* start codon [44]) remained unaltered. As expected, the production and outer membrane localization of YadA$_{\Delta 29\text{-}81}$ did not appear to be affected in comparison with wild-type YadA (see below).

Expression of *yadA*$_{\Delta 29\text{-}81}$ in *E. coli* and *Y. enterocolitica* O:8. In yersinae the expression of *yadA* is positively regulated by VirF (24). To achieve a high expression of *yadA* in *E. coli*, we cloned the 5-kb *Bam*HI fragment of pYVO8 encoding VirF in the low-copy-number vector pRK290B (18), generating pB8-5. Plasmid pB8-5 was conjugated into *E. coli* strains harboring wild-type YadA or mutated YadA on a multicopy plasmid, as described elsewhere (18). The production of YadA in *E. coli* was verified by SDS-PAGE and Western blotting (immunoblotting) (data not shown). For expression of *yadA*$_{\Delta 29\text{-}81}$ in *Y. enterocolitica* we transferred the *yadA*$_{\Delta 29\text{-}81}$ gene into the YadA-negative strain WA(pYVO8-A-0) (35). The *yadA*$_{\Delta 29\text{-}81}$ gene fragment was cut out of pUC-A$_{\Delta 29\text{-}81}$ with *Eco*RI and *Sph*I and ligated into the mobilizable suicide vector pGP704, resulting in pGP-A$_{\Delta 29\text{-}81}$. For pGP704 derivatives *E. coli* SM10 λpir was used as the host strain. In order to obtain a better selection, a 1.8-kb Sm^r Spc^r Ω fragment was subsequently ligated into the *Eco*RI site of pGP-A$_{\Delta 29\text{-}81}$, resulting in pGPS-A$_{\Delta 29\text{-}81}$. Plasmid pGPS-A$_{\Delta 29\text{-}81}$ was mobilized into WA(pYVO8-A-0) by conjugation and inserted into pYVO8-A-0 by homologous recombination. Transconjugants harboring cointegrates (pYVO8-A-0::pGPS-A$_{\Delta 29\text{-}81}$) were selected for nalidixic acid, kanamycin, and spectinomycin resistance. The resulting clones [named WA(pYVO8-A$_{\Delta 29\text{-}81}$)] were characterized by PCR and restriction enzyme analysis. The integration of the suicide vector pGPS-A$_{\Delta 29\text{-}81}$ occurred upstream of the *yadA*::Km gene.

Immunoblotting. YadA-containing outer membrane preparations were obtained as described elsewhere (16) and resuspended in electrophoresis buffer (1% SDS and 0.25% 2-mercaptoethanol). The samples were boiled for 5 min or incubated at 37°C for 2 h and separated by discontinuous SDS-PAGE (11% polyacrylamide) (23). The proteins were electrophoretically transferred to nitrocellulose sheets (BA85; Schleicher and Schüll, Inc.) (49) and blocked with 3% bovine serum albumin fraction V in phosphate-buffered saline (PBS) overnight at 4°C. For immunostaining of YadA, the YadA-specific monoclonal antibody 801 was used (35) with subsequent incubation with anti-mouse immunoglobulin G-alkaline phosphatase conjugate (Sigma) as described elsewhere (14).

Immunofluorescence. To analyze the surface exposure of YadA$_{\Delta 29\text{-}81}$, WA(pYVO8-A$_{\Delta 29\text{-}81}$) and WA-314 were grown at 37°C for 6 h, harvested by centrifugation, and washed with PBS. Bacteria were incubated with rabbit anti-YadA serum [preabsorbed with the YadA-negative strain WA(pYVO8-A-0)] at 37°C for 45 min. After being washed three times with PBS, bacteria were coated onto glass slides and fixed with methanol (−20°C). Fluorescein-conjugated anti-rabbit immunoglobulin (Sigma) was used to detect surface bound anti-YadA.

ECM binding, HEp-2 cell adherence, serum resistance, and autoagglutination. The assays for studying YadA-mediated binding to ECM proteins and to HEp-2 cells were done as described elsewhere (35) and carried out in an *E. coli* background. Collagens I and IV were purchased from Sigma; laminin was purchased from ICN. The test for survival in 50% normal human serum (NHS) was done as described elsewhere (17); the autoagglutination assay of yersiniae was performed as described by Kapperud et al. (22).

Affinity blot of YadA to factor H. The affinity blot was done with modifications as described elsewhere (8). Briefly, outer membrane protein preparations of WA-314 or WA(pYVO8-A$_{\Delta 29\text{-}81}$) were separated by SDS-PAGE and electrotransferred to nitrocellulose sheets (49). Strips of these nitrocellulose sheets were incubated with 10% NHS diluted in PBS for 3 h at 27°C. After three washes with PBS, 0.5% Tween 20-bound factor H was visualized with purified rabbit anti-factor H serum diluted 1:100 and subsequent incubation with anti rabbit immunoglobulin G-alkaline phospatase conjugate (14). Anti-factor H serum and purified human factor H were gifts from Rolf D. Horstmann (Bernhard-Nocht-Institut, Hamburg, Germany). For affinity purification of the anti-factor H serum, factor H was subjected to SDS-PAGE and electrotransferred to nitrocellulose. Nitrocellulose strips containing the immobilized factor H were used as an affinity matrix for purification of anti-factor H serum as described elsewhere (45).

CL, phagocytosis, and killing assays with PMN. PMN were isolated from peripheral blood obtained from healthy volunteers by a one-step separation method using the Mono-Poly resolving medium (Flow Laboratories, Irvine, United Kingdom) (11). The activation of the neutrophil oxidative burst was measured as luminol-enhanced CL and monitored with a microplate-chemiluminometer (Hamamatsu Photonics, Herrsching, Germany) as described elsewhere (39). Bacteria were tested unopsonized or preopsonized with 5% NHS and mixed with PMN at a multiplicity of infection of 40:1. Bacterial concentrations were adjusted by measuring the optical densities at 600 nm and checked by serial dilutions from every sample and plating on Mueller-Hinton agar. Agar plates were incubated at 27°C for 36 h, and CFU were determined. For restimulation of PMN, opsonized zymosan (250 μg/ml) was added and the secondary CL signal was measured for 75 min. Each assay was performed in duplicate and repeated at least three times. Only representative and reproducible CL graphs are shown.

Adherence, phagocytosis, and killing experiments were performed in parallel to the CL assays. Bacteria were incubated with PMN at 37°C in microplates at a multiplicity of infection of 20:1. For adherence studies PMN were washed at a given time with PBS and centrifuged onto glass slides by a cytocentrifuge (Shandon, Pittsburgh, Pa.). Slides were air dried, fixed with methanol, and stained with Giemsa. Three hundred PMN from three independent experiments (100 PMN from each experiment) were examined by light microscopy. The number of PMN associated with bacteria and the number of bacteria per PMN were determined.

To discriminate between intra- and extracellularly located bacteria, the double-immunofluorescence technique described by Heesemann and Laufs (19) was used. For evaluation of the ratio of killed yersiniae, PMN were lysed with ice-cold distilled water containing 0.5% Tergitol after incubation with bacteria for 1.5 h at 37°C. Serial dilutions were plated on Mueller-Hinton agar, and CFU were counted after 36 h of incubation at 26°C. Control samples, containing no PMN, were treated in parallel under the same conditions. The killing rate was quantified as mean percent (± standard deviation) killed bacteria of the total number of bacteria for at least three independent experiments.

Virulence test. The virulence of WA(pYVO8$_{\Delta 29\text{-}81}$) was tested in the orogastric and intravenous mouse infection models as described previously (35). For the oral infection route, groups of six C57BL/6 mice (female, 6 to 8 weeks old; Charles River WIGA, Sulzfeld, Germany) were infected with 5×10^8 bacteria. For the intravenous infection route, groups of five BALB/c mice (female, 6 to 8 weeks old; Charles River WIGA) were infected with 4×10^4 bacteria. At the indicated days postinfection, the numbers of bacteria in the organs were determined by plating serial dilutions. (Animal licensing committee permission no. 621-2531.01-52/95.)

RESULTS

Construction of YadA$_{\Delta 29\text{-}81}$. The YadA protein of *Y. enterocolitica* O:8 can be sectioned into different domains. The signal sequence (aa 1 to 25) and hydrophobic domains (aa 80 to 101 and 377 to 455) are involved in cellular transport and surface polymerization of YadA, respectively (48). The stretch between the signal sequence and the N-terminal hydrophobic domain (aa 26 to 79) is not thought to be involved in either YadA traffic and surface polymerization or binding to collagen or HEp-2 cells. To investigate the functional role of aa 29 to 81, we constructed a deletion variant of the YadA protein as outlined in Fig. 1. aa 29 to 81 correspond to nucleotides 437 to 595 (GenBank accession no. X13881) of the *yadA* gene and are located between a *Cla*I and a *Pst*I restriction site. The

TABLE 2. Binding of *E. coli* DH5α mutants to immobilized collagen types I and IV and laminin[a]

Strain	A_{590}[b]		
	Coll I	Coll IV	Laminin
DH5α(pUC-A-1, pB8-5)	0.956 ± 0.04	0.870 ± 0.034	0.49 ± 0.08
DH5α(pUC-A-0, pB8-5)	0.202 ± 0.027	0.212 ± 0.034	0.088 ± 0.04
DH5α(pUC-$A_{\Delta 29\text{-}81}$, pB8-5)	0.798 ± 0.09	0.768 ± 0.086	0.45 ± 0.055

[a] Bacteria were incubated in enzyme-linked immunosorbent assay plates coated with collagen (Coll) or laminin as indicated. Adherent bacteria were detected with specific antiserum, subsequent incubation with anti-immunoglobulin G-alkaline phosphatase, and development with *p*-nitrophenyl phosphate as substrate.
[b] The data are means ± standard deviations for four independent experiments.

*Cla*I-*Pst*I fragment was removed from the gene and replaced by an appropriate PCR product. The resulting $yadA_{\Delta 29\text{-}81}$ was studied in *E. coli* or *Y. enterocolitica* O:8. To achieve expression of *yadA* in *E. coli*, the gene *virF* encoding the transcriptional activator of *yadA* (24) was cloned as a 5-kb *Bam*HI fragment in pRK290B. The resulting plasmid pB8-5 was transferred into the *E. coli* strains harboring wild-type *yadA* or mutated *yadA* on a second plasmid. For studying $yadA_{\Delta 29\text{-}81}$ in *Y. enterocolitica* O:8, the mutated *yadA* gene was cloned into a suicide plasmid (pGPS-$A_{\Delta 29\text{-}81}$) and mobilized into the YadA-negative strain WA(pYVO8-A-0), resulting in WA(pYVO8-$A_{\Delta 29\text{-}81}$). The deletion caused the loss of 5 positively charged aa and 5 negatively charged aa. The calculated pI value of the YadA protein changed from 7.09 (wild type) to 8.95 (mutant).

Expression and surface localization of $YadA_{\Delta 29\text{-}81}$. Subjection of YadA to SDS-PAGE reveals three different forms of YadA (28). Preincubation of YadA in sample buffer at 100°C results in a monomeric form (~50 kDa) and an oligomeric form (~200 kDa) (named oligomer A), whereas preincubation at room temperature results in an oligomeric form of about 116 kDa (named oligomer B). Outer membrane proteins of WA-314 and WA(pYVO8-$A_{\Delta 29\text{-}81}$) were prepared and preincubated with sample buffer at 27 or 100°C, respectively. The samples were separated by SDS-PAGE, and the presence of monomeric and oligomeric forms of YadA was shown by immunoblotting (Fig. 2). As expected, the monomeric form of $YadA_{\Delta 29\text{-}81}$ produced by WA(pYVO8-$A_{\Delta 29\text{-}81}$) is about 5 kDa smaller than the wild-type YadA. The oligomeric form B of wild-type YadA could be seen at about 116 kDa as a fuzzy band; the corresponding oligomeric band of $YadA_{\Delta 29\text{-}81}$ appeared at about 100 kDa in reduced quantity. Analysis of cell membrane proteins revealed that $YadA_{\Delta 29\text{-}81}$ was localized in the outer membrane fraction, indicating that the deletion of aa 29 to 81 had no significant effect on localization of $YadA_{\Delta 29\text{-}81}$. WA(pYVO8-$A_{\Delta 29\text{-}81}$) and WA-314 were subjected to slide agglutination and indirect immunofluorescence (without fixation of the bacteria) by using a purified polyclonal anti-YadA rabbit serum (50) to further characterize the localization of $YadA_{\Delta 29\text{-}81}$. The results of these tests were similar for both strains (agglutination titer, 1:800; immunofluorescence titer, 1:600). Moreover, like the wild-type YadA, $YadA_{\Delta 29\text{-}81}$ was able to confer on *E. coli* and *Y. enterocolitica* O:8 the ability to autoagglutinate. Altogether, $YadA_{\Delta 29\text{-}81}$ was found to be expressed on the bacterial surface in an amount comparable to that for the wild-type YadA and to expose at least the majority of epitopes required for antiserum-mediated agglutination, thus indicating that the three-dimensional structure of the truncated YadA was not severely affected.

Binding to extracellular matrix proteins and HEp-2 cells. Two domains of YadA are known to be involved in the binding to ECM proteins and/or HEp-2 cells. These are the hydrophobic domain comprising aa 80 to 101 (48) and the histidine residues at positions 156 and 159 (35). Therefore, we studied

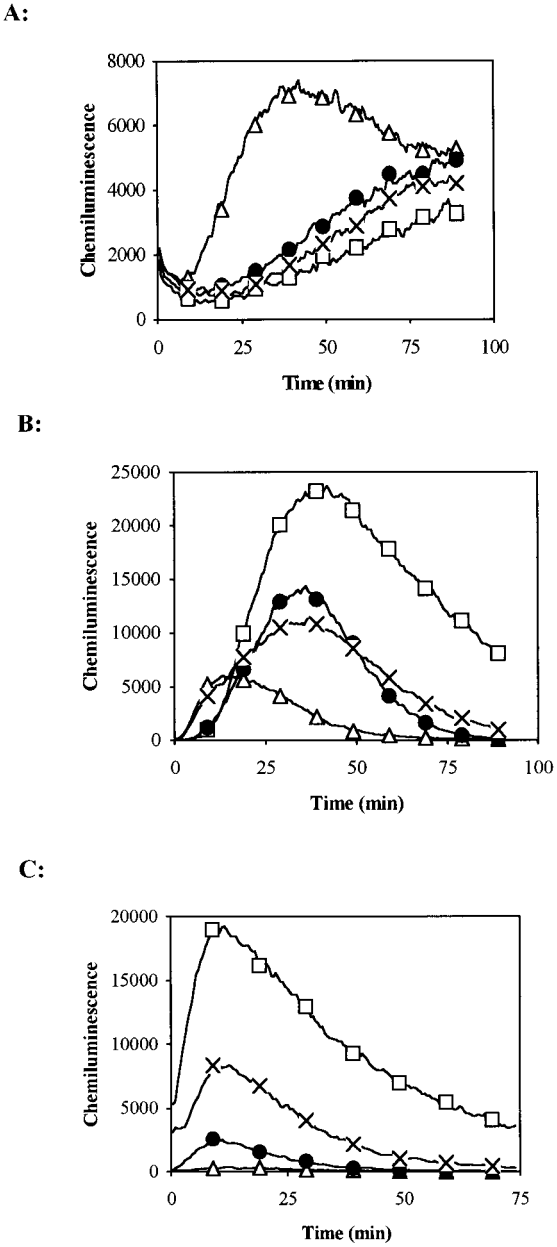

FIG. 3. Different WA strains were incubated with PMN at a ratio of 40:1, and the primary or secondary CL response was measured as described in Materials and Methods. (A) Unopsonized bacteria were incubated with PMN, and the primary CL response was measured. (B) The bacteria were opsonized with 5% NHS and incubated with PMN. The primary CL response was measured. (C) The bacteria were opsonized with 5% NHS and incubated with PMN. After 90 min the PMN were restimulated with opsonized zymosan, and the secondary CL response was measured. □, WA-C; △, WA-314; ●, WA(pYVO8-A-0); ×, WA(pYVO8-$A_{\Delta 29\text{-}81}$).

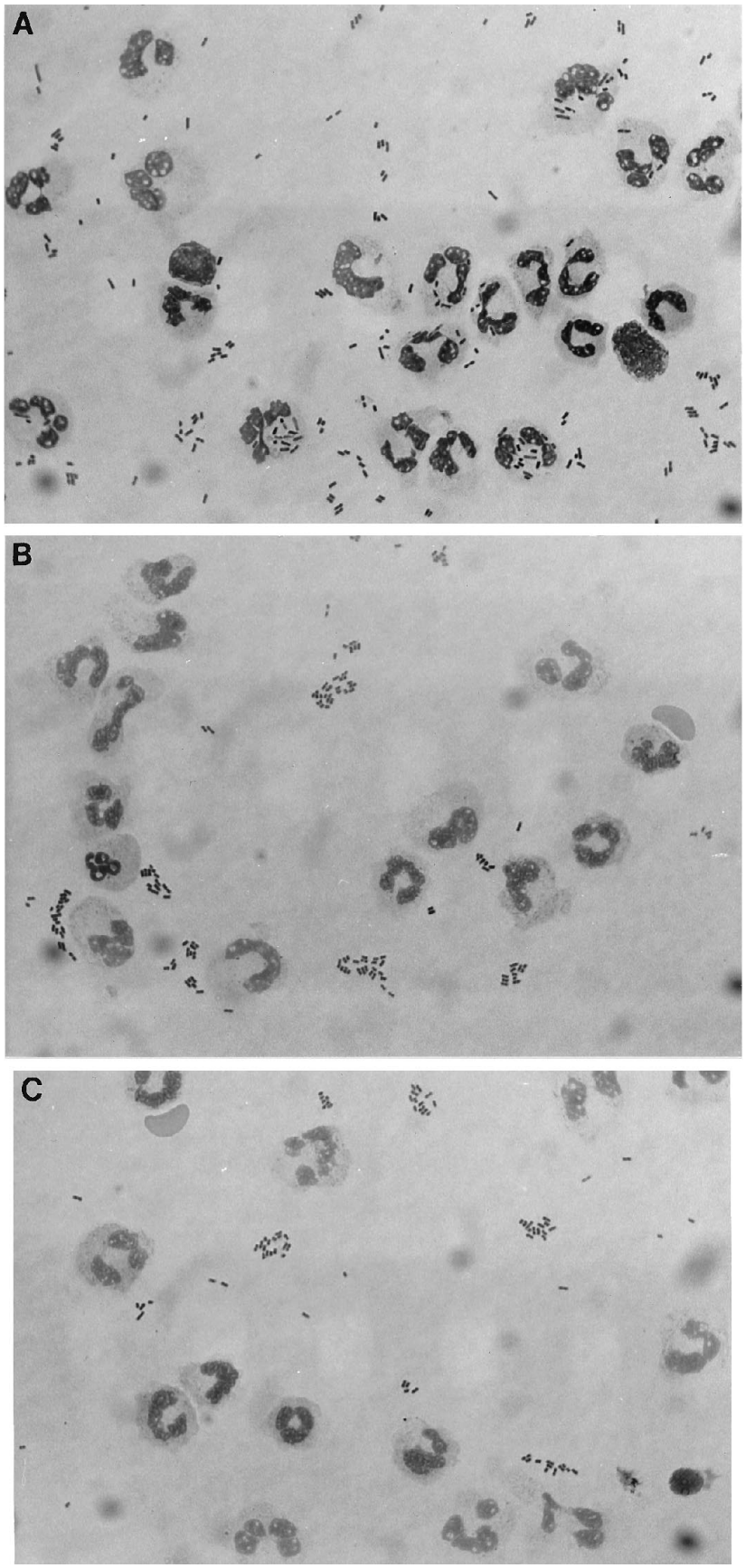

FIG. 4. Giemsa stain. Different WA strains were incubated nonopsonized with PMN. After 30 min the cells were washed in PBS, centrifuged on glass slides, and stained with Giemsa. (A) WA-314; (B) WA(pYVO8-A-0); (C) WA(pYV-$A_{\Delta 29\text{-}81}$). Only WA-314 is interacting with PMN.

TABLE 3. Interaction of different WA strains with PMN opsonized with 5% NHS 5, 15, and 30 min after infection[a]

Strain	PMN associated with bacteria (%)			No. of bacteria/PMN		
	5 min	15 min	30 min	5 min	15 min	30 min
WA-314	54 ± 4	68 ± 8	68 ± 7	2.6 ± 0.7	3.7 ± 1.3	4.2 ± 1.0
WA(pYVO8-A-0)	19 ± 5	60 ± 12	98 ± 1	1.5 ± 0.2	3.3 ± 0.2	9.9 ± 3.5
WA(pYVO8-$A_{\Delta 29\text{-}81}$)	65 ± 9	69 ± 10	98 ± 1	3.0 ± 5	3.5 ± 0.2	9.2 ± 1.7
WA-C	15 ± 4	75 ± 6	89 ± 6	1.7 ± 0.3	2.9 ± 0.5	5.7 ± 2.5

[a] Bacteria were opsonized with 5% NHS and incubated with PMN at a ratio of 20:1 at 37°C with gentle shaking. Five, 15, and 30 min after infection PMN were washed with PBS, centrifuged onto glass slides, and stained with Giemsa. The number of PMN associated with bacteria was determined and expressed as a percentage. The number of cell-associated bacteria was determined and divided by the number of PMN associated with bacteria. The data are means for three independent experiments ± standard deviations.

the properties of binding of the $YadA_{\Delta 29\text{-}81}$ mutant to collagen types I and IV, to laminin, and to HEp-2 cells. As shown in Table 2, strain DH5α(pUC-$A_{\Delta 29\text{-}81}$, pB8-5), producing $YadA_{\Delta 29\text{-}81}$, bound to the different ECM proteins similarly to strain DH5α(pUC-A-1, pB8-5), producing wild-type YadA. Also, the ability of HEp-2 cell binding was conferred on *E. coli* by $YadA_{\Delta 29\text{-}81}$ just as by the wild-type YadA (data not shown).

Interaction of WA(pYVO8-$A_{\Delta 29\text{-}81}$) with PMN. The expression of YadA is necessary for yersinia strains to interact with PMN under nonopsonized conditions (39). We tested strain WA(pYVO8-$A_{\Delta 29\text{-}81}$) for adherence to PMN and induction of CL without opsonization in comparison with YadA-positive and YadA-negative strains. As shown in Fig. 3A, WA(pYVO8-$A_{\Delta 29\text{-}81}$) did not induce a significant CL signal. The low CL response was associated with the inability of WA(pYVO8-$A_{\Delta 29\text{-}81}$) to attach to PMN (Fig. 4). This kind of adherence deficiency is known for YadA-negative mutants (39).

After opsonization with 5% NHS, WA-314 (wild-type YadA) induced a low primary CL response (11, 27) and a suppressed secondary CL response (restimulation with opsonized zymosan) (39). The YadA-negative strain generated a stronger primary CL signal and was hampered in suppressing the secondary CL response (39). As shown in Fig. 3B and C, opsonized WA(pYVO8-$A_{\Delta 29\text{-}81}$) induced a primary and a secondary CL signal of PMN, similarly to the YadA-negative strain WA(pYVO8-A-0). The different intensities in the CL response of PMN to the yersinia strains correlated with the degree of interaction between the bacteria and the PMN (Table 3). In contrast to the wild-type WA-314, the interaction of WA(pYVO8-$A_{\Delta 29\text{-}81}$) with PMN led to increased phagocytosis. As could be demonstrated by indirect double immunofluorescence, 41% ± 9% of WA(pYVO8-$A_{\Delta 29\text{-}81}$) organisms were located intracellularly 1 h after infection (phagocytosis of the YadA-negative mutant, 44% ± 7%; phagocytosis of the wild-type strain WA-314, 12% ± 3% [39]). Not surprisingly, the result of the killing assay of WA(pYVO8-$A_{\Delta 29\text{-}81}$) was also comparable to that for WA(pYVO8-A-0) (39): 40% ± 15% of the given bacteria were killed by the PMN after 60 min, whereas WA-314 was not killed in the same period.

Serum resistance and binding of factor H. YadA is involved in the ability of yersiniae to resist the bacteriolytic effect of human serum (2, 34). YadA inhibits the activation and deposition of complement components on the bacterial surface, possibly because of the binding of the serum complement-inhibiting factor H to YadA (8). We tested serum resistance in 50% NHS of strains WA-314, WA(pYVO8-A-0), and WA(pYVO8-$A_{\Delta 29\text{-}81}$). As shown in Table 4, the deletion of aa 29 to 81 within the YadA protein did not cause a decrease of the number of survivors in serum.

The binding of serum factor H to YadA or $YadA_{\Delta 29\text{-}81}$ was tested in an affinity blot. Serum factor H bound to YadA irrespectively of the presence or absence of aa 29 to 81 (Fig. 5).

Mouse virulence. The effect of deleting aa 29 to 81 in the YadA protein on virulence was tested in the orogastric and intravenous mouse infection models. For orogastric infection, groups of six C57BL/6 mice were infected with 5×10^8 bacteria of strain WA-314 or WA(pYVO8-$A_{\Delta 29\text{-}81}$). At days 1 and 5 postinfection the numbers of surviving bacteria in the small intestine, Peyer's patches, mesenteric lymph nodes, and spleen were determined. The results are shown in Table 5. The numbers of reisolated bacteria (CFU) of strain WA(pYVO8-$A_{\Delta 29\text{-}81}$) at day 1 were more than 50 times smaller than those of the wild-type WA-314. Obviously, WA(pYVO8-$A_{\Delta 29\text{-}81}$) had a reduced ability to colonize the gut. Five days after infection the numbers of CFU obtained from Peyer's patches and from the lumen of the small intestine remained 20 to 100 times smaller for WA(pYVO8-$A_{\Delta 29\text{-}81}$) than for the wild-type WA-314. Strikingly, WA(pYVO8-$A_{\Delta 29\text{-}81}$) was not able to disseminate from Peyer's patches to other lymphatic organs. The results from the intravenous infection route were in good correlation with those of the orogastric infection model. As shown in Table 6, strain WA(pYVO8-$A_{\Delta 29\text{-}81}$) was rapidly eliminated from spleen, approximately as fast as the YadA-negative mutant WA(pYVO8-A-0). Yersinae reisolated from the spleen 4 days after intravenous infection carried the unaltered recombinant plasmid pYVO8-$A_{\Delta 29\text{-}81}$ (as shown by restriction fragment analysis), demonstrating genetic stability.

DISCUSSION

Two genetic approaches have been described for dissecting the multifunctional features of YadA: nucleotide substitution and oligonucleotide deletions within the *yadA* gene (35, 48). In this study we examined the effect of an N-terminal truncation in YadA (deletion of aa 29 to 81) on the protein's function. The stretch of deleted amino acids is located downstream from the signal sequence of YadA and upstream from the N-terminal hydrophobic domain (aa 80 to 101), which is required for collagen binding (48). Expression of the truncated $yadA_{\Delta 29\text{-}81}$ resulted in unaltered surface localization demonstrated by indirect immunofluorescence and slide agglutination. Moreover,

TABLE 4. Survival of WA strains in 50% NHS at 37°C[a]

Strain	Survival (%)	
	60 min	120 min
WA-314	102 ± 6	118 ± 8
WA(pYVO8-A-0)	40 ± 13	1 ± 5
WA(pYVO8-$A_{\Delta 29\text{-}81}$)	115 ± 10	130 ± 8

[a] After the indicated time, the number of viable bacteria was determined by plating serial dilutions onto Mueller-Hinton agar. One hundred percent is 5×10^5 bacteria per ml. The data are means ± standard deviations for three independent experiments.

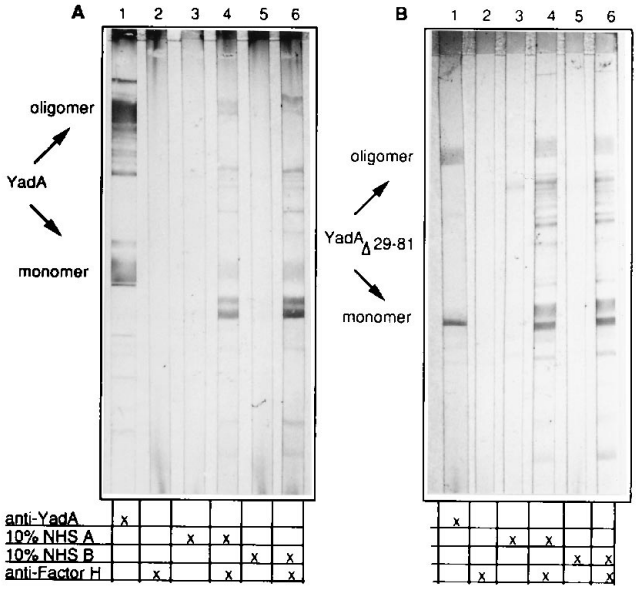

FIG. 5. Factor H affinity blot. Outer membrane fractions of WA-314 (A) or WA(pYV-$A_{\Delta 29\text{-}81}$) (B) were separated on SDS-PAGE and transferred to nitrocellulose sheets. The sheets were cut in strips. The presence of YadA and $YadA_{\Delta 29\text{-}81}$ was demonstrated in strips 1 by incubation with anti-YadA rabbit serum. Strips 3 to 6 were incubated for 3 h with 10% NHS of two different seronegative persons. After a wash, monospecific anti-factor H rabbit serum was added to strips 2, 4, and 6. After another wash, bound antibodies were detected with anti-rabbit immunoglobulin G-alkaline phosphatase conjugate. Binding of factor H to YadA, $YadA_{\Delta 29\text{-}81}$, and obviously to other yersinia proteins, too, could be detected in strips 4 and 6. Strips 2 demonstrated that anti-factor H did not react with YadA. Strips 3 and 5 showed that the sera of the two persons did not have interfering antibodies, which might have caused a false-positive reaction.

the amount of $YadA_{\Delta 29\text{-}81}$ on the bacterial surface was comparable to that of the wild-type YadA. As shown by SDS-PAGE, $YadA_{\Delta 29\text{-}81}$ was able to form monomeric and oligomeric protein bands resembling the pattern of wild-type YadA. Thus, oligomerization and probably three-dimensional structure seem to be unaffected by this deletion. This is supported by the ability of $YadA_{\Delta 29\text{-}81}$ to mediate autoagglutination, serum resistance, HEp-2 cell adherence, and binding to ECM proteins.

Recently, Ruckdeschel et al. (39) demonstrated that YadA plays a specific role during the interaction of *Y. enterocolitica* and PMN: YadA mediated rapid attachment to PMN under nonopsonized conditions (YadA-negative yersiniae practically do not interact with PMN). Also under opsonized conditions the presence of YadA apparently favors translocation of YopH and YopE into the PMN via an attachment function, resulting in a low primary CL response and in suppressed secondary zymosan-induced CL response. As the YadA-negative mutant [WA(pYVO8-A-0)] is hampered in PMN attachment and consequently in the polarized translocation of Yops, the primary and secondary CL responses of PMN to the YadA-negative mutant are markedly higher (39). For this reason we examined the interaction of WA(pYVO8-$A_{\Delta 29\text{-}81}$) with PMN. Under nonopsonized condition, WA(pYVO8-$A_{\Delta 29\text{-}81}$) behaved like the YadA-negative mutant WA(pYVO8-A-0): both mutants practically did not adhere to PMN (Fig. 4). These results suggest that wild-type YadA probably recognizes a specific receptor on the surface of PMN and that the binding domain of YadA may be located within the polypeptide segment aa 29 to 81. The results of CL experiments obtained under opsonized conditions supported this conclusion: the primary and secondary CL curves induced by WA(pYVO8-$A_{\Delta 29\text{-}81}$) were comparable to those of the YadA-negative mutant (Fig. 3). Obviously, proper adherence to PMN via YadA is required for Yop translocation and subsequent inhibition of CL response and bactericidal activity of PMN. The phagocytosis and killing experiments are also in line with this assumption: mutant WA(pYVO8-$A_{\Delta 29\text{-}81}$) was phagocytosed and killed by the PMN to a similar degree as was the YadA-negative mutant WA(pYVO8-A-0). In one aspect the CL curve of WA(pYVO8-$A_{\Delta 29\text{-}81}$) differed from that of a YadA-negative mutant: opsonized WA(pYVO8-$A_{\Delta 29\text{-}81}$) induced an early CL response comparable to that of the wild-type WA-314 (appearance of a shoulder [Fig. 3]). To examine the early phase of interaction in more detail, PMN were Giemsa stained 5, 15, and 30 min after infection with bacteria. As shown in Table 3, the appearance of a shoulder in the CL curves of strain WA-314 and WA(pYVO8-$A_{\Delta 29\text{-}81}$) corresponds to a more extensive interaction of these strains with PMN during the first 5 min after infection in comparison with the YadA-negative strains

TABLE 5. Numbers of WA bacteria per organ on days 1 and 5 in C57BL/6 mice after orogastric infection[a]

Site[b]	$\log_{10}$ CFU[c]				
	WA(pYVO8-A-1)		WA(pYVO8-$A_{\Delta 29\text{-}81}$)		WA(pYVO8-A-0)[d]
	Expt 1	Expt 2	Expt 1	Expt 2	
Day 1					
SI	4.1 ± 0.5	4.12 ± 0.6	2.34 ± 0.4	2.44 ± 0.36	1.44 ± 0.04
PP	4.2 ± 0.9	4.4 ± 0.26	2.7 ± 0.7	2.5 ± 0.58	1.16 ± 0.06
MLN	<0.5	<0.5	<0.5	<0.5	<0.5
S	<1.25	<1.25	<1.25	<1.25	<1.25
Day 5					
SI	6.72 ± 0.6	6.5 ± 0.4	4.42 ± 0.36	4.38 ± 0.52	<0.5
PP	6.26 ± 0.27	6.24 ± 0.16	5.14 ± 0.2	4.25 ± 0.42	<0.5
MLN	3.91 ± 0.5	4.14 ± 0.41	<0.5	<0.5	<0.5
S	4.44 ± 0.4	4.8 ± 0.32	<1.25	<1.25	<1.25

[a] Groups of six C57BL/6 mice were infected with 5×10^8 bacteria at day 0 as described in Materials and Methods. At day 1 or 5 the numbers of colony-forming bacteria in the organs were determined by plating.
[b] PP, Peyer's patches; SI, small intestine; MLN, mesenteric lymph nodes; S, spleen.
[c] The data are means ± standard deviations.
[d] Additionally, see reference 35.

TABLE 6. Numbers of WA bacteria in the spleen and liver in BALB/c mice after intravenous infection with 4×10^4 bacteria

Strain	$\log_{10}$ CFU[a]	
	Spleen	Liver
WA-314		
Day 2	5.8 ± 0.44	5.25 ± 0.71
Day 4	8.0 ± 0.32	7.0 ± 0.61
WA(pYVO8$_{\Delta 29\text{-}81}$)		
Day 2	3.0 ± 0.61	1.4 ± 0.64
Day 4	2.0 ± 0.97	<1.25
WA(pYVO8-A-0)		
Day 2	2.4 ± 0.82	1.25 ± 0.25
Day 4	<1.25	<1.25

[a] The data are means ± standard deviations for five animals.

WA-C and WA(pYVO8-A-0). As wild-type YadA and mutant YadA$_{\Delta 29\text{-}81}$ bind the complement-inhibiting factor H (8) (Fig. 5), we can assume similar low C3b depositions on the surfaces of WA-314 and WA(pYVO8-A$_{\Delta 29\text{-}81}$) and thus comparable interactions of these two strains with the C3b receptor of PMN. However, strain WA-314 can additionally interact with PMN via YadA. In contrast, the mutant cannot, because of truncated YadA, which has lost PMN adherence functions. WA-314 and WA(pYVO8-A$_{\Delta 29\text{-}81}$) are good ECM protein-binding strains in contrast to WA-C and WA(pYVO8-A-0). Presumably, this feature is responsible for the very early interaction (first 5 min) with about 55 to 65% of the PMN population. On the other hand, it is plausible that mutant WA(pYVO8-A$_{\Delta 29\text{-}81}$) is able to compensate this defect in specific PMN adherence by improved interaction caused by opsonization and increase of the pI value of YadA (calculated increase in pI, 1.9), which may enhance unspecific electrostatic interaction of charged surfaces.

The altered interaction with PMN of strain WA(pYVO8-A$_{\Delta 29\text{-}81}$) should be associated with an alteration in the virulence of the strain. With respect to strain WA-314 we observed reduced colonization of the gut and Peyer's patches and lack of dissemination of the mutant WA(pYVO8-A$_{\Delta 29\text{-}81}$) in the oral mouse infection model. In the intravenous mouse infection model, strain WA(pYVO8-A$_{\Delta 29\text{-}81}$) was attenuated to the same degree as the YadA-negative strain WA(pYVO8-A-0). These results indicate that, in spite of the preserved abilities of YadA$_{\Delta 29\text{-}81}$ to autoagglutinate and bind to ECM proteins and to HEp-2 cells, this deletion caused a strong attenuation of virulence. Recently, we have shown that inhibition of bactericidal activity of PMN requires specific interaction of yersiniae via YadA and translocation of YopH and YopE. If YadA-mediated attachment is impaired, yersiniae may be killed by oxidative burst products and defensins released by activated PMN. Obviously, the complete functional repertoire of YadA is absolutely required for conferring full virulence.

In summary, differential functions of YadA can be impaired by designed mutations. In this case deletion of aa 29 to 81 in the YadA protein did not affect features such as autoagglutination, serum resistance, and binding to ECM proteins, to HEp-2 cells, and to factor H, whereas the interaction with PMN was impeded and mouse virulence was attenuated. This and previous studies also demonstrate that specific functions of YadA are required at distinct stages of the infection process. This knowledge should make it possible to create live vaccine strains for induction of mucosal immunity, e.g., by directing mutants to Peyer's patches, where they can multiply to produce the desired antigen but cannot disseminate (35).

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