

## Molecular Analysis of Transport and Oligomerization of the *Yersinia enterocolitica* Adhesin YadA

Andreas Roggenkamp,\* Nikolaus Ackermann, Christoph A. Jacobi, Konrad Truelzsch, Harald Hoffmann, and Jürgen Heesemann

Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig Maximilian University, and Medical Centre Grosshadern, 81377 Munich, Germany

Received 6 March 2003/Accepted 16 April 2003

The *Yersinia* adhesin YadA is the prototype of a novel class of bacterial adhesins which form oligomeric lollipop-like structures and are anchored in the outer membrane by the C terminus. For YadA, six different regions (R) or domains (D) are predicted from the amino acid sequence: the N-terminal leader sequence, head-D, neck-D, stalk-D, linking-R, and a C-terminal transmembrane region consisting of four  $\beta$ -strands. To identify structural and functional features of these domains, we performed in-frame deletion mutagenesis and constructed N-terminally tagged YadA variants. Diverse YadA variants were analyzed for outer membrane localization, surface exposure, oligomerization/adhesion properties, and ability to protect against complement-mediated lysis. We demonstrated that (i) the C-terminal region (amino acids [aa] 353 to 422) is sufficient for outer membrane insertion and formation of trimers in the outer membrane; (ii) the head, neck, and stalk domains (aa 26 to 330) are surface exposed, forming a passenger domain; and (iii) the linking region (aa 331 to 369) is responsible for outer membrane translocation of the passenger domain. Thus, YadA meets all the criteria of an autotransporter. The same may be true for all other members of the YadA family, forming a subfamily of surface-attached oligomeric autotransporters. Moreover, in-frame truncation mutagenesis suggested that the head and neck domains together form the YadA-binding module which is located on the top of the stalk. However, the YadA-binding module did not confer serum resistance. Mutants lacking the head and neck domain were resistant to complement-mediated lysis. In-frame truncation of the stalk domain did not result in significant attenuation of the mutant in an orogastric mouse infection model.

The outer membrane protein YadA (*Yersinia* adhesin A) is an important virulence factor for enteropathogenic *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* (for a review, see reference 11). YadA mediates attachment to the surface of host cells and protects yersiniae against complement and defensin bactericidal activity. Thus, YadA contributes significantly to the extracellular life style. Moreover, YadA-mediated cell adherence supports the function of the protein secretion-translocation machinery to inject yersinia outer membrane proteins (Yops) into host cells, resulting in paralysis of professional phagocytes (7).

The *yadA* gene is located on the virulence plasmid pYV of enteropathogenic *Yersinia* species. According to the *yadA* gene polymorphism the size of YadA varies between 41 and 44 kDa. From sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and ultracentrifugation sedimentation studies of membrane-extracted YadA, a trimeric structure was suggested for native YadA (13, 21). More structural details of surface-exposed YadA were revealed by electron microscopy and amino acid sequence analysis (18). YadA could be visualized as a lollipop-shaped projection on the outer membrane of yersiniae. From the YadA amino acid sequence, a tripartite organization was predicted, composed of an N-ter-

minal head domain, a coiled-coil stalk, and a C-terminal membrane anchor. However, the exact number of YadA molecules forming this particular surface projection of YadA and the orientation of the C-terminal membrane anchor remain to be elucidated.

Sequence comparison suggested that YadA is the prototype of a novel class of nonfimbrial adhesins. According to the common oligomeric structure of coiled coils that this novel class of adhesins produced, we will call it the Oca family. YadA-homologous proteins are produced in free-living  $\alpha$ -Proteobacteria (such as *Sinorhizobium meliloti*) as well as in human- and plant-pathogenic  $\beta$ -Proteobacteria (such as *Neisseria meningitidis*) and  $\gamma$ -Proteobacteria (such as *Escherichia coli*, *Moraxella catarrhalis*, and *Xanthomonas* spp.) (5, 18, 25). A similar structure is predicted from the protein sequence of this class of outer membrane proteins. Conserved features or structural elements are (i) an N-terminal Sec-dependent secretion signal, (ii) a highly conserved neck domain, (iii) a stalk domain that varies in length, with a high probability of coiled-coil formation, and (iv) a C-terminal membrane anchor domain consisting of 35 amino acids (aa) with a left-handed coiled-coil segment (2 to 3 heptamers, termed the linking region), followed by four amphipathic transmembrane  $\beta$ -strands at the very end of the protein.

The proteins of the Oca family which have been characterized biochemically form heat-stable oligomers in SDS-PAGE, reflecting the oligomerization capacity of the proteins on the cell surface (3, 5, 6, 9, 13, 21, 28). This oligomerization is probably associated with the conserved membrane anchor domain of the C terminus (34). The absence of cysteine residues

\* Corresponding author. Mailing address: Max von Pettenkofer Institute for Hygiene and Medical Microbiology, Ludwig Maximilian University Munich, Medical Centre Grosshadern, Marchioninistrasse 15, 81377 Munich, Germany. Phone: 49-(0)89-2180-78202. Fax: 49-(0)89-2180-78207. E-mail: Andreas.Roggenkamp@mpk.med.uni-muenchen.de.

in the mature polypeptides is another feature common to Oca members and may indicate that intermolecular disulfide bridging could lead to abrogation of translocation of the N terminus across the outer membrane (3, 5, 9, 28, 31).

Besides structural characteristics, the Oca proteins also share functional characteristics. Known functions are binding to the eukaryotic cell surface and extracellular matrix (ECM) proteins (3, 5, 15, 20, 27–30). The binding domains mediating adherence are probably located in nonconserved regions of the N terminus of Oca family members. Moreover, it has been demonstrated that Oca proteins are able to confer resistance to complement and defensin lysis and thus protect the pathogen against the host immune response (1, 9, 24, 28, 35). The rapidly growing Oca family has been included in conserved domain databases such as Pfam (St. Louis, version 7.5; 03895, YadA) with YadA as the prototype.

The contribution of the different domains to proper insertion into the outer membrane, oligomerization to lollipop-shaped structures, and virulence-associated features of YadA is mostly unknown. To identify protein segments and motifs involved in the topology of YadA (e.g., outer membrane localization, surface exposure, and oligomerization), we constructed truncated forms of YadA and Flag-tagged YadA. Preserving the N-terminal signal sequence and the C-terminal  $\beta$ -sheets, we generated in-frame deletions of the head domain (H) and neck domain (N) and repeats of the stalk domain (S) and linking region (L). From our results, we conclude that the head, neck, and stalk domains resemble surface-exposed passenger domains of autotransporters. Moreover, the linking region preceding the four  $\beta$ -strands of the C terminus is required for translocation of the passenger domain across the outer membrane, and the C-terminal transmembrane region is responsible for outer membrane insertion and oligomerization. Finally, the highly conserved neck domain is a part of the ECM-cell surface binding module, and YadA-mediated “serum resistance” requires the four  $\beta$ -strands and the stalk domain of YadA but not the head domain.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains used in this study are listed in Table 1. Strains were cultivated in Luria-Bertani (LB) medium: *Escherichia coli* at 37°C, and *Yersinia enterocolitica* at 27°C. For induction of *yadA* expression, overnight cultures at 27°C were diluted 1:40 in RPMI medium and grown for 2 h (for collagen and cell binding assays) or 6 h (for outer membrane protein preparations) at 37°C. Antibiotics were used at the following concentrations: ampicillin, 100  $\mu$ g/ml; kanamycin, 25  $\mu$ g/ml; nalidixic acid, 60  $\mu$ g/ml; and spectinomycin, 50  $\mu$ g/ml.

**DNA manipulations and PCR.** Restriction endonuclease digestion, DNA ligations, transformations, DNA sequencing, and PCR were performed according to standard techniques (2). Conjugations were done as described before (26). Plasmid DNA preparations and isolation of DNA fragments from agarose gels were performed with Macherey-Nagel kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany) as recommended by the manufacturer. *Pfu* DNA polymerase (Stratagene, Amsterdam, Netherlands) was used in cloning procedures. The 30-bp Flag epitope oligonucleotides were synthesized with a phosphate group at the 5' ends. For annealing, the Flag oligonucleotides were dissolved at high concentration (10 optical density at 260 nm [OD<sub>260</sub>] units/100  $\mu$ l) in STE buffer (10 mM Tris [pH 8.0], 50 mM NaCl, 1 mM EDTA), mixed together in equal molar amounts, heated to 94°C, and gradually cooled to room temperature.

**Construction of different *yadA* in-frame deletions and expression in *Y. enterocolitica* O8.** Mutations were performed with pUC-A-1, harboring the *yadA* gene and the flanking regions as a 5-kb *EcoRI*-*HindIII* fragment. pUC-A-1 was digested with *ClaI* (cutting at positions 177 and 194, GenBank accession no. X13881) and *SphI* (cutting at position 2185) to remove the wild-type *yadA*,

generating a pUC-A-1 *ClaI*-*SphI* backbone. Truncated *yadA* genes were generated with two separate PCR fragments, one with *ClaI* and *SacI* restriction sites and the other with *SacI* and *SphI* restriction sites at the ends, and ligated in the pUC-A-1 *ClaI*-*SphI* backbone. The oligonucleotides used in this study are listed in Table 2.

For construction of A-H and the different A-HNS mutants, the *ClaI*-*SacI* PCR product spanned positions 195 to 438 (forward primer A-Cla-f; reverse primer A-87-r), the *SacI*-*SphI* product spanned different lengths, depending on the intended deletion [(forward primer variable: A-565-f (H), A-733-f (HNS1), A-778-f (HNS2), A-868-f (HNS3), A-991-f (HNS4), and A-1057-f (HNS5); reverse primer, A-Sph-r]. For the A-S mutants, the same strategy was used with another *ClaI*-*SacI* PCR product spanning positions 195 to 1035 (forward primer A-Cla-f; reverse primer A-684-r) and the same *SacI*-*SphI* products as above.

For the A-N mutant, a *ClaI*-*SacI* PCR product spanning positions 195 to 918 (forward primer A-Cla-f; reverse primer A-567-r) and a *SacI*-*SphI* PCR product spanning positions 997 to 2185 (forward primer A-646-f; reverse primer A-Sph-r), for YadA-L1 a *ClaI*-*SacI* PCR product spanning positions 195 to 1341 (forward primer A-Cla-f; reverse primer A-990-r), and a *SacI*-*SphI* PCR product spanning positions 1408 to 2185 (forward primer A-1057-f; reverse primer A-Sph-r), and for YadA-L2 a *ClaI*-*SacI* PCR product spanning positions 195 to 1407 (forward primer A-Cla-f; reverse primer A-1056-r) and a *SacI*-*SphI* PCR product spanning positions 1459 to 2185 (forward primer A-1108-f; reverse primer A-Sph-r) were used. The oligonucleotides used in this study are listed in Table 2. The different PCR product pairs were ligated into the pUC-A-1 *ClaI*-*SphI* backbone and transformed into *E. coli* DH5 $\alpha$ .

For subsequent expression studies in *Y. enterocolitica*, the in-frame truncated *yadA* gene fragments were cut out from the pUC vectors with *EcoRI* and *SphI* and ligated into the mobilizable suicide vectors pGP704. For better selection, a 1.8-kb *Sm<sup>r</sup> Spe<sup>r</sup>  $\Omega$*  fragment was additionally ligated into the *EcoRI* site of the different pGP constructs, resulting in pGPS-A-N, pGPS-A-S1 to -S4, pGPS-A-H, pGPS-A-HNS1 to -HNS5, pGPS-A-L1, and pGPS-A-L2. Additional mutants were created by using the suicide plasmid as starter vector. To fuse the neck domain to the truncated stalk variants of the pGPS-HNS derivatives, the neck domain encoding aa 189 to 228 of *yadA* was amplified with primers A-Cla-f and A-684-r with pUC-H as the template DNA. The neck PCR fragment was ligated into pGPS-HNS1, pGPS-HNS2, pGPS-HNS3, and pGPS-HNS4 with *ClaI* and *SacI*, resulting in pGPS-A-N1, pGPS-A-N2, pGPS-A-N3, pGPS-A-N4, respectively. The Flag epitope was synthesized with oligonucleotides and inserted into the *SacI* site of pGPS-HNS2, pGPS-HNS3, pGPS-HNS4, and pGPS-HNS5. The various mutations were verified by sequencing.

For pGP704 derivatives, *E. coli* SM10 *λπ*ir was used as the host strain. The plasmids were mobilized into WA(pYVO8-A-0) by conjugation and inserted into pYVO8-A-0 by homologous recombination. Selection and characterization of the clones were performed as described elsewhere (26). For the formation of YadA heterooligomers, pGPS derivatives carrying truncated *yadA* genes were conjugated into the wild-type strain WA-314. Integration of the suicide vector and the truncated *yadA* gene resulted in a merodiploid virulence plasmid that harbored a wild-type copy of *yadA* and a truncated *yadA*. The constructs were verified by restriction enzyme analysis of plasmid preparations and PCR.

**Immunofluorescence.** To ascertain the surface exposure of the YadA mutants, they were grown at 37°C for 6 h, harvested by centrifugation, and washed with phosphate-buffered saline (PBS). Bacteria were incubated with YadA-specific monoclonal antibodies (MAbs) (8D1 or anti-Flag antibody, Sigma-Aldrich, Taufkirchen, Germany) at 37°C for 30 min. After three washes with PBS, fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin (Sigma-Aldrich) diluted 1:50 in PBS was used to detect surface-bound MAbs. Bacteria were used to coat glass slides and visualized by fluorescence microscopy.

**Immunoblotting.** Outer membrane preparations of YadA were obtained as described previously (18). The samples were resuspended in electrophoresis buffer (1% SDS and 0.25% 2-mercaptoethanol), boiled for 10 min or incubated at 37°C for 120 min, and separated by discontinuous SDS-PAGE (11% polyacrylamide), followed by electrophoretic transfer to nitrocellulose sheets (BA85; Schleicher and Schüll, Inc.) and blocking with 3% bovine serum albumin (fraction V) in PBS–0.5% Tween overnight at 4°C. For immunostaining of YadA, polyvalent YadA-specific rabbit antiserum, monoclonal antibody 9A6, 9H7, 9H11, or 8D1, or anti-Flag antibody was used. Antigen-antibody complexes were detected with an anti-rabbit immunoglobulin G (IgG)-alkaline phosphatase conjugate (Sigma-Aldrich) diluted 1:5,000 in PBS–0.5% Tween for rabbit antiserum or with an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) diluted 1:5,000 in PBS–0.5% Tween for the MAbs, and subsequent development with indoxylphosphate-tetrazolium (Sigma-Aldrich) as described elsewhere (26).

**Binding assays and autoagglutination.** Binding to collagen and HEp-2 cells and the assay for autoagglutination were performed as described previously (26).

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid         | Description   | Source or reference |
|---------------------------|---|---------------------|
| <i>Y. enterocolitica</i>  |   |                     |
| WA-314                    | Clinical isolate of serotype O8, carrying virulence plasmid pYV08   | 16                  |
| WA-c                      | Plasmid-cured derivative of WA-314  | 16                  |
| <i>E. coli</i>            |   |                     |
| DH5 $\alpha$              | <i>endA1 supE44 hsdR17</i> ( $r_K^- m_K^+$ ) <i>thi-1 recA1 gyrA relA1</i> $\Delta(lacZYA-argF)U169$ ( $\phi 80 lacZ\Delta M15$ ) | 14                  |
| Sm10 $\lambda$ pir        | <i>thi thr leu tonA lacY supE recA::RP4-2-TC::Mu-Kan</i> ( $\lambda$ pir)   | 23                  |
| Plasmids                  |   |                     |
| pUC-A-1                   | pUC13 with 5-kb <i>EcoRI-HindIII</i> insert fragment of pYV08 carrying <i>yadA</i> (A-1 fragment)                                 | 23                  |
| pUC-A-N                   | pUC-A-1, bp 568–645 deleted in <i>yadA</i> (corresponding to neck region, aa 190–215)   | This study          |
| pUC-A-S1                  | pUC-A-1, bp 685–732 deleted in <i>yadA</i> (corresponding to 1st 15-mer repeat, aa 229–244)                                       | This study          |
| pUC-A-S2                  | pUC-A-1, bp 685–777 deleted in <i>yadA</i> (corresponding to 1st and 2nd 15-mer repeats, aa 229–259)                              | This study          |
| pUC-A-S3                  | pUC-A-1, bp 685–867 deleted in <i>yadA</i> (corresponding to first four 15-mer repeats, aa 229–289)                               | This study          |
| pUC-A-S4                  | pUC-A-1, bp 685–990 deleted in <i>yadA</i> (corresponding to all 15-mer repeats, aa 229–330)                                      | This study          |
| pUC-A-H                   | pUC-A-1, bp 88–564 deleted in <i>yadA</i> (corresponding to head region, aa 30–188)   | This study          |
| pUC-A-HNS1                | pUC-A-1, bp 88–732 deleted in <i>yadA</i> (corresponding to head region, neck region, and 1st 15-mer repeat, aa 30–244)           | This study          |
| pUC-A-HNS2                | pUC-A-1, bp 88–777 deleted in <i>yadA</i> (corresponding to head region, neck region, and 1st and 2nd 15-mer repeats, aa 30–259)  | This study          |
| pUC-A-HNS3                | pUC-A-1, bp 88–867 deleted in <i>yadA</i> (corresponding to head region, neck region, and first four 15-mer repeats, aa 30–289)   | This study          |
| pUC-A-HNS4                | pUC-A-1, bp 88–990 deleted in <i>yadA</i> (corresponding to head region, neck region, and all 15-mer repeats, aa 30–330)          | This study          |
| pUC-A-HNS5                | pUC-A-1, bp 88–1056 deleted in <i>yadA</i> (corresponding to head, neck and stalk regions, aa 30–352)                             | This study          |
| pUC-A-L1                  | pUC-A-1, bp 991–1056 deleted in <i>yadA</i> (corresponding to three 7-mer repeats, aa 331–352)                                    | This study          |
| pUC-A-L2                  | pUC-A-1, bp 1057–1107 deleted in <i>yadA</i> (corresponding to linking region, aa 353–369)  | This study          |
| pHP45 $\Omega$            | Origin of spectinomycin resistance cassette   | Sanonnetti          |
| pGP704                    | Suicide vector, R6K <sup>2</sup> replicon, requires $\pi$ protein in <i>trans</i> by $\lambda$ pir-containing host                | 23                  |
| pGPS-A-1                  | pGP704, 1.8-kb <i>Spc'</i> cassette in <i>EcoRI</i> site, carrying <i>yadA</i> as an <i>EcoRI-SphI</i> fragment from pUC-A-1      | 23                  |
| pGPS-A-N                  | pGPS, <i>EcoRI-SphI</i> A-N fragment of pUC-A-N   | This study          |
| pGPS-A-S1                 | pGPS, <i>EcoRI-SphI</i> A-S1 fragment of pUC-A-S1   | This study          |
| pGPS-A-S2                 | pGPS, <i>EcoRI-SphI</i> A-S2 fragment of pUC-A-S2   | This study          |
| pGPS-A-S3                 | pGPS, <i>EcoRI-SphI</i> A-S3 fragment of pUC-A-S3   | This study          |
| pGPS-A-S4                 | pGPS, <i>EcoRI-SphI</i> A-S4 fragment of pUC-A-S4   | This study          |
| pGPS-A-H                  | pGPS, <i>EcoRI-SphI</i> A-H fragment of pUC-A-H   | This study          |
| pGPS-A-HNS1               | pGPS, <i>EcoRI-SphI</i> A-HNS1 fragment of pUC-A-HNS1   | This study          |
| pGPS-A-HNS2               | pGPS, <i>EcoRI-SphI</i> A-HNS2 fragment of pUC-A-HNS2   | This study          |
| pGPS-A-HNS3               | pGPS, <i>EcoRI-SphI</i> A-HNS3 fragment of pUC-A-HNS3   | This study          |
| pGPS-A-HNS4               | pGPS, <i>EcoRI-SphI</i> A-HNS4 fragment of pUC-A-HNS4   | This study          |
| pGPS-A-HNS5               | pGPS, <i>EcoRI-SphI</i> A-HNS5 fragment of pUC-A-HNS5   | This study          |
| pGPS-A-L1                 | pGPS, <i>EcoRI-SphI</i> A-L1 fragment of pUC-A-L1   | This study          |
| pGPS-A-L2                 | pGPS, <i>EcoRI-SphI</i> A-L2 fragment of pUC-A-L2   | This study          |
| pGPS-A-N1                 | pGPS-A-HNS1 carrying neck region ( <i>yadA</i> bp 568–684) in front of truncated <i>yadA</i> of pGPS-A-HNS1                       | This study          |
| pGPS-A-N2                 | pGPS-A-HNS2 carrying neck region ( <i>yadA</i> bp 568–684) in front of truncated <i>yadA</i> of pGPS-A-HNS2                       | This study          |
| pGPS-A-N3                 | pGPS-A-HNS3 carrying neck region ( <i>yadA</i> bp 568–684) in front of truncated <i>yadA</i> of pGPS-A-HNS3                       | This study          |
| pGPS-A-N4                 | pGPS-A-HNS4 carrying neck region ( <i>yadA</i> bp 568–684) in front of truncated <i>yadA</i> of pGPS-A-HNS4                       | This study          |
| pGPS-A-HNS2-Flag          | pGPS-A-HNS2 carrying 32-bp Flag epitope sequence in front of truncated <i>yadA</i> of pGPS-A-HNS2                                 | This study          |
| pGPS-A-HNS3-Flag          | pGPS-A-HNS3 carrying 32-bp Flag epitope sequence in front of truncated <i>yadA</i> of pGPS-A-HNS3                                 | This study          |
| pGPS-A-HNS4-Flag          | pGPS-A-HNS4 carrying 32-bp Flag epitope sequence in front of truncated <i>yadA</i> of pGPS-A-HNS4                                 | This study          |
| pGPS-A-HNS5-Flag          | pGPS-A-HNS5 carrying 32-bp Flag epitope sequence in front of truncated <i>yadA</i> of pGPS-A-HNS5                                 | This study          |
| pYV08                     | Virulence plasmid of WA-314   | 16                  |
| pYV08-A-0                 | pYV08 <i>yadA</i> , Km-GenBlock inserted in <i>PstI</i> sites of <i>yadA</i> by allelic exchange                                  | 23                  |
| pYV08-A-1                 | pYV08-A-0 with integrated pGPS-A-1, wild-type <i>yadA</i>   | 23                  |
| pYV08-A- $\Delta_{29-81}$ | pYV08-A-0 with integrated pGPS-A-A- $\Delta_{29-81}$  | 23                  |
| pYV08-A-N                 | pYV08-A-0 with integrated pGPS-A-N  | This study          |
| pYV08-A-S1                | pYV08-A-0 with integrated pGPS-A-S1   | This study          |
| pYV08-A-S2                | pYV08-A-0 with integrated pGPS-A-S2   | This study          |
| pYV08-A-S3                | pYV08-A-0 with integrated pGPS-A-S3   | This study          |
| pYV08-A-S4                | pYV08-A-0 with integrated pGPS-A-S4   | This study          |
| pYV08-A-S5                | pYV08-A-0 with integrated pGPS-A-S5   | This study          |
| pYV08-A-H                 | pYV08-A-0 with integrated pGPS-A-H  | This study          |
| pYV08-A-HNS1              | pYV08-A-0 with integrated pGPS-A-HNS1   | This study          |
| pYV08-A-HNS2              | pYV08-A-0 with integrated pGPS-A-HNS2   | This study          |
| pYV08-A-HNS3              | pYV08-A-0 with integrated pGPS-A-HNS3   | This study          |
| pYV08-A-HNS4              | pYV08-A-0 with integrated pGPS-A-HNS4   | This study          |
| pYV08-A-L1                | pYV08-A-0 with integrated pGPS-A-L1   | This study          |
| pYV08-A-L2                | pYV08-A-0 with integrated pGPS-A-L2   | This study          |
| pYV08-A-N1                | pYV08-A-0 with integrated pGPS-A-N1   | This study          |
| pYV08-A-N2                | pYV08-A-0 with integrated pGPS-A-N2   | This study          |
| pYV08-A-N3                | pYV08-A-0 with integrated pGPS-A-N3   | This study          |
| pYV08-A-N4                | pYV08-A-0 with integrated pGPS-A-N4   | This study          |
| pYV08-A-HNS2-Flag         | pYV08-A-0 with integrated pGPS-A-HNS2-Flag  | This study          |
| pYV08-A-HNS3-Flag         | pYV08-A-0 with integrated pGPS-A-HNS3-Flag  | This study          |
| pYV08-A-HNS4-Flag         | pYV08-A-0 with integrated pGPS-A-HNS4-Flag  | This study          |
| pYV08-A-HNS5-Flag         | pYV08-A-0 with integrated pGPS-A-HNS5-Flag  | This study          |

TABLE 2. Oligonucleotides used in this study

| Primer               | Description   | Sequence (5' → 3')                 |
|----------------------|---|------------------------------------|
| PCR-1                | PCR-1: <sup>5'</sup> <i>Clal</i> - <sup>3'</sup> <i>SacI</i> fragments  |                                    |
| A- <i>Clal</i> -f    | Constant forward primer for PCR-1, start is 167 bp in front of <i>YadA</i> start codon  | TTTAAAGATCGATTAGTGCTGT             |
| A-87-r               | Reverse, used for <i>YadA</i> -H(NS)-mutants, includes <i>yadA</i> signal sequence plus first 4 aa of <i>yadA</i> : N26-N27-D28-E29 + L introduced by <i>SacI</i> | TACTGGAGCTCGTCATTATTGGCAAA         |
| A-567-r              | Reverse, used for <i>YadA</i> -N, end: bp 567 (=L189) of <i>yadA</i> , + E-L introduced by <i>SacI</i>  | GAAGAGCTCAAGGCTTTCATGACCAGGA       |
| A-684-r              | Reverse, used for <i>YadA</i> -S mutants, end: bp 684 (=E228) of <i>yadA</i> + L introduced by <i>SacI</i>  | CGAGAGCTCAGCTGATTTTTTATTGTCAT      |
| A-990-r              | Reverse, used for <i>YadA</i> -L1, end: bp 990 (=Y330) of <i>yadA</i> + E-L introduced by <i>SacI</i>   | ATGGAGCTCGTATTGATTCGATTACGGATTG    |
| A-1056-r             | Reverse, used for <i>YadA</i> -L2, end: bp 1056 (=G352) of <i>yadA</i> + E-L introduced by <i>SacI</i>  | CCAGAGCTCACCTTTGTCAACTCGTGTGTC     |
| PCR-2                | PCR-2: <sup>5'</sup> <i>SacI</i> - <sup>3'</sup> <i>SphI</i> fragments  |                                    |
| A-565-f              | Forward, used for <i>YadA</i> -H, <i>SacI</i> site, start: bp 565 (=L189) of <i>yadA</i>  | GCTAAGAGCTCAATCGCCAATTGACACATCTTG  |
| A-646-f              | Forward, used for <i>YadA</i> -N, <i>SacI</i> site, start: bp 646 (=E216) of <i>yadA</i>  | AAGGAGCTCGAAAAAACACAGGAAAATGCAAA   |
| A-733-f              | Forward, used for <i>YadA</i> -HNS1 and <i>YadA</i> -S1, <i>SacI</i> site, start: bp 733 (=L245) of <i>yadA</i>   | TAGTAAAAGTGCTGAGCTCTTGAAAAATGCGCGT |
| A-778-f              | Forward, used for <i>YadA</i> -HNS2 and <i>YadA</i> -S2, <i>SacI</i> site, start: bp 778 (=L260) of <i>yadA</i>   | TGACCTGTCTAACGAGCTCTTGATATGCCAAAA  |
| A-868-f              | Forward, used for <i>YadA</i> -HNS3 and <i>YadA</i> -S3, <i>SacI</i> site, start: bp 868 (=L290) of <i>yadA</i>   | AAAAAATGAGCTGAGCTCTTAGCAAGCGCTAAT  |
| A-990-f              | Forward, used for <i>YadA</i> -HNS4 and <i>YadA</i> -S4, <i>SacI</i> site, start: bp 991 (=T331) of <i>yadA</i>   | AGACAGAGCTCACAGATCATAAATTCATCAAC   |
| A-1057-f             | Forward, used for <i>YadA</i> -L1, <i>SacI</i> site, start: bp 1057 (=L353) of <i>yadA</i>  | CGATTGAGCTCTTAGCCAGTTCAGCCGCTTFA   |
| A-1108-f             | Forward, used for <i>YadA</i> -L2, <i>SacI</i> site, start: bp 1108 (=K370) of <i>yadA</i>  | AAAGAGCTCAAAGATAACTTTCTCGCAGGTG    |
| A- <i>SphI</i> -r    | Constant reverse primer for PCR-2, end: 569 bp after <i>yadA</i> stop codon, 30 bp after <i>SphI</i> site   | GTCAATACAGAGATAGAACAGCT            |
| FLAG- <i>SacI</i> -f | Construction of 30-bp Flag insert dsDNA, deleting a <i>SacI</i> site after insertion  | GGACTATAAGGACGATGATGACAAACAGCT     |
| FLAG- <i>SacI</i> -r | Construction of 30-bp Flag insert dsDNA, deleting a <i>SacI</i> site after insertion  | GTTTGTATCATCGTCCTTATAGTCCAGCT      |

Briefly, collagen II was allowed to bind to Microlon 600 96-well plates (Greiner, Frickenhausen, Germany) in 50  $\mu$ l with a concentration of 20  $\mu$ g/ml in PBS for 1 h at 37°C. Nonspecific binding sites were blocked by incubation with 200  $\mu$ l of coating buffer (PBS, 0.5% bovine serum albumin) for 1 h at 37°C. The wells were washed five times with PBS-0.1% Tween 20 and incubated with the different WA(pYVO8-A) *yadA* mutant strains (OD<sub>500</sub> 0.5) in PBS-0.01% sodium azide for 1 h at 37°C. After they had been washed five times with PBS-0.1% Tween 20, the binding of the bacteria was detected by immunostaining of bacteria with a polyclonal 1:10,000-diluted rabbit anti-WA-c antiserum overnight at 4°C, subsequent incubation with peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) for 1 h at 37°C, and addition of 1 mg of *p*-nitrophenyl phosphate per ml in H<sub>2</sub>O as a substrate at 37°C. The reaction was stopped with 0.5 N sodium hydroxide. The absorbance at 405 nm was determined.

Monolayers of HEP-2 cells grown in Click RPMI 1640 medium (Biochrom KG, Berlin, Germany) were incubated with 5  $\times$  10<sup>7</sup> bacteria per ml for 30 min at 37°C on glass slides. Slides were stained in Giemsa solution, and cell binding was assessed microscopically. Autoagglutination was observed as clearance of the medium as bacteria formed aggregates at the bottom.

**Serum resistance test.** The serum resistance test was performed as described previously (26). Bacteria were grown overnight in RPMI medium at 37°C, pelleted by centrifugation, washed in PBS-MgCl<sub>2</sub> (5 mM), and then incubated at 37°C in 50% normal human serum pooled from healthy blood donors. Surviving bacteria were determined by plating out serial dilutions after 0 and 90 min to determine the CFU. The value at 0 min was set as 100%.

**Virulence test.** The virulence of WA(pYVO8-A-S3) was tested in the orogastric mouse infection model as described previously (26). Groups of six BALB/c mice (female, 6 to 8 weeks old; Charles River WIGA, Sulzfeld, Germany) were infected with 3  $\times$  10<sup>7</sup> bacteria. Four 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 in-frame deletions in *yadA* of *Y. enterocolitica* serotype O8.** A PCR-based strategy was used to delete different parts of the *yadA* gene. One PCR product generated

from the 5' end of the *yadA* gene was ligated to a second PCR product generated from the 3' end of the *yadA* gene. The distance between the two PCR products determined the size of the deletion. The two PCR products were ligated via a restriction site (*SacI*) introduced by the primers at the corresponding ends. Together, both PCR products were inserted into the vector backbone via appropriate restriction sites (*Clal* and *SphI*) and transformed into *E. coli*. All deletions were verified by DNA sequencing. The deletions constructed in this study are schematically shown in Fig. 1 and outlined in Table 1. For expression studies and further functional analysis, the mutated *yadA* genes were introduced into the *yadA*-negative strain *Y. enterocolitica* WA(pYVO8-A-0) by using a  $\lambda$ pir suicide vector (26).

**Expression of in-frame deleted *YadA* and localization of binding epitopes of *YadA*-specific monoclonal antibodies.** The level of *YadA* expression was determined in whole-cell lysates of mid-logarithmic-phase cultures by Western blotting with *YadA*-specific rabbit antisera or MAbs (12, 26). With two exceptions, the level of *YadA* expression by the different constructs was comparable to that of the wild-type WA-314 (data not shown). These results are surprising because point mutations introduced into the head region of *yadA* significantly reduced the production of *YadA* (A. Roggenkamp, unpublished data). The two mutants carrying deletions in the linking region [WA(pYVO8-A-L1 and WA(pYVO8-A-L2)] could not be detected by SDS-PAGE or immunoblot (data not shown). Presumably, these *YadA* variants were rapidly degraded due to ineffective transport and misfolding. These results suggest that

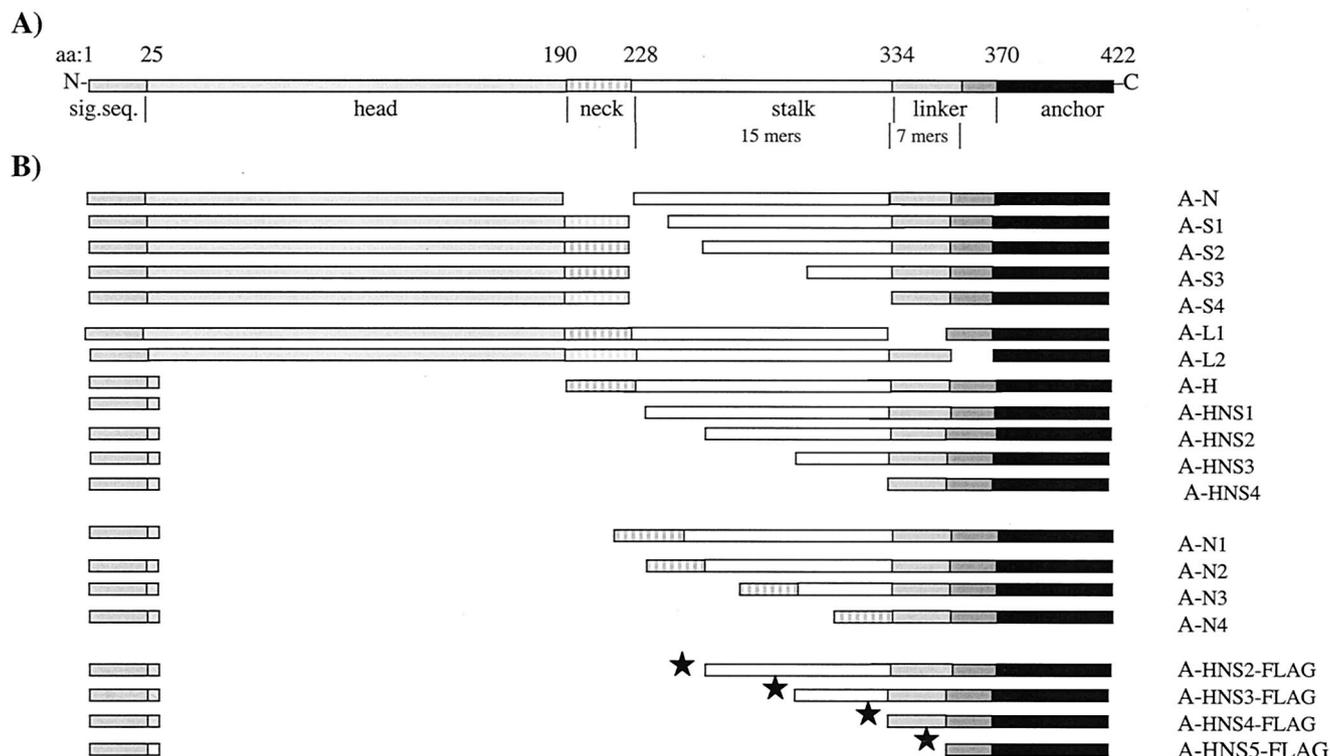


FIG. 1. (A) Schematic drawing of YadA protein of *Y. enterocolitica*. The putative domains suggested by Hoiczky et al. are depicted. sig. seq., signal sequence. (B) Schematic drawing of the YadA deletions constructed in this study. The designations of the mutations are given on the right. Stars indicate the position of the Flag tag.

in contrast to the head, neck, and stalk regions, the linking region is indispensable for stable YadA production.

The truncated forms of YadA are useful candidates for mapping a set of YadA-specific mouse (MAbs) by immunoblotting. MAbs 9A6 and 9H11 recognized the N-terminal region between aa 29 and 81. The epitope of MAb 9H7 is localized between aa 245 and 259, and MAb 8D1 binds within the region of aa 290 and 330 (data not shown). Interestingly, neither the MAbs nor the polyvalent rabbit anti-YadA serum recognized the C-terminal membrane anchor of YadA. The absence of antigenic epitopes from the membrane anchor of YadA was also noticed by testing the Flag-YadA chimeras (see below).

**Surface localization and oligomerization of in-frame deleted YadA mutants.** The surface localization of the truncated YadA mutants was analyzed by Western blotting with outer membrane preparations and by immunofluorescence of whole yersinia cells. All truncated YadA variants except the above-mentioned mutants with deleted linking region copurified with the outer membrane fraction (Fig. 2). Moreover, all these YadA mutants were exposed on the cell surface, which could be demonstrated by accessibility to the YadA-specific MAbs 9A6, 9H7, and 8D1 in immunofluorescence assays with unfixed whole bacteria (data not shown). The surface exposure of mutants encoding the neck domain was also verified by slide agglutination with rabbit anti-YadA serum, as described by Sory et al. (32). Mutants lacking the neck domain did not agglutinate, indicating that the neck domain carries the surface-exposed agglutinogenic epitopes (see also below). These results also demonstrate that transport across the cytoplasmic

membrane, insertion into the outer membrane, and surface exposure of the N-terminal part of YadA do not require the head, neck, or stalk domain.

Oligomerization of YadA can be visualized by the appear-

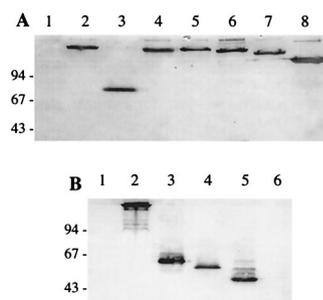


FIG. 2. Expression, outer membrane localization, and formation of high-molecular-weight complexes of different YadA mutants. Outer membrane fractions were prepared from different strains grown at 37°C for 6 h, and 8 µg of each sample was solubilized in sample buffer for 2 h at 37°C, separated by SDS-PAGE, transferred to nitrocellulose sheets, and probed with polyclonal anti-YadA serum. (A) Lanes: 1, WA(pYVO8-A-0); 2, WA(pYVO8-A-1); 3, WA(pYVO8-A-H); 4, WA(pYVO8-A-N); 5, WA(pYVO8-A-S1); 6, WA(pYVO8-A-S2); 7, WA(pYVO8-A-S3); 8, WA(pYVO8-A-S4). (B) Lanes: 1, WA(pYVO8-A-0); 2, WA(pYVO8-A-1); 3, WA(pYVO8-A-HNS1); 4, WA(pYVO8-A-HNS2); 5, WA(pYVO8-A-HNS3); 6, WA(pYVO8-A-HNS4). Note that mutant WA(pYVO8-A-HNS4) was not properly recognized by the antiserum. The positions of molecular size markers are shown on the left (in kilodaltons).

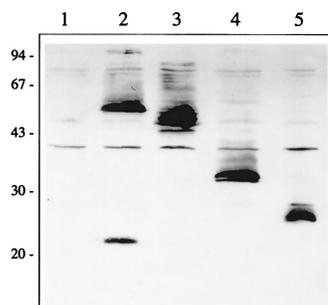


FIG. 3. Outer membrane localization and trimerization of truncated YadA-Flag chimera. Outer membrane fractions were prepared from different strains grown at 37°C for 6 h, and 8  $\mu$ g of each sample was solubilized in sample buffer for 2 h at 37°C, separated by SDS-PAGE, transferred to nitrocellulose sheets, and probed with the anti-FLAG MAb. Lanes: 1, WA(pYVO8-A-1); 2, WA(pYVO8-A-HNS2-Flag); 3, WA(pYVO8-A-HNS3-Flag); 4, WA(pYVO8-A-HNS4-Flag); 5, WA(pYVO8-A-HNS5-Flag). Note that all constructs are present in their trimeric form. The calculated sizes of the monomeric forms of the truncated YadA variants are 19.36 kDa for HNS2-Flag in lane 2, 16.06 kDa for HNS3-Flag in lane 3, 11.55 kDa for HNS4-Flag in lane 4, and 9.13 kDa for HNS5-Flag in lane 5. The positions of molecular size markers are shown on the left (in kilodaltons).

ance of higher-molecular-weight complexes in SDS-PAGE. It is known that proteins harboring coiled-coil structures tend to form aggregates in SDS-PAGE. On the other hand, the C-terminal membrane anchor has been suggested to direct oligomerization and formation of higher-molecular-weight complexes in SDS-PAGE. As shown in Fig. 2, YadA mutants formed higher-molecular-weight complexes irrespective of the presence of the head, neck, or stalk domain. Even the shortest YadA mutants [WA(pYVO8-A-HNS4-Flag) and WA(pYVO8-A-HNS5-Flag)] formed higher-molecular-weight complexes of the predicted sizes (see below; Fig. 3, lanes 4 and 5).

These results demonstrate that the membrane anchor (aa 331 to 422) determines the formation of higher-molecular-weight complexes of YadA. The stalk domain may support oligomerization but is not absolutely necessary. However, disintegration of the oligomeric forms with an increased concentration of urea (1 to 8 M) did not demonstrate a contribution of the head, neck, or stalk domain to stabilization of YadA oligomers, because all the mutants dissociated into monomers at 3 M urea and 100°C (data not shown).

**Linking region of YadA is responsible for membrane translocation.** As shown above, the head, neck, and stalk domains of YadA were found to be surface exposed. We speculated that the linking region might be indispensable for membrane translocation of the N-terminal part of YadA. To test the hypothesis, we introduced a tag sequence into the truncated YadA mutants A-HNS2, A-HNS3, A-HNS4, and A-HNS5 at positions 260, 290, and 330, and 352, generating A-HNS2-Flag, A-HNS3-Flag, A-HNS4-Flag, and A-HNS5-Flag. The constructs were expressed and transported to the outer membrane and formed oligomers when analyzed by SDS-PAGE (Fig. 3). The sizes of the different YadA oligomers are consistent with the prediction of YadA trimers. Immunofluorescence analysis of unfixed cells demonstrated that the Flag epitopes at aa 260, 290, and 330 were surface exposed. However, the Flag at aa 352 was not recognized by the anti-FLAG antibody (data not

shown). In contrast to the proximal part of the linking region (aa 330 to 351), the distal part (aa 352) might be hidden beneath the outer membrane or directed to the periplasmic space. These results suggest that the linking region is involved in translocation of the N terminus across the outer membrane. Moreover, the distal 70 aa of YadA are probably sufficient to direct oligomerization of YadA.

**Trimerization of YadA.** SDS-PAGE analysis of outer membrane preparations of different Flag-YadA chimera indicated that the higher-molecular-weight complexes of YadA are trimers (Fig. 3). Mack et al. and Gripenberg-Lerche et al. (13, 21) also suggested that YadA forms a trimer on the cell surface. However, estimation of the volume of YadA by electron microscopy predicted a YadA tetramer (18). Following the experiments of Gripenberg-Lerche et al., we analyzed the ability of YadA to form heterooligomers by coexpression of complete YadA and in-frame truncated YadA mutants. For the construction of merodiploid virulence plasmids, truncated *yadA* genes located on suicide vectors (pGPS) were integrated into pYVO8 plasmids of the parental strain WA-314. By SDS-PAGE, we detected four YadA oligomeric bands, indicating trimeric forms (Fig. 4). Similar results were also obtained by coexpression of wild-type YadA and the shortest YadA mutant, A-HNS4 [encoding only the linking region and the membrane anchor, strain WA(pYVO8::pGPS-A-HNS4); data not shown]. The fact that the C terminus of YadA (aa 331 to 422) was able to form heterooligomers with full-length YadA pointed to the importance of the membrane anchor for the oligomerization of YadA. SDS-PAGE analysis of the truncated Flag-YadA chimera and the formation of YadA heterooligomers strongly suggested that YadA forms trimers after boiling.

**Adherence phenotypes of YadA mutants.** The multifunctional binding domain(s) of YadA which mediates adherence to eukaryotic cells and extracellular matrix proteins (e.g., collagen) is located in the globular head domain (aa 26 to 190) (10, 26, 34). Moreover, a hydrophobic region of the head

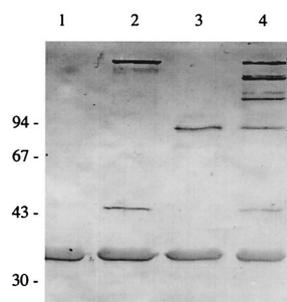


FIG. 4. Formation of heterooligomers by wild-type YadA A-1 and the truncated YadA mutant A-H. Whole-cell lysates of different strains grown at 37°C for 6 h were separated by SDS-PAGE, transferred to nitrocellulose sheets, and probed with MAb 8D1. Lanes: 1, WA(pYVO8-A-0); 2, WA(pYVO8); 3, WA(pYVO8-A-H); 4, WA(pYVO8::pGPS-A-H). In lane 4, four different oligomeric bands are visible. This constellation can only be explained by the formation of trimers. The different homo- and heterooligomers are marked by symbols: ●, homooligomer, three times wild-type A-1 mass; ▲, homooligomer, three times truncated A-H mass; ▼, heterooligomer, two times A-1 mass and one time A-H mass; ◆, heterooligomer, one time A-1 mass and two times A-H mass. The positions of molecular size markers are shown (in kilodaltons).

TABLE 3. Survival of WA strains in 50% pooled normal human serum at 37°C<sup>a</sup>

| Strain           | Mean survival (%)<br>± SD at 90 min |
|------------------|-------------------------------------|
| WA(pYVO8-A-1)    | 82 ± 41                             |
| WA(pYVO8-A-0)    | 7 ± 26                              |
| WA(pYVO8-A-N)    | 102 ± 41                            |
| WA(pYVO8-A-S1)   | 164 ± 43                            |
| WA(pYVO8-A-S2)   | 90 ± 38                             |
| WA(pYVO8-A-S3)   | 111 ± 43                            |
| WA(pYVO8-A-S4)   | 1 ± 1                               |
| WA(pYVO8-A-H)    | 104 ± 11                            |
| WA(pYVO8-A-HNS1) | 81 ± 56                             |
| WA(pYVO8-A-HNS2) | 0.084 ± 0.176                       |
| WA(pYVO8-A-HNS3) | 0.08 ± 0.186                        |
| WA(pYVO8-A-HNS4) | 6 ± 24                              |
| WA(pYVO8-A-N1)   | 112 ± 7                             |
| WA(pYVO8-A-N2)   | 0.086 ± 0.134                       |
| WA(pYVO8-A-N3)   | 6 ± 5                               |
| WA(pYVO8-A-N4)   | 5 ± 5                               |

<sup>a</sup> After 90 min, the number of surviving bacteria was determined by plating out serial dilutions onto Luria-Bertani agar. A value of 100% is equal to  $5 \times 10^5$  bacteria per ml. The data are means ± standard deviations for three independent experiments.

domain is responsible for the YadA-mediated autoagglutination phenotype (34). Here we confirmed these previous results with YadA head region deletion mutants. Mutants lacking the head domain failed to bind to epithelial cells or collagen and lost the capacity to mediate autoagglutination (data not shown). Surprisingly, the mutant carrying a deletion of the neck domain (aa 190 to 221) also lost the adherence function. On the other hand, in-frame deletions of different numbers of 15-mer repeats leading to truncation of the stalk domain did not cause YadA to lose the ability to bind to epithelial cells or collagen. The neck domain is probably important for correct folding of the globular head. The head and neck domains may form a functional unit (binding module), whereas the length of the stalk does not seem to be essential for binding.

**Serum resistance of YadA mutants.** It is well known that YadA mediates the serum resistance of *Y. enterocolitica* strains (11). However, YadA structure-function relationships have not been analyzed. It has been suggested that binding of complement factor H may be involved in inactivation of the complement attack (4, 27). However, a direct binding of purified factor H or factor H-like protein (FHL-1) and YadA could not be demonstrated (P. F. Zipfel and A. Roggenkamp, unpublished data). We analyzed whether a set of truncated YadA mutants could confer the ability to survive 50% pooled human serum on yersiniae. The serum resistance and survival rates are shown in Table 3. Surprisingly, the head and neck domains of YadA were dispensable for YadA-mediated serum resistance. The shortest construct mediating serum resistance consisted of a stalk with five 15-mer repeats, the linking region, and the membrane anchor [WA(pYVO8-A-HNS1)]. Moreover, YadA mutants carrying in-frame deletions only in the stalk domain conferred serum resistance, demonstrating that the four N-terminal 15-mer repeats were also dispensable for serum resistance [WA(pYVO8-A-S1), WA(pYVO8-A-S2), and WA(pYVO8-A-S3)]. Therefore, serum resistance could not be ascribed to a single YadA domain.

Considering the geometrical data obtained from electron

microscopy of YadA structural units, we assume that a surface projection of a certain length may be sufficient to promote serum resistance. For WA(pYVO8-A-HNS1), the calculated length of the truncated stalk was about 15 nm; for WA(pYVO8-A-S3), the calculated diameter of the head was 5 nm and of the truncated stalk was 8 nm, for a total of about 13 nm.

An alternative is that YadA stabilizes the outer membrane by building stable oligomers on the cell surface. To test this hypothesis, we constructed additional mutants. The neck domain is highly conserved in the class of YadA-homologous proteins and may function as a stabilizer of stalk domains. We fused the neck domain (aa 189 to 228) to truncated YadA mutants carrying stalks of different lengths [see Fig. 1, mutants WA(pYVO8-A-N1), WA(pYVO8-A-N2), WA(pYVO8-A-N3), and WA(pYVO8-A-N4)]. All these constructs were produced in amounts comparable to the wild type, were detected in the outer membrane, and were found to be surface exposed (data not shown). Moreover, mutants carrying the neck domain agglutinated with polyvalent anti-YadA rabbit serum in slide agglutination tests (see also above). However, the neck domains did not confer serum resistance on the YadA mutants encoding only short stalk domains of YadA (Table 3).

**Virulence of stalk mutant WA(pYVO8-A-S3).** We wondered whether the length of the stalk is important for the ability of YadA to contribute to the virulence of *Y. enterocolitica*. To address this question, we analyzed the virulence of the mutant WA(pYVO8-A-S3), which carries a deletion in the stalk domain of 60 aa (four pentadecamer repeats), in comparison to the wild-type strain WA(pYVO8-A-1). Two groups of BALB/c mice were intragastrically infected with  $3 \times 10^7$  bacteria. Four days postinfection, all mice in both groups appeared ill, and the bacterial load in the organs was determined (Fig. 5). Obviously, the mutant WA(pYVO8-A-S3) retained the ability to colonize the small intestine, multiply in the Peyer's patches, and disseminate to the spleen and liver. In this infection model, no significant difference between the wild type and the stalk mutant could be detected. The truncation of the stalk region did not result in significant attenuation.

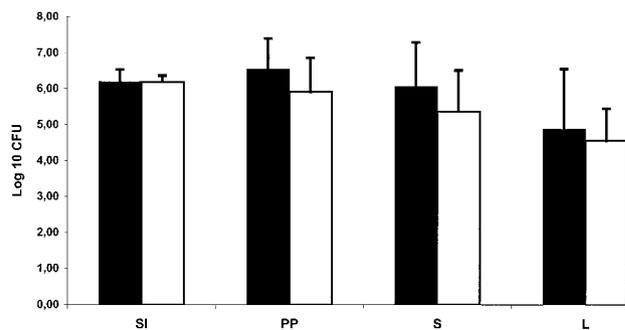


FIG. 5. Virulence of *Y. enterocolitica* serotype O8 strains WA (pYVO8-A-1) (wild type, black bars) and WA(pYVO8-A-S3) (deletion of 60 aa in the stalk domain, white bars) in intragastrically infected BALB/c mice. Groups of six mice were infected with  $3 \times 10^7$  bacteria. After 4 days, the number of bacteria in the small intestine (SI), Peyer's patches (PP), spleen (S), and liver (L) was determined. The data are means ± standard deviations.

## DISCUSSION

In this study, we analyzed YadA for translocation into the outer membrane, oligomerization, and pathogenic functions. We confirmed that the C terminus, probably consisting of four  $\beta$ -strands, is required for integration into the outer membrane. Secreted or outer membrane proteins that contain all the information required for transport to the outer membrane and translocation through the cell envelope are classified as autotransporters (17, 36). YadA fulfills these criteria. YadA is composed of (i) an N-terminal Sec-dependent secretion signal necessary for translocation of the cytoplasmic membrane (aa 1 to 25) (31), (ii) a surface-exposed passenger domain (aa 26 to 330, containing the head, neck, and stalk domains) that remains covalently attached after outer membrane translocation, (iii) a linking region (aa 331 to 369) necessary for translocation of the passenger domain through the outer membrane, and (iv) a C-terminal transmembrane region consisting of four amphipathic  $\beta$ -sheets.

On the cell envelope, the YadA passenger domains appear as lollipop-like surface projections (18). For this structure, the formation of a YadA homooligomer is necessary. Deletion mutagenesis, formation of heterooligomers, and YadA-Flag fusions supported the suggestions of Mack et al. and Gripenberg-Lerche et al. (13, 21) that YadA forms trimers. Moreover, our present study demonstrates that the C-terminal membrane anchor domain is responsible for the topology of YadA in the outer membrane. Deletion of the C-terminal domain abolished membrane insertion of the truncated YadA (34).

Taking into account that one YadA molecule contains four amphipathic  $\beta$ -sheets, the formation of a 12-stranded  $\beta$ -barrel in the outer membrane after trimerization has to be postulated. A comparable  $\beta$ -barrel structure is known from the TolC protein (19). The  $\beta$ -barrel of TolC is assembled from three protomers with four amphipathic  $\beta$ -sheets each (10 to 13 residues long, 40 Å high), resulting in a cylinder with an internal diameter of about 36 Å. Analogous to TolC, it seems likely that the C terminus of YadA (aa 370 to 422) forms a pore in the outer membrane. In the case of TolC, only the loop domains are surface exposed; the major part of the protein forms the channel through the periplasm. However, the major part of YadA is exposed on the cell surface. Our results indicate that the linking region is responsible (aa 331 to 369) for the translocation of the passenger domain. The linking region contains a segment with a high probability of forming a left-handed coiled coil (two to three heptamers) (18). Flag fusions demonstrated that the N-terminal part of this domain is surface exposed, whereas the outer membrane hides the rest of this domain. We speculate that the left-handed coiled-coil segment of the linking region crosses the outer membrane through the pore formed by the trimeric C-terminal membrane anchor of YadA. The residues between the left-handed coiled-coil domain and the membrane anchor may form a short hairpin loop.

The importance of the linking domain is supported by the fact that deletions in this region resulted in degradation of the whole protein, possibly by periplasmic proteases. Moreover, deletions in all other surface-exposed domains of YadA did not prevent the surface exposure of the truncated protein. A domain functionally identical to the linking region of YadA is known in the autotransporter family (22). Sequence homolo-

gies between the two types of linking regions could not be detected. This may be because conventional autotransporters are monomers, whereas the linking region of YadA crosses the outer membrane as a trimer.

Oligomerization and surface exposure of passenger domain are to a great extent determined by the membrane anchor domain of YadA (the C-terminal 91 aa, linking region, and four antiparallel amphipathic membrane-spanning  $\beta$ -sheets). This is exactly the domain with the highest similarity in the class of Oca proteins. Conserved domain databases such as Pfam define the class of YadA-homologous Oca proteins via their homology in the membrane anchor domain, suggesting that the other members of the Oca family may also be autotransporters that form oligomers in the outer membrane.

Based on other sequence homologies, the Oca members UspA1 and UspA2 of *Moraxella catarrhalis* and Hia of *Haemophilus influenzae* have been included in the family of autotransporters (17). For Hia, experimental results support this classification (33). However, the structural predictions given for the Hia membrane anchor are not in accordance with our results. St Geme and Cutter (33) postulated that the Hia  $\beta$ -barrel is formed by 14 antiparallel amphipathic membrane-spanning  $\beta$ -sheets encoded by the C-terminal 319 aa of one Hia molecule. We postulate that all Oca proteins form oligomeric  $\beta$ -barrels in the outer membrane due to the conserved C-terminal 91 aa (linking region and four antiparallel amphipathic membrane-spanning  $\beta$ -sheets). Hia formed high-molecular-weight complexes in SDS-PAGE, indicating oligomerization (3). This observation supported our assumption. However, other models for the C-terminal membrane anchor domain of Hia may also be possible, and a detailed analysis of the  $\beta$ -barrel has to be done.

As part of the passenger domain, a sequence of about 20 aa has been identified in YadA that shows high similarity to conserved regions in other members of this protein family. This stretch is located between the head and stalk domains and was termed the neck domain (18). The distribution and the high degree of conservation suggested an important contribution of the neck domain to structure or assembly in this class of proteins. However, the function of this domain was unknown. In-frame deletion mutagenesis indicated that the neck domain, together with the head domain, forms the binding module of YadA. The neck domain is dispensable for surface localization, stalk formation, stabilization, and oligomerization of YadA on the cell surface. Moreover, the neck domain is not involved in the serum resistance that is conferred by some members of this class of proteins. In contrast to the stalk domain, deletion of the neck domain abolished the binding ability of YadA, which is encoded in the head domain. As has been demonstrated for YadA, the neck domain might be the platform for the individual binding units of the other Oca proteins.

It has been suggested that the head domain of YadA may aggregate to a densely packed coat on the surface of the pathogen, forming a “quasi-periplasmic space” between the outer membrane and the head domains (18). Lipopolysaccharides and other proteins of the outer membrane localized in the quasi-periplasmic space may be masked. However, a recent study by Eitel and Dersch suggested that even maximally expressed YadA does not cover the bacterium totally (8). Other

and smaller proteins are functional in the presence of YadA (e.g., the invasin Inv).

Corresponding to this study, the results of our study did not substantiate the postulation of a surface coat. (i) The results of the mouse infection model showed that a truncation of 75% of the stalk domain [mutant WA(pYVO8-A-S3)], more or less eliminating the quasi-periplasmic space, did not result in significant attenuation of the mutant. However, this has to be postulated if the formation of a protective shield is a virulence-associated feature of YadA. On the hand, other proteins (e.g., the invasin Inv) may have taken over essential virulence functions in WA(pYVO8-A-S3). (ii) YadA and some other members of the family are involved in serum resistance. The formation of a protective shield may be one strategy to promote this feature. However, YadA was able to confer serum resistance without the globular head domain. This might also be true for other members of this family which are involved in serum resistance. The nature of YadA-mediated serum resistance is far from clear. Most likely, the oligomeric structures in the outer membrane prevent insertion of the terminal lytic complex of serum. Lack of insertion has been demonstrated in YadA-positive but not in YadA-negative *Y. enterocolitica* strains (24).

In conclusion, electron microscopy and sequence analysis predict that YadA and homologous proteins consist of distinct protein domains forming different modules. Using in-frame deletion mutagenesis, we confirmed this prediction and analyzed the different modules in more detail. Our results suggest that YadA is the first example of an autotransporter that forms trimers in the outer membrane. The C-terminal membrane anchor domain is most important in this context. YadA-homologous proteins, which we term Oca proteins, may also belong to this new subfamily of autotransporter proteins.

#### ACKNOWLEDGMENT

We thank G. Anding for excellent technical assistance.

#### REFERENCES

- Aebi, C., E. R. Lafontaine, L. D. Cope, J. L. Latimer, S. L. Lumbley, G. H. McCracken, Jr., and E. J. Hansen. 1998. Phenotypic effect of isogenic *uspA1* and *uspA2* mutations on *Moraxella catarrhalis* 035E. *Infect. Immun.* **66**:3113–3119.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1989. Current protocols in molecular biology. John Wiley & Sons, New York, N.Y.
- Barenkamp, S. J., and J. W. St. Geme III. 1996. Identification of a second family of high-molecular-weight adhesion proteins expressed by non-typable *Haemophilus influenzae*. *Mol. Microbiol.* **19**:1215–1223.
- China, B., M. P. Sory, B. T. N'Guyen, M. De Bruyere, and G. R. Cornelis. 1993. Role of the YadA protein in prevention of opsonization of *Yersinia enterocolitica* by C3b molecules. *Infect. Immun.* **61**:3129–3136.
- Comanducci, M., S. Bambini, B. Brunelli, J. Adu-Bobie, B. Arico, B. Capocchi, M. M. Giuliani, V. Masignani, L. Santini, S. Savino, D. M. Granoff, D. A. Caugant, M. Pizza, R. Rappuoli, and M. Mora. 2002. NadA, a novel vaccine candidate of *Neisseria meningitidis*. *J. Exp. Med.* **195**:1445–1454.
- Cope, L. D., E. R. Lafontaine, C. A. Slaughter, C. A. Hasemann, Jr., C. Aebi, F. W. Henderson, G. H. McCracken, Jr., and E. J. Hansen. 1999. Characterization of the *Moraxella catarrhalis* *uspA1* and *uspA2* genes and their encoded products. *J. Bacteriol.* **181**:4026–4034.
- Cornelis, G. R., A. Boland, A. P. Boyd, C. Geuijen, M. Iriarte, C. Neyt, M. P. Sory, and I. Stainier. 1998. The virulence plasmid of *Yersinia*, an antihost genome. *Microbiol. Mol. Biol. Rev.* **62**:1315–1352.
- Eitel, J., and P. Dersch. 2002. The YadA protein of *Yersinia pseudotuberculosis* mediates high-efficiency uptake into human cells under environmental conditions in which invasin is repressed. *Infect. Immun.* **70**:4880–4891.
- Elkins, C., K. J. Morrow, Jr., and B. Olsen. 2000. Serum resistance in *Haemophilus ducreyi* requires outer membrane protein DsrA. *Infect. Immun.* **68**:1608–1619.
- El Tahir, Y., P. Kuusela, and M. Skurnik. 2000. Functional mapping of the *Yersinia enterocolitica* adhesin YadA. Identification of eight NSVAIG-S motifs in the amino-terminal half of the protein involved in collagen binding. *Mol. Microbiol.* **37**:192–206.
- El Tahir, Y., and M. Skurnik. 2001. YadA, the multifaceted *Yersinia* adhesin. *Int. J. Med. Microbiol.* **291**:209–218.
- Emödy, L., J. Heesemann, H. Wolf-Watz, M. Skurnik, G. Kapperud, P. O Toole, and T. Wadström. 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. *J. Bacteriol.* **171**:6674–6679.
- Gripenberg-Lerche, C., M. Skurnik, and P. Toivanen. 1995. Role of YadA-mediated collagen binding in arthritogenicity of *Yersinia enterocolitica* serotype O:8: experimental studies with rats. *Infect. Immun.* **63**:3222–3226.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**:557–580.
- Heesemann, J., and L. Grüter. 1987. Genetic evidence that the outer membrane protein Yop1 of *Yersinia enterocolitica* mediates adherence and phagocytosis resistance to human epithelial cells. *FEMS Microbiol. Lett.* **40**:37–41.
- Heesemann, J., C. Keller, R. Morawa, N. Schmidt, H. J. Siemens, and R. Laufs. 1983. Plasmids of human strains of *Yersinia enterocolitica*: molecular relatedness and possible importance for pathogenesis. *J. Infect. Dis.* **147**:107–115.
- Henderson, I. R., F. Navarro-Garcia, and J. P. Nataro. 1998. The great escape: structure and function of the autotransporter proteins. *Trends Microbiol.* **6**:370–378.
- Hoiczky, E., A. Roggenkamp, M. Reichenbecher, A. Lupas, and J. Heesemann. 2000. Structure and sequence analysis of *Yersinia* YadA and *Moraxella* UspAs reveal a novel class of adhesins. *EMBO J.* **19**:5989–5999.
- Koronakis, V., A. Sharf, E. Koronakis, B. Luisi, and C. Hughes. 2000. Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405**:914–919.
- Lafontaine, E. R., L. D. Cope, C. Aebi, J. L. Latimer, G. H. McCracken, Jr., and E. J. Hansen. 2000. The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella catarrhalis* to human epithelial cells in vitro. *J. Bacteriol.* **182**:1364–1373.
- Mack, D., J. Heesemann, and R. Laufs. 1994. Characterization of different oligomeric species of the *Yersinia enterocolitica* outer membrane protein YadA. *Med. Microbiol. Immunol. (Berlin)* **183**:217–227.
- Maurer, J., J. Jose, and T. F. Meyer. 1999. Characterization of the essential transport function of the AIDA-1 autotransporter and evidence supporting structural predictions. *J. Bacteriol.* **181**:7014–7020.
- Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
- Pilz, D., T. Vocke, J. Heesemann, and V. Brade. 1992. Mechanism of YadA-mediated serum resistance of *Yersinia enterocolitica* serotype O3. *Infect. Immun.* **60**:189–195.
- Ray, S. K., R. Rajeshwari, Y. Sharma, and R. V. Sonti. 2002. A high-molecular-weight outer membrane protein of *Xanthomonas oryzae* pv. *oryzae* exhibits similarity to non-fimbrial adhesins of animal pathogenic bacteria and is required for optimum virulence. *Mol. Microbiol.* **46**:637–647.
- Roggenkamp, A., H. R. Neuberger, A. Flugel, T. Schmoll, and J. Heesemann. 1995. Substitution of two histidine residues in YadA protein of *Yersinia enterocolitica* abrogates collagen binding, cell adherence and mouse virulence. *Mol. Microbiol.* **16**:1207–1219.
- Roggenkamp, A., K. Ruckdeschel, L. Leitritz, R. Schmitt, and J. Heesemann. 1996. 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. *Infect. Immun.* **64**:2506–2514.
- Sandt, C. H., and C. W. Hill. 2000. Four different genes responsible for nonimmune immunoglobulin-binding activities within a single strain of *Escherichia coli*. *Infect. Immun.* **68**:2205–2214.
- Schulze-Koops, H., H. Burkhardt, J. Heesemann, T. Kirsch, B. Swoboda, C. Bull, S. Goodman, and F. Emmrich. 1993. Outer membrane protein YadA of enteropathogenic yersiniae mediates specific binding to cellular but not plasma fibronectin. *Infect. Immun.* **61**:2513–2519.
- Schulze-Koops, H., H. Burkhardt, J. Heesemann, K. von der Mark, and F. Emmrich. 1992. Plasmid-encoded outer membrane protein YadA mediates specific binding of enteropathogenic yersiniae to various types of collagen. *Infect. Immun.* **60**:2153–2159.
- Skurnik, M., and H. Wolf-Watz. 1989. Analysis of the *yopA* gene encoding the Yop1 virulence determinants of *Yersinia* spp. *Mol. Microbiol.* **31**:1443–1462.
- Sory, M. P., J. Tollenaere, C. Laszlo, T. Biot, G. R. Cornelis, and G. Wauters. 1990. Detection of pYV<sup>+</sup> *Yersinia enterocolitica* isolates by P1 slide agglutination. *J. Clin. Microbiol.* **28**:2403–2408.
- St. Geme, J. W., III, and D. Cutter. 2000. The *Haemophilus influenzae* Hia

- adhesin is an autotransporter protein that remains uncleaved at the C terminus and fully cell associated. *J. Bacteriol.* **182**:6005–6013.
34. **Tamm, A., A. M. Tarkkanen, T. K. Korhonen, P. Kuusela, P. Toivanen, and M. Skurnik.** 1993. Hydrophobic domains affect the collagen-binding specificity and surface polymerization as well as the virulence potential of the YadA protein of *Yersinia enterocolitica*. *Mol. Microbiol.* **10**:995–1011.
35. **Visser, L. G., P. S. Hiemstra, M. T. van den Barselaar, P. A. Ballieux, and R. van Furth.** 1996. Role of YadA in resistance to killing of *Yersinia enterocolitica* by antimicrobial polypeptides of human granulocytes. *Infect. Immun.* **64**:1653–1658.
36. **Yen, M. R., C. R. Peabody, S. M. Partovi, Y. Zhai, Y. H. Tseng, and M. H. Saier.** 2002. Protein-translocating outer membrane porins of Gram-negative bacteria. *Biochim. Biophys. Acta* **1562**:6–31.