Trimer stability of YadA is critical for virulence of Yersinia enterocolitica

Schütz M. ¹#, Weiss E.-M. ¹, Schindler M. ¹, Hallström T. ², Zipfel P. F. ², Linke D. ³, Autenrieth, I.B. ¹

1) Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Elfriede-Aulhornstr. 6, Tübingen D-72076, Germany
2) Leibniz Institute for Natural Product Research and Infection Biology-Hans Knöll Institute, Jena D-07745, Germany
3) Max-Planck-Institute for Developmental Biology, Department Protein Evolution, Spemannstr. 35-39, Tübingen D-72076, Germany

# Correspondence: Dr. Monika Schütz, Institute for Medical Microbiology and Hygiene, University Hospital Tübingen, Elfriede-Aulhornstr. 6, Tübingen D-72076, Germany; telephone: (+49) 7071 29 81527, fax: (+49) 7071 29 5440, email: monika.schuetz@med.uni-tuebingen.de

Running title: Trimer stability of YadA is important for virulence
Abstract

Yersinia adhesin A (YadA) is a trimeric autotransporter adhesin with multiple functions in host-pathogen interactions. The aim of this study was to dissect the virulence functions promoted by YadA in vitro and in vivo. To accomplish this we generated Yersinia enterocolitica (Ye) O:8 mutants expressing point mutations in YadA G389, a highly conserved residue in the membrane anchor of YadA, and analyzed their impact on YadA expression and virulence functions. We found that point mutations of YadA G389 led to impaired transport, stability and surface display of YadA. YadA mutants G389A and G389S showed comparable YadA surface expression, autoagglutination and adhesion, compared to the YadA wildtype, but displayed reduced trimer stability and complement resistance in vitro, and were 10-1,000 fold attenuated in experimental Ye infection in mice. The mutants G389T, G389N, and G389H lost trimer stability, exhibited strongly reduced surface display, autoagglutination, adhesion properties and complement resistance, and were avirulent (>10,000 fold attenuation) in mice. Our data demonstrate that G389 is a critical residue of YadA required for optimal trimer stability, transport, surface display and serum resistance. We also show that stable trimeric YadA protein is essential for virulence of Ye.

Introduction

Enteropathogenic Yersinia species Yersinia enterocolitica (Ye) and Yersinia pseudotuberculosis (Yps) are foodborne pathogens causing diarrhea, mesenteric lymphadenitis and reactive arthritis (10). Upon ingestion of contaminated food the bacteria colonize the intestine and may invade M cells which overlie the Peyer’s patches (17). After translocation by M cells, Ye multiplies extracellularly in the adjacent tissue. A major virulence determinant of Ye is the Yersinia adhesin A (YadA) (13). YadA belongs to the family of trimeric autotransporter adhesins (TAAs) (29). TAAs consist of a N-terminal...
head domain connected via an extended stalk to the C-terminal membrane anchor domain. The head domain of YadA is involved in binding to collagen or host cells whereas the stalk domain is involved in serum resistance (34). Three membrane anchor domains of one YadA trimer build up a beta-barrel pore which facilitates the transition of the passenger domains (stalk, head) onto the outer membrane (21). The exact mechanism of trimeric autotransport remains yet unclear. There do exist several models, amongst which the hairpin and the threading model propose that the membrane anchor domains integrate into the OM and build up a beta barrel-pore. After that the passenger domains traverse the pore starting with either the N- or C-terminus (5).

Numerous efforts have attempted to elucidate the function of YadA in establishing an infection with Ye. The domain-function relationships of YadA have been analysed in detail (35, 36). There is striking evidence that the head domain of YadA is involved in binding to extracellular matrix proteins like collagen and in binding to neutrophils (14, 20, 27, 34). Also the autoagglutination capacity seems to involve at least parts of the head domain (44). In addition to adherence and autoagglutination, serum resistance seems to be an important function mediated by YadA for virulence of Ye in vivo. Via the binding of serum complement factor H and C4 binding protein (C4BP), YadA may prevent deposition of C3b on the bacterial surface and therefore blocks formation of membrane attack complexes and killing of the bacteria (6, 23). However, this function has so far only been mapped for factor H which binds to the stalk domain of YadA (7). The binding region for C4BP is still unknown.

Recent studies by Ackermann et al. (1) have shown that only the factual membrane anchor domain of YadA is able to confer serum resistance and mouse virulence to Ye when expressing chimeric fusion proteins of the N-terminal YadA passenger domain and C-terminal membrane anchor domains of the TAAs UspA1 (Moraxella catharralis), EibA (Escherichia coli), and Hia (Haemophilus influenzae).
We previously showed that YadA and most other known TAAs have a G-residue in the second beta-strand of the membrane anchor domain which forms the beta-barrel translocator pore; in the exceptional TAAs it is either A, S, T, or N (19). This residue (G389 in YadA of Ye O:8) faces the lumen of the beta-barrel. According to a YadA beta-barrel model that was generated based on the Haemophilus influenzae Hia 3D structure the free space around G389 should be large enough to accommodate either A, S, C, D, P, N, T, V, E, or Q (in ascending order of side chain volume) without constraining the conformation of adjacent residues. An H-residue would reach the limit of the pocket size but might still be accommodated with only minor local structural adjustments. We previously substituted the G389 with the four naturally occurring residues (A, S, T, and N) and also with H in order to explore the upper size limit of the pocket. The side chain size of the residues increases in the order G<A<S<T<N<H and polarity in the order A<G<S<T<N<H. When expressed in E. coli these G389 substitutions were shown to influence the YadA surface display and stability to different degrees (19).

To elucidate which of the many functions of YadA in host-pathogen interaction in vitro is of relevance for the pathogenicity of Ye in vivo, we introduced the G389 substitution mutations into Ye background, and thoroughly analysed trimer stability, accessibility by trypsin, outer membrane localisation, adhesion to collagen and HeLa cells, Yop-mediated suppression of cytokine responses, autoagglutination, serum resistance, binding of complement regulatory factors and virulence in mice. From our results we conclude that the stability of YadA trimers is decisive for the YadA mediated serum resistance and the virulence of Ye in mice.

**Materials and Methods**

**Mice**

From 6- to 8-week-old female C57BL/6 or BALB/c mice were purchased from Harlan Winkelmann (Borchen, Germany). The mice were kept under specific-pathogen-free
conditions in positive-pressure cabinets (Techniplast) and provided with sterile food and water ad libitum.

**Bacterial Strains and Culture Conditions**

The bacterial strains used in this study are listed in Table 1. The strains of Ye were grown overnight in Luria–Bertani broth at 27°C supplemented with nalidixic acid (10 µg/ml). The YadA deficient mutant YadA0 was additionally supplemented with kanamycin (50 µg/ml). Ye strains expressing mutant versions of YadA were grown in the presence of nalidixic acid, kanamycin and spectinomycin (50 µg/ml). A 1:20 dilution of the overnight Ye culture was incubated for additional 2-3 h at 37°C. The bacteria were washed once with PBS (Invitrogen) and the optical density at 600 nm was determined.

**DNA manipulations and PCR**

Exchanges of single amino acids in the Y. enterocolitica yadA gene were introduced as described previously (19) using the plasmid pUC-A-1 (35) and were verified by DNA sequencing. The primer pairs used are available upon request. Using the EcoRI and SphI restriction sites the point mutated yadA genes were subcloned into the mobilizable suicide vector pGP704 resulting in the constructs pGP-YadAwt, pGP-YadA G389A, pGP-YadA G389S, pGP-YadA G389T, pGP-YadA G389N, pGP-YadA G389H. Subsequently a spectinomycin resistance cassette was inserted into the EcoRI restriction site resulting in the plasmids pGPS-YadAwt, pGPS-YadA G389A, pGPS-YadA G389S, pGPS-YadA G389T, pGPS-YadA G389N, pGPS-YadA G389H. After transformation into E. coli S17-1 λpir the plasmids were mobilized into the YadA deficient strain Ye YadA0 (35). Transconjugants harbouring cointegrates (e.g. pYVO8-A-0::pGPS-YadAwt) were selected for kanamycin and spectinomycin resistance and verified by PCR and western blot.
Sample preparation for Western blot analysis

Bacterial pellets were lysed in SDS sample buffer (5 x 10^7 Ye cells were loaded per lane) and incubated for 10 min at 95°C prior to loading if not otherwise indicated. For the preparation of unheated samples, alkaline lysis of approximately 1.5 x 10^9 Ye bacteria was performed using 180 µl of buffer 1 of the peqGOLD plasmid miniprep kit (PEQLAB-Biotechnologie GmbH, Erlangen, Germany) and 20 µl of 1 M NaOH. After adjustment to pH 7.0, 20 µl of DNase I incubation buffer (10x) and 2 µl of DNase (10 U/ml; Roche Diagnostics GmbH, Mannheim, Germany) were added and left for 20 min at room temperature before SDS sample buffer was added. For denaturation of trypsin digested proteins the samples were heated in 5x Laemmli buffer supplemented with 6M urea for 10 min at 95°C.

Trypsin Digestion

The overnight cultures of Y. enterocolitica grown at 27°C were diluted to an OD600 of 0.1 in fresh medium and grown to exponential phase for 2 h at 27°C. After that bacteria were grown for 2 h at 37°C to induce YadA expression. 1.5 x 10^8 bacteria were pelleted and washed once with PBS. Bacteria were then resuspended in PBS containing 0.1 mg/ml trypsin (Applichem) and incubated in ice for 1 h. To stop tryptic digestion trypsin inhibitor (0.2 mg/ml final concentration; Sigma) was added. Bacteria and supernatant were separated by centrifugation. The bacterial pellet was resuspended in an equal volume of 5x concentrated Laemmli buffer, heated and subjected to SDS-PAGE.

Western blot analysis

After SDS-PAGE, proteins were transferred onto nitrocellulose membranes. The membranes were blocked overnight with phosphate-buffered saline (PBS)-5 % milk powder at 4°C. For the detection of YadA, a purified IgG fraction of polyclonal rabbit YadA antiserum (diluted 1:1,000) and a peroxidase-conjugated secondary anti-rabbit antibody (diluted 1:10,000;
Dianova) were added and left for 1 h and 45 min at room temperature, respectively. Detection of bound antibodies was carried out using the ECL detection kit (Amersham Biosciences).

**Surface localisation of YadA by immunofluorescence**

Coverslips were coated overnight at 4°C with 10 µg/ml human collagen type I (Calbiochem/Merck, Darmstadt, Germany) in PBS. After washing with PBS, 2 x 10^7 bacteria were centrifuged onto the coverslips at 300 rpm for 5 min. After 1 h of incubation at 37°C, the coverslips were washed three times with PBS and then fixed with 4 % paraformaldehyde (PFA). We tested if a periplasmic protease was stained in fixed but non-permeabilized cells. This was not the case, therefore, only extracellular proteins were stained in this assay.

Bacteria were incubated with a polyclonal rabbit antibody (IgG fraction) directed against YadAO:8 (diluted 1:200) and a Cy2-conjugated secondary anti-rabbit antibody (diluted 1:200; Dianova) in PBS at room temperature for 1 h and 45 min, respectively. Fluorescence images were obtained with a DMRE fluorescence microscope (Leica, Wetzlar, Germany).

**Quantification of YadA surface localisation by flow cytometry**

Overnight cultures were grown at 27°C. Next day bacteria were diluted 1:20 and grown for 2 hours at 37°C. After 2 h, 1 x 10^9 bacteria were harvested by centrifugation. Cells were washed once with PBS, fixed with 4 % PFA, washed again, and resuspended in PBS. Samples were stained with a purified IgG fraction of a rabbit anti-YadA antiserum (1:200) and Cy2-conjugated secondary antibody (1:100; Dianova) for 1 h at room temperature and then washed twice with PBS. Surface localization of YadA was measured by flow cytometry on a FACSCalibur (BectonDickinson), and data were analyzed with WinMDI (J. Trotter) software. Data are means of 3 independent experiments.

**Autoagglutination**
The autoagglutination of Ye was assayed essentially as described previously (Laird and Cavanaugh, 1980). The overnight cultures of Ye strains were diluted 1:20 in RPMI-1640 supplemented with 10 % FCS and 25 mM HEPES and grown for 24 h at 37°C. Then bacteria were allowed to settle at room temperature. Autoagglutination was recorded as a reduction of optical density (OD600) in the culture supernatant in 15 min intervals. The clearance of the medium and aggregated bacteria at the bottom of the tubes were additionally recorded with a digital camera. For microscopical investigation 5 µl of the aggregates were pipetted onto a slide, sealed with a coverslip and examined under phase contrast.

Adhesion to collagen coated slides

Coverslips were coated overnight at 4°C with 10 µg/ml human collagen type I (Calbiochem/Merck, Darmstadt, Germany) in PBS. Bacteria were grown and harvested as described before. After washing with PBS, 2 x 10^7 bacteria were centrifuged onto the coverslips at 300 rpm for 5 min. After 1 h of incubation at 37°C, the coverslips were washed three times with PBS and then fixed with 4 % PFA. Bacteria were stained with DAPI and counted in three randomly selected fields of view obtained at a magnification of x 100 under a fluorescence microscope. Wildtype adhesion was set to 100 %.

Adhesion to HeLa cells

1 x 10^5 human HeLa cervical epithelial cells (ATCC CCL-2.1) cells per well were seeded on coverslips in a 24well microplate and grown overnight in RPMI-1640 supplemented with 10 % fetal calf serum (FCS) and antibiotics. Next day cells were washed once with prewarmed PBS and grown for another 1 hour in RPMI-1640 10 % FCS without antibiotics. Two wells were trypsinized and the number of cells per well was determined. Bacteria grown overnight at 27°C were diluted 1:20 into fresh medium with antibiotics and grown for 2-3 h at 37°C to induce the expression of YadA. Afterwards bacteria were harvested by centrifugation (5 min,
3000 rpm in a tabletop centrifuge), washed once with prewarmed PBS and resuspended in RPMI-1640 10 % FCS. OD600 was determined and the HeLa cells were infected with bacteria at MOI 50. Bacteria were spun down on the cells by a short centrifugation step (1 min, 300 rpm) and incubated for 30 min at 37°C. Non-adhering bacteria were removed by 3 washing steps with prewarmed PBS. HeLa cells with the attached bacteria were fixed with 4 % PFA in PBS for 10 min at room temperature. The fixed samples were washed once with PBS and stained with an aqueous solution of fuchsine for 2 minutes. The stained samples were washed once in PBS and air dried. Finally the coverslips were mounted in entellan and examined with a light microscope. Pictures of randomly chosen areas were taken at a magnification of x 100. ~6 images comprising about 100 cells on average were used to count cells and adhering bacteria. From these data the number of bacteria per cell was calculated for each strain. The numbers given in diagrams are averages from three experiments. Adherence of YeYadAwt was set to 100 % and all other values refer to this.

Cell culture and IL-8 ELISA

HeLa cells were cultivated in RPMI-1640 medium supplemented with 2 mM glutamine and 10 % fetal calf serum (experiments were also performed with serum starved cells and gave comparable results). Infection experiments and interleukin-8 (IL-8) enzyme-linked immunosorbent assay (ELISA) were carried out as described previously (39), using a multiplicity of infection (MOI) of 50. IL-8 concentrations were calculated using recombinant human IL-8 (BD Biosciences Pharmingen) as a standard.

Gentamicin killing assay

Bacterial invasion was assessed 3 h after infection (MOI 50). 1 hour after the infection the cells were washed three times with phosphate-buffered saline, fresh medium with 100 µg of gentamicin/ml was added, and the cells were incubated for another 2 h at 37°C and then lysed
with 1% Triton X-100 and plated on selective LB agar plates in appropriate dilutions.

Serum resistance test

Pooled normal human serum (NHS) from healthy donors was purchased from the transfusion medicine department of the university hospital Tuebingen, aliquoted and stored at -80°C. Ye were grown to exponential phase at 37°C, washed once with PBS and OD600 was determined. 3 x 10^6 bacteria were incubated in 25% NHS for 1 h at 37°C. As a control, bacteria were incubated in heat inactivated serum (HIS). Complement activity was stopped by placing the samples on ice and by the addition of 1 volume of brain heart infusion medium. Serial dilutions (10^{-1} - 10^{-6}) of the bacteria were plated on selective agar and incubated at 27°C for 48 h. The serum bactericidal effect was calculated as the survival percentage, taking the bacterial counts obtained with bacteria incubated in HIS as 100%. The killing experiment was repeated for each strain at least three times, starting from independent cultures.

Quantification of FactorH/FHL-1 and C4BP binding by flow cytometry

The capacity of Ye WAP, Ye YadA0, the reconstituted strain Ye YadAw and the different YadA G389 point mutants to bind factor H/FHL-1 or C4BP from heat-inactivated normal human serum (HIS), purified factor H or purified C4BP (each 200 µg/ml; Complement Technology, Tyler, TE) was analyzed by flow cytometry. The different strains from overnight cultures (grown at 27°C) were grown until mid log phase and washed once in PBS containing 2% BSA. Bacteria (10^8) were incubated with HIS for 1 h at 37°C. After washings, bacteria were incubated with rabbit anti-factor H antiserum directed against short consensus repeats (SCRs) 1-4 (24) or rabbit anti-C4BP pAb (Complement Technology, Tyler, TE) for 30 min on ice, followed by incubation with the Alexafluor 488-conjugated swine anti-rabbit pAb. After two additional washes, bacteria were analyzed in a flow cytometer (FACScan LRII, Becton-Dickinson, Mountain View, California, USA). All incubations were done in a final volume of
100 µl PBS-2 % BSA, and the washings were done with the same buffer. The primary and secondary pAbs were added separately as a negative control for each strain analyzed.

**Cofactor assay**

*Ye WAP, Ye YadA0, Ye YadAwt and Ye G389A (5x10⁸)* were incubated with Factor H (100 mg/ml) in DPBS for 1 h at 37°C. After thorough washing in the same buffer, the bacteria were incubated with C3b (2.4 µg/ml) and Factor I (4.8 µg/ml) for 15 min at 37°C. The reactions were terminated by the addition of SDS-PAGE sample buffer (RotiLoad1, Carl Roth GmbH, Karlsruhe, Germany). The samples were analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. C3b degradation was analyzed using goat anti-human C3 pAb (Complement Technology), followed by HRP-conjugated rabbit anti-goat pAb (Dakopatts). After additional washings, development was performed with ECL Western blotting detection reagent (Applichem).

**Virulence test**

For systemic infection bacteria were grown in Luria Bertani broth at 27°C, harvested during the log phase, and frozen in 1 ml aliquots at -80°C. Prior to each experiment, an aliquot was thawed, washed, and resuspended in sterile phosphate-buffered saline (PBS), pH 7.4. Mice were then injected with 1 x 10⁵ bacteria in a volume of 100 µl into their tail vein. 24 h after infection mice were sacrificed. Serum was collected and the spleen was removed. Half of the spleen was used to determine the bacterial load by plating their homogenates in serial dilutions onto selective agar plates. The other half was used for immunohistology. For orogastric infection bacteria were grown for 18h at 27°C, diluted to an OD₆₀₀ 0.1 and grown for another 2-3 h at 27°C, washed with PBS, and diluted to the appropriate infectious dose in PBS. Mice were intragastrically infected with 1x10⁹ bacteria and sacrificed on day 3 after infection.
infection (animal licensing committee permission No. H5/08). Organs were homogenized in 5 ml (spleen) or 3 ml (MLN and PP) PBS containing 0.1% Tergitol TMN10 (Fluka) and 0.1% BSA (Biochrom). The number of bacteria was determined by plating 0.1 ml of serial dilutions of the homogenates on Yersinia selective agar. The lower limit of detectable CFUs per organ by this method was 50 ($\log_{10} 50 = 1.7$) in spleen and 30 ($\log_{10} 30 = 1.5$) in PPs and MLNs (PPs and MLNs were pooled separately and are referred to as one organ). Higher values for the detection limits (Fig. 7) result from the fact that CFUs per gram of organ are shown.

To assure presence of the recombinated virulence plasmid bacteria were plated in duplicate on CIN agar and CIN supplemented with the appropriate antibiotics. In all experiments bacterial counts were comparable on agar with or without selective antibiotics indicating that the plasmid was still present. The CFU was determined after 2 d of incubation as described previously (3).

**Determination of CXCL1 production in serum**

The blood of sacrificed mice was collected immediately and left on wet ice for at least 1 h. Serum and clotted blood components were separated by centrifugation at 4°C and 2500 x g for 10 min. The blood clot was removed and the resulting serum was aliquoted and stored at -80°C. Serum CXCL1 levels of infected mice were determined by using a capture enzyme-linked immunosorbent assay (KC-ELISA Duo set; R&D Systems, Wiesbaden, Germany).

**Immunohistology**

For immunohistological analysis the tissues were embedded in Tissue-Tek OCT compound (Nunc, Roskilde, Denmark), snap-frozen in liquid nitrogen, and stored at -80°C. Frozen sections were prepared and stained by an immunoperoxidase method using 3,3-diaminobenzidine-tetrahydrochloride acid (DAB; Sigma, Deisenhofen, Germany) as chromogenic substrate. Nonspecific binding sites were blocked by the incubation of the
sections with PBS containing 10% fetal calf (FCS) and 5% normal goat serum (NGS). Anti-

*Yersinia* antibody WA-vital (antibody risen against whole bacteria) was diluted 1:200 in PBS
containing 5% FCS and 5% NGS for 1 h at room temperature. The secondary antibody was
peroxidase-conjugated affinity purified F(ab’)2 fragment goat anti rabbit IgG (Jackson
ImmunoResearch; diluted 1:400). Isotype-matched irrelevant rabbit IgG was used in controls
and revealed no staining signal. The sections were counterstained with Mayer’s hemataun,
mounted, and assessed microscopically by two independent investigators. Immunostaining of
controls was negative for all groups tested.

Statistics
The data shown in the figures are from representative experiments. Comparable results were
obtained in at least two additional experiments. Differences between mean values were
analyzed using the one-way ANOVA test and a Bonferroni post test at a confidence interval
of 95%. A *p* value of < 0.05 was considered statistically significant (*), a *p* value of 0.001-
0.01 was considered to be very significant (**) and a *p* value < 0.001 to be extremely
significant (***). Error bars represent values ± SEM.

Results
Generation of Ye expressing point mutated versions of YadA
In our previous work we demonstrated that the replacement of a highly conserved glycine
residue within the YadA membrane anchor domain by amino acids with larger side chain size
affects both the trimer stability and the surface display of YadA expressed by *E. coli
BL21(DE3)Omp8* (19). In order to gain knowledge about the actual virulence functions of
YadA, the point mutated versions of YadA in which G389 was exchanged by A, S, T, N, H
were expressed in Ye and analyzed for virulence functions *in vitro* and *in vivo*. 
Site directed mutagenesis and generation of the *Yersinia* strains was performed essentially as described (19, 35). Fragments including the whole *yadA* gene and adjacent regions were subcloned into the suicide plasmid pGP-704. For latter selection of recombined clones a spectinomycin resistance cassette was introduced. These plasmids were transformed into *E. coli* SM17λpir. As a recipient strain we used *Ye* YadA0, in which the *yadA* gene was disrupted by the insertion of a kanamycin cassette. The wildtype *yadA* gene and the mutated *yadA* genes, respectively, were introduced into the recipient strain via homologous recombination and cointegrate formation. By the selection for resistance against spectinomycin and kanamycin, the transconjugants harbouring cointegrates were identified. Single colonies were screened for the presence of the intact full length *yadA* gene and PCR products were sequenced to verify the point mutations.

**Expression, trimer stability and surface display of YadA is reduced in G389 mutants**

To assess expression, trimer formation and trimer stability of YadA G389 mutants expressed in *Ye*, heated or unheated whole cell lysates of *Ye* expressing YadA wildtype or G389 mutants were subjected to Western blot analysis. Figure 1A shows that a band of approximately 200 kD could be observed for YadAwt representing the trimeric form of YadA despite the heating of samples. Mutant proteins G389A and G389S also formed trimers, but the stability was reduced because in the heated samples only the monomeric form of YadA could be detected. We were not able to detect the mutants G389T, G389N and G389H by the Western blot analysis (Fig. 1A). Recent work suggests that this might be due to degradation of accumulated YadA mutant protein in the periplasmic space by proteases such as DegP (19).

To investigate whether the YadA mutants are actually transported and exposed onto the bacterial surface we performed trypsin digestion experiments (Fig. 1B). *Yersinia* strains expressing YadAwt, YadA G389A and YadA G389S were treated with trypsin and analysed by Western blot. Trypsin digestion resulted in one major truncation leading to a shift from
~200 kD to ~150 kD for the trimer of YadAwt, YadA G389A and YadA G389S (upper panel) and to a shift from ~50 kD to ~45 kD for the monomers (lower panel). These findings demonstrate that YadAwt, YadA G389A and YadA G389S are accessible for trypsin and therefore transported and exposed on the bacterial outer membrane. Additionally, in order to find out if the mutants revealed comparable truncations as the YadA wildtype protein, digested samples were treated with 6M urea prior to loading to disrupt YadA trimers. As you can see in Fig. 1B (lower panel), the molecular weight of the resulting digestion products suggests that these proteins experienced comparable truncations.

Immunofluorescence staining for YadA without the permeabilization of the bacteria and subsequent microscopic analysis revealed that Ye WAP strain (parental YadA wildtype strain) and the mutant strains expressing YadAwt, YadA G389A and YadA G389S displayed a strong, homogenous ring-shaped fluorescence pattern (Fig. 2A). In contrast, Ye YadA G389T, Ye YadA G389N and Ye YadA G389H revealed a weak and irregular staining pattern. This may reflect an irregular distribution of YadA at the bacterial surface for yet unknown reasons.

To quantify fluorescence intensity we also performed flow cytometry analysis (Fig. 2B). The data revealed comparable mean fluorescence intensities for Ye WAP (93 ± 15 %), Ye G389A (98 ± 16 %) and Ye G389S (90 ± 10 %) compared to Ye YadAwt. In contrast, Ye G389T (46 ± 7 %), Ye G389N (32 ± 9 %) and Ye G389H (15 ± 4 %) showed a significant reduction in mean fluorescence intensity when compared to Ye YadAwt.

Together, these data demonstrate that single point mutations of YadA G389 affect trimer stability and/or surface display of YadA, and that Western blot as well as immunofluorescence and flow cytometry analysis are required to address the various features of YadA.

YadA G389 point mutations affect autoagglutination and adhesion of Ye to host cells
YadA has been demonstrated to promote autoagglutination of Ye (43). The ability of YadA-expressing Ye to form cell aggregates and macrocolonies may affect colonization, growth and thus virulence (15). To address whether G389 point mutations affect autoagglutination, bacteria were grown in RPMI 1640 medium supplemented with 10% heat inactivated FCS or minimal medium without FCS or LB medium at 37°C for 24 h according to the protocol published by Laird and Cavanaugh (25). Thereafter sedimentation of bacteria, clearance of the culture medium and the formation of bacterial aggregates was monitored macroscopically, densitometrically and by microscopy. The same results were obtained with minimal medium or LB broth (data not shown). We found that Ye WAP rapidly settled from the suspension and formed a pellet in the culture tube (Fig. 3). The microscopic examination of the pellet revealed large clumps of aggregated bacteria. Autoagglutination could also be observed with Ye WAP Inv- (carrying a disrupted invasin gene) demonstrating that invasin is dispensable for autoagglutination. The control strain Ye WAC (which lacks the virulence plasmid including yadA) did not form a pellet and single, highly mobile bacteria could be observed by microscopy. Accordingly, Ye WAC Inv- did not show autoagglutination. Surprisingly, Ye YadA0, lacking YadA expression rapidly agglutinated and a pellet consisting of large clumps of bacteria was observed. Likewise, Ye YadAwt, Ye G389A and Ye G389S displayed autoagglutination comparable to Ye WAP. In contrast, Ye G389T, Ye G389N, and Ye G389H did not display autoagglutination. YadA specifically binds with high affinity to the alpha1 chain of collagen type I of triple-helical conformation and also to triple-helical collagen-like peptides (27, 41). This binding activity is conformation dependent and resides within the YadA head domain (35). Adhesion to extracellular matrix proteins has been implicated to be an important prerequisite for Yersinia virulence in vivo. Therefore, we wanted to test if Ye mutant strains which display reduced YadA surface levels are still able to bind to collagen type I and HeLa cells, respectively. For this purpose, coverslips coated with collagen type I or confluent with HeLa
cells were incubated with Ye wild type and mutant strains. Adherent Ye were stained with DAPI or fuchsine and counted on microscopic images (Fig. 4). We found that the adhesion to both collagen type I and HeLa cells was unaltered for Ye YadAwt, Ye G389A, Ye G389S as compared with Ye WAP, whereas adhesion was largely abolished in Ye YadA0 mutant strain. Adhesion was significantly reduced in Ye G389T, Ye G389N (p < 0.01) and Ye G389H mutants (p < 0.001).

Taken together, these results suggest that: (i) YadA may promote autoagglutination in all tested strains except for Ye YadA0 and the degree of autoagglutination is associated with the amount of YadA surface display. (ii) There may be other yet unknown factors encoded by the virulence plasmid which, in the absence of YadA, also can mediate autoagglutination. However, YadA, even if present on the bacterial surface at low quantities, may mask this effect. This assumption remains speculative at present, and needs further exploration in future studies. (iii) Similar to autoagglutination, adhesion to collagen I or HeLa cells is abolished in those YadA mutants with significant reduced YadA surface display.

Mutations of G389 of YadA do not affect the ability of Ye to suppress host cell cytokine responses and host cell invasion

Upon the engagement of host cells, several components from Ye including YadA, invasin protein, lipopolysaccharide (LPS) and YopB have been demonstrated to activate NF-κB, which subsequently gives rise to a proinflammatory host cell response including production of, e.g., IL-8 (17, 39, 45-47). NF-κB is a key transcription factor involved in innate immune responses to pathogens (9). However, virulent Ye may suppress NF-κB activation and cytokine production of host cells (2). The ability to suppress cytokine responses has been assigned to the presence of the Yersinia outer protein P (YopP) (37, 38).

Sufficient attachment to host cells is believed to be prerequisite for cell invasion and the
injection of Yops (11). To assess whether Ye G389 mutants with unaltered or reduced adherence to HeLa cells are affected in their ability to suppress host cell cytokine responses and invasion, HeLa cells were exposed to Ye wild type and G389 mutant strains and IL-8 levels were determined in the cell culture supernatants after 6 hours (Fig. 5A). The numbers of internalized bacteria were determined by a gentamicin killing assay. In agreement with previously published data (18) the avirulent strain Ye WAC (without pYV plasmid encoding YadA) triggered production of IL-8 (~ 1500 pg/ml) and was internalized efficiently while the virulent strain Ye WAP totally suppressed production of IL-8 and was significantly less internalized (~ 2.4 % (WAC) versus ~ 0.3 % (WAP) invasion as percent of inoculum after 3h). Moreover, the absence of YadA did not affect the suppression of cytokine production by Yops. In fact, Ye YadA0 as well as all Ye G389 mutant strains efficiently suppressed IL-8 production in HeLa cells. In addition, compared to Ye WAC, all other mutant strains were significantly less internalized. Taken together, this data suggest that despite reduced YadA surface display and strongly reduced host cell adhesion, all YadA mutant strains could inject enough Yops into host cells to suppress IL-8 release and to resist internalization by host cells.

Mutations of YadA G389 interfere with serum resistance and binding to factor H and C4BP

YadA, in addition to Ail mediates protection of Ye against the host complement system (6-8, 12, 23, 33). Furthermore, LPS may also contribute to complement resistance of Ye. A protective effect has been shown for LPS of Ye serotype O:9 (42), but not for Ye strains of serotype O:3 and O:8 (4, 8). To test the ability of YadA G389 mutant proteins to protect from complement killing, bacteria were incubated for 1 hour in 25 % of normal human serum (NHS) or heat inactivated serum (HIS) and thereafter plated on LB agar. Survival was calculated as percentage referred to bacterial counts obtained with HIS (100 % survival). The data depicted in Fig. 6A demonstrate that compared to Ye WAP, Ye YadAwt displayed an...
increased ability to survive in NHS, whereas Ye G389A and Ye G389S displayed a decreased ability to survive in NHS; Ye G389T, Ye G389N and Ye G389H were as sensitive to complement killing as the YadA deficient strain Ye YadA0.

Recently, it has been shown that the ability of YadA to protect Ye from killing by complement depends on its ability to bind complement regulatory components factor H and C4BP. By this means Ye prevents deposition of activated C3 on the bacterial membrane (6, 23). To investigate the binding of factor H and C4BP to Ye strains we both tested whole serum and purified proteins. Because binding of purified protein was much lower than binding of serum derived Factor H and C4BP we carried out all binding studies with HIS as a source of complement factors. To address if a disturbed binding of factor H and/or C4BP in YadA G389 mutants is decisive for their survival in serum, we analysed the binding of serum factor H and C4BP by flow cytometry. For analysis of factor H binding we also performed binding assays by ELISA and obtained comparable results (not shown). The results reveal that Ye WAP, Ye YadAwt, Ye YadA G389A and Ye G389S bound factor H to a similar manner (Fig. 6C). Compared to these strains, Ye YadA0 and all the other mutant strains (G389T, G389N and G389H) displayed virtually no factor H binding. Similar to factor H, C4BP was bound by Ye WAP and Ye YadAwt and Ye G389A in comparable and substantial amounts and to lower extents by Ye G389S, whereas only residual binding of C4BP could be observed with the strains Ye G389T, G389N and G389H. Factor H regulates the alternative pathway activity by serving as a cofactor for factor I in the degradation of C3b. Ye G389A revealed impaired serum resistance though factor H was bound at wildtype level by this mutant. Therefore we wanted to analyze if factor H bound to Ye G389A actually has cofactor activity. We also tested Ye WAP and Ye YadA0 as positive and negative control, because it has been shown previously for Ye O:3, that YadA serves as the main factor H binder (6). Bacteria were incubated in the presence or absence of factor H, intensively washed and afterwards factor I and C3b were added. Following incubation for 15 min, all lysates were separated by SDS-
The cleavage of C3b was then analyzed by Western blotting. The cleavage products generated in the presence of surface bound factor H and factor I showed comparable size as the fragments generated by factor I in combination with factor H in fluid phase. When C3b was incubated in the presence of factor H that has bound to the bacterial surface, the cleavage products of α43 and 41 kDa appeared (Fig. 6D). However, almost no cleavage products were seen with bacteria only. Factor H bound to the surface of Ye WAP and Ye YadAwt was functionally active and exhibited substantial cofactor activity, whereas Ye YadA0 did not bind factor H and therefore no factor H mediated C3b cleavage could be detected. Ye G389A bound factor H retained its cofactor activity to degrade C3b comparable to that of Ye WAP and Ye YadAwt. Taken together, the most interesting finding of all these assays is that Ye G389A shows reduced serum resistance although it recruits similar amounts of Factor H and C4BP when compared to Ye YadAwt.

**Mutation of YadA G389 leads to significantly reduced virulence of Ye in mice**

To reveal the importance of YadA G389 mutations for the virulence of Ye in vivo, mice were intravenously infected with Ye wild type and mutant strains and the bacterial burden in the spleen was determined (Fig. 7A). Ye WAP and Ye YadAwt resulted in a bacterial burden of log_{10} CFU per gram of tissue of 6.7-7.5 while Ye YadA0 did not colonize the spleen (p < 0.01). Compared to Ye YadAwt, Ye G389A was significantly attenuated with a bacterial burden reduced by ~84 % (5.6-6.3 log_{10} CFU). Ye G389S was even more attenuated resulting in a bacterial burden reduced by ~99.8 % (~4.1 log_{10} CFU). Mutant strains Ye G389T, N and H were totally avirulent. CXCL1 (KC) is a functional homologue of human IL-8 and is associated with neutrophil recruitment and inflammation. Influx of neutrophils from the blood and their interaction with vascular endothelium at the site of infection is an early step of acute inflammation (16). As the Ye YadA G389 mutants testified to be significantly reduced in virulence and abscess formation, we wanted to find out if they induce a systemic
inflammatory reaction in infected mice. Therefore we analysed CXCL1 levels in the serum of systemically infected mice by ELISA (Fig. 7B). We observed comparably increased levels of CXCL1 in serum of mice infected with Ye WAP (~3500 pg/ml), Ye YadAwt (~2900 pg/ml) or Ye G389A (~2800 pg/ml). In contrast, Ye G389S triggered a significantly lower level of CXCL1 (~600 pg/ml), although this level was higher compared to uninfected controls. Only background levels of CXCL1 could be detected in mice infected with Ye G389T, Ye G389N, Ye G389H and Ye YadA0. To further analyse the consequences of YadA G389 mutations for the natural route of a Yersinia infection, we orogastrically administered $5 \times 10^9$ bacteria to mice (Fig. 7C). Three days after infection, mice were sacrificed. Peyer’s patches (PP), mesenteric lymph nodes and spleens were removed and the bacterial load of each organ was determined. PP revealed comparable bacterial loads in mice infected with the Ye YadA wildtype strain ($\log_{10}$ CFU ~ 8), Ye G389A ($\log_{10}$ CFU ~ 7) and Ye G389S ($\log_{10}$ CFU ~ 6). Nevertheless, only Ye YadAwt ($\log_{10}$ CFU ~ 5.5) could be detected in mesenteric lymph nodes indicating that efficient dissemination was impaired in the mutant strains. Accordingly, only Ye YadAwt ($\log_{10}$ CFU ~ 3.5) but not Ye G389A and Ye G389S colonized the spleens of orally infected mice.

In conclusion, the data indicate that virulence of Ye YadA wildtype and Ye G389 mutant strains in mice is closely associated with their ability to cause systemic infection including significant bacterial burden in spleen, abscess formation and systemic CXCL1 serum levels.

After the systemic infection Ye may induce formation of abscesses in the spleen that consist of granulocytes, macrophages and bacteria. To investigate whether and how YadA G389 mutations may affect abscess morphology, cryosections from the infected spleen of mice were prepared and subjected to immunohistochemistry analysis including anti-Yersinia antibodies (Fig. 8). Numerous abscesses with diameters of approximately 40-100 µm could be observed in mice infected with Ye WAP and Ye YadAwt. Abscesses were made up of a central mass of...
bacteria infiltrated with inflammatory cells. The whole area was surrounded by a broad border of inflammatory cells and was in the most cases located adjacent to lymphoid follicles. In mice infected with Ye G389A only less and small abscesses could be detected. Upon infection with Ye G389S only very few and very small abscesses were found; however, lymphoid follicles were infiltrated by numerous polymorphonuclear leukocytes. Neither abscesses nor signs of neutrophil inflammatory infiltrations were seen in mice infected with Ye YadA0, Ye G389T, Ye G389N and Ye G389H.

Discussion

In the previous work we identified a highly conserved glycine residue (G389) within the second beta-sheet of the YadA membrane anchor domain and demonstrated that it is involved in YadA autotransport, trimer stability and YadA mediated serum resistance when expressed in E. coli (19). In the present study we wanted to clarify two issues: (i) what is the impact of G389 for the YadA-associated in vitro virulence functions of Ye? To address this we we introduced the G389 substitution mutations G389A, G389S, G389T, G389N and G389H into Ye and tested the bacteria for YadA expression, trimer stability, trypsin accessibility, outer membrane localization, autoagglutination, adherence, host cell cytokine induction, serum resistance and the binding of the serum complement regulatory components factor H and C4BP. (ii) Are the in vitro virulence assays performed with the YadA point mutants relevant for the virulence of Ye in the mouse model? To elucidate this, we infected mice with Ye wildtype bacteria and mutants expressing the YadA point mutants and compared bacterial burden in several organs, serum cytokine levels and morphological changes in spleen. The most salient finding of this study is that all YadA mutants displayed reduction of trimer stability; however, their ability to mediate autoagglutination, adherence, serum resistance and virulence differed significantly in the various yadA mutant strains (a summary of the
phenotypes of all strains is given in table 2). Moreover, the data obtained by both the intravenous and the orogastric mouse infection model suggest that only few of the *in vitro* virulence assays actually are closely associated with the virulence of *Ye* in mice, thus challenging their meaningfulness. In fact, apparently only trimer stability and serum resistance, but not the other *in vitro* virulence assays, are closely associated with the virulence functions *in vivo*. Finally, we can conclude that minor changes in the membrane anchor of YadA may, directly or indirectly, affect the complement resistance of *Ye*, the key virulence function mediated by YadA. However, at present we can only speculate about the molecular basis for the reduced virulence and serum resistance. The current YadA membrane anchor model is based on the Hia structure (19, 30) and suggests that at G389 larger side chain residues could be accommodated. The reason why and how the larger side chains disturb the protein stability and the autotransport needs further investigation. Thus, the molecular structure of the YadA membrane anchor needs to be elucidated either by x-ray crystallography or by NMR experiments. From the high conservation of the Gly residue (19), it can be assumed, that the absence of a large side chain might be very important either in the folding process, the autotransport process, or in the final thermodynamic stability (or a combination of those factors). How mutations of this conserved residue finally may affect the recruitment of complement regulatory factors remains to be elucidated.

When analyzing the expression of YadA, we were not able to detect the mutant versions G389N, G389T and G389H by Western blots of whole cell lysates of *Yersinia*. When overloading the SDS gels, occasionally very faint bands of YadA trimer or monomer could be observed in non-heated, but not in heated samples of G389N, G389T and G389H (data not shown) suggesting that G389N, G389T and G389H have reduced trimer stability and are readily degraded in *Ye*. Nevertheless we could demonstrate expression of G389N, G389T and G389H by immunofluorescence microscopy and flow cytometry indicating that these methods are more sensitive than Western blotting. In *E. coli* we were able to rescue expression of
G389 yadA mutants by knocking out the periplasmic protease DegP (19). DegP is part of the periplasmic stress response and responsible for degradation of misfolded proteins including autotransporter proteins accumulating in the periplasm (22, 32). Analogous to our experiments with E. coli future experiments with a Ye ΔhtrA mutant strain will have to demonstrate whether the closely related periplasmic protease HtrA (28) is involved in degradation of YadA G389 mutants.

YadA is the major adhesin of Ye and has been demonstrated to mediate autoagglutination (43). The exact mechanism of this phenomenon is not understood, but most likely due to the hydrophobic nature of the bacterial surface and due to homologous interaction of highly concentrated outer membrane proteins, YadA expressing bacteria readily settle out of a suspension. However, the role of autoagglutination per se for virulence of Ye is not known.

Bacteria bearing the pYV virulence plasmid and expressing YadA in 3D collagen gels grow as densely packed microcolonies while bacteria carrying the pYV plasmid, but lacking YadA, grow as loosely packed microcolonies (15). There appears to be a correlation between the autoagglutination phenotype and growth behaviour in collagen gels (15). Formation of densely packed microcolonies, however, did not depend on the ability of YadA to bind collagen (15). It is conceivable that densely packed microcolonies might protect bacteria from the complement attack and from phagocytosis. The results of the present study clearly demonstrate that the autoagglutination may also occur in the absence of YadA, and that the autoagglutination of Ye wildtype or mutant strains is not associated with the ability to exert a systemic infection in an experimental mouse infection model and to induce tissue abscesses including microcolonies in vivo. In fact, Ye WAP, Ye WAP Inv-, Ye YadAwt, Ye G389A and Ye G389S displayed autoagglutination suggesting that YadA, but not invasin, contributes to this effect. Ye G389T, Ye G389N and Ye G389H showed no significant sedimentation, most probably due to the low quantity of YadA available for hydrophobic interactions. Ye YadA0, however, in our hands displayed rapid autoagglutination. To rule out that this rather
unexpected result emanated from our experimental setting we repeated it under serum-free
conditions with minimal medium and LB but gained the same outcome. Therefore, we can
exclude that autoagglutination of Ye YadA0 was mediated by serum-originating components.
We rather think that there are other factors on the surface of Ye which are also capable of
mediating autoagglutination; these factors are masked by YadA. If there are only some
residual molecules of YadA present like in G389T, Ye G389N and Ye G389H, efficient
interaction of these unknown autoagglutination factors is blocked. The identification of these
factors is subject of future experiments.

In vitro, YadA induces cytokine production (e.g. IL-8) in host cells (39, 40). This host cell
response can be suppressed by injection of Yops via the type III secretion system into the host
cells. To our surprise, a YadA-deficient strain also efficiently suppressed secretion of IL-8 by
HeLa cells. Accordingly, all of our mutant strains only differing in the amount of YadA
presented on their surface did not result in significant IL-8 secretion suggesting that few
adhering bacteria seem to transclocate enough effector proteins to exert inhibition of IL-8
secretion. Thus, in vitro YadA does not play an essential role for Yop translocation if
sufficient adherence is ensured. This result is in accordance to the observation that low
amounts of YadA can mediate adhesion to host cells. Assumed that YadA is a major
determinant for virulence and that a YadA deficient Ye strain might also be able to translocate
Yop effectors efficiently in vivo but is nevertheless severely attenuated, other YadA mediated
effects must be decisive for YadA dependent survival of Ye in the mouse model. Surprisingly,
all of our mutant strains displayed comparable invasion behaviour. This might be explained
by the fact that invasion reflects the net balance of adhesion (triggering uptake) and Yop
injection (preventing uptake by disrupting the host cell actin cytoskeleton). Although we
observed significantly reduced adhesion to HeLa cells with some of our mutants, these strains
seemed to have injected enough Yops to efficiently suppress internalization.
The action of complement is one of the first host immune barriers to invading Ye.
Complement resistance is thus a crucial feature of invasive, extracellularly located pathogens like Ye. Consequently, numerous pathogenic microorganisms have developed mechanisms to evade the complement system (26, 48). An important function of YadA is the prevention of membrane attack complex (MAC) formation on the bacterial surface. Ye evades the complement system by the recruitment of two major complement regulatory proteins namely factor H and C4BP. Factor H prevents C3 deposition on the bacterial surface in vitro by inhibiting the binding of Factor B to C3b, by supporting the dissociation of the C3bBb complex (decay accelerating activity) and by its function as a cofactor for the cleavage of C3b by Factor I (1, 6, 8, 12, 23, 33). C4BP is a fluid-phase complement regulator that downregulates classical and lectin pathway complement activity by preventing the assembly and accelerating the decay of the C3 convertase.

It has been shown in vitro that both the stalk and the membrane anchor are involved in serum resistance (34). By means of the Ye YadA G389 mutants we were able to test whether the quantity of YadA is decisive for complement resistance. Serum resistance of Ye WAP and Ye YadAwt was comparable. All the other mutant strains were significantly reduced in serum resistance, irrespective of the amount of YadA displayed on the outer membrane. Therefore, it is assumed that the exchange of G389 abrogates efficient interaction of YadA with complement regulators and thus leads to complement killing of Ye. Obviously this complement regulatory factor (CRF) is neither factor H nor C4BP, because both are bound to almost wildtype level by Ye G389A and at least factor H exhibits full cofactor activity when bound to this strain. Nevertheless, interaction with other CRFs could be disturbed by modulation of binding to YadA. It has been shown for Ail, that single point mutations within loop2, which is exposed on the surface can abrogate serum resistance (31). Studies by Ackermann et al. (1) support a scenario where specific interaction between the C-terminus of YadA and complement inhibitory factors is inhibited. Analysis of serum resistance of Ye expressing no YadA, wildtype YadA, or chimeras revealed that the exchange of the YadA C-
terminus with that of UspA1 (Moraxella catarrhalis), EibA (Escherichia coli) or Hia (Haemophilus influenzae), respectively, resulted in a significant loss of survival in normal human serum. These results demonstrated that the C-terminal membrane anchor domain is involved in serum resistance either directly or indirectly, a finding supported also by this study.

Systemic infection of mice with Ye YadA wt and YadA G389 mutant strains revealed that only YadA wt was highly pathogenic in vivo associated with high splenic bacterial burden, abscess formation and high CXCL1 serum levels while Ye YadA0, Ye YadA G389T, N and H did not induce significant changes. Both Ye YadA G389A and G389S were attenuated but retained their ability to induce abscesses and increased CXCL1 serum levels. Thus, Ye YadA0, Ye YadA G389T, G389N and G389H are rapidly killed by the first line of host defense (most likely complement), are not able to establish abscess formation and do not lead to major inflammatory events including cytokine production. Ye YadA G389A and G389S cannot be killed as efficiently probably due to their still present even though reduced serum resistance. In an orogastric mouse infection model Ye YadA wt, G389A and G389S were able to colonize the Peyer’s patches even though Ye YadA G389A and G389S were found in slightly reduced numbers. Interestingly, only Ye YadA wt but not G389A and G389S were found in mesenteric lymph nodes and could disseminate into the spleen at the time points investigated. Although this may be the result of (i) a less efficient uptake by M-cells, (ii) a more efficient killing by the host or (iii) a delayed bacterial growth within the host tissue, scenario (ii) might be the most tempting one and will be pursued by exploring the virulence of YadA mutants in mice deficient in complement system components.

In summary, we could demonstrate that the exchange of the highly conserved single amino acid glycine within the YadA membrane anchor abrogates serum resistance. Moreover, virulence tests in the mouse model have shown that YadA mediated serum resistance is decisive for Ye virulence rather than other YadA mediated functions like adhesion as we have
shown in vitro. This data suggests that in vivo assays provide the most sensitive assays to address minor changes of virulence factors.

Acknowledgements

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Figure Legends

Figure 1

Expression, stability and trypsin accessibility of YadA and mutant versions expressed by Ye. Whole cell-lysates were prepared from Ye grown for 2-3 h at 37°C. (A) Samples were either heated or not heated before separation by SDS-PAGE and YadA was detected by Western Blot. Wildtype YadA appears as a trimer at ~ 180 kDa, monomers at 50 kDa. (B) Bacteria were grown as described above and incubated with or without 0.1 mg/ml trypsin for 1 h on ice. Afterwards the bacterial pellet was resuspended in sample buffer. To disrupt protein trimers samples were heated with 6 M urea to 95 °C prior to loading where indicated.

Figure 2

Immunofluorescence microscopy and flow cytometry analysis of Ye expressing wildtype and mutant YadA. (A) Bacteria were grown for 2 h at 37°C to induce expression of YadA. YadA on the surface of bacteria was detected with anti-YadA antibodies and Cy2-coupled secondary antibodies. Ye YadA0 served as a negative control. (B) Histogram overlays of
fluorescence intensity distribution in samples of bacteria expressing YadAwt or mutants. Histograms of bacteria expressing YadAwt (gray filling) were overlaid with the corresponding histogram of the individually indicated strain. Fluorescence of Ye YadA0 was also determined (not shown). The minute fluorescence of these samples was considered as blank/background and substracted from all other values. Mean fluorescence intensity of YadAwt was set to 100 %. Histograms depict one representative experiment. Values are YadA0 background substracted means ± standard deviation of three independent experiments.

Figure 3

Autoagglutination of Ye. Cultures were grown overnight at 37°C in medium containing FCS (the same results were obtained with medium without FCS; see supplementary figure 1). Then tubes were incubated at room temperature without agitation and bacteria were allowed to settle. Photographs of culture tubes were taken after 90 min of settling. WAP = Ye wildtype strain, WAC = Ye without virulence plasmid, WAP Inv- = Ye wildtype strain with disrupted inv gene, WAC Inv- = virulence plasmid cured strain with disrupted inv gene. Middle columns show microscopic pictures of bacterial aggregates which were taken from the sediment of the culture tubes. Right columns show time courses of bacterial sedimentation. Optical density of bacterial culture supernatants was determined after bacteria were allowed to settle for the indicated time.

Figure 4

Adhesion of Ye to collagen type I and HeLa cells. Ye expressing wildtype and mutant YadA were allowed to adhere to collagen coated coverslips (A) or HeLa cells (B), respectively. Weakly bound bacteria were washed off and adhering bacteria were counted. Ye YadAwt adherence was set to 100 %. The values are means ± standard deviations and are representative of three independent experiments.
Figure 5

**Yersinia-induced IL-8 secretion and cell invasion in vitro.** (A) HeLa cells were incubated with Ye for 1 h, then bacteria were killed and cells were incubated for further 6 h. IL-8 levels in the cell culture supernatants were determined by ELISA. TNF-alpha was used as a positive control. As a negative control cells were incubated without bacteria. Values and standard deviations are means from 2 independent experiments. (B) Uptake of bacteria into HeLa cells was analyzed by determining the number of intracellularly viable bacteria using gentamicin killing.

Figure 6

**Serum resistance and binding of complement regulatory components factor H and C4BP of Ye mediated by YadA.** (A) Equal numbers of bacteria were incubated with 25 % NHS or HIS for 1 h at 37°C and plated on LB agar. The bacterial survival was calculated as a percentage, taking the bacterial counts obtained with HIS as 100 %. (B) Binding of serum derived factor H and C4BP to bacteria expressing or not expressing YadA was assessed by FACS. Histograms of control samples (stained with primary and secondary antibodies only; light lines) were overlaid with histograms of samples specifically stained for factor H and C4BP (bold lines), respectively. The figure depicts the histograms of one representative experiment. (C) Binding of serum derived factor H and C4BP was assessed for all strains. Bars show values of background substracted mean fluorescence intensities. Background substracted signal intensities for both Factor H and C4BP staining of Ye YadA0, G389T, G389N and G389H sometimes reached values below zero due to variable signal noise. To make bars visible in the diagram these values were set to 50. The data are means ± standard deviations of two independent experiments. (D) Cofactor activity of factor H bound to Ye WAP, Ye YadA0, Ye YadAwt and Ye G389A was assessed. The bacteria were incubated with
(+ or without (-) factor H and intensively washed. Afterwards the bacteria were exposed to factor I and C3b. Control reactions (controls) were carried out without bacteria. C3b and the cleavage products resulting from membrane bound factor H cofactor activity were detected with an polyclonal antibody directed against human C3b. Inactivation of C3b is indicated by the appearance of C3b $\alpha^\prime$ chain cleavage fragments of 68, 43 and 41 kDa and by reduction of the intensity of the C3b $\alpha^\prime$ chain band.

**Figure 7**

**Virulence test and CXCL1 cytokine levels in serum of infected mice.** (A) The number of bacteria (expressed as log$_{10}$ CFU per gram of tissue) in the spleen of C57BL/6 mice 1 d post intravenous infection with $10^5$ bacteria of different *Yersinia* strains. (B) Cytokine levels in sera of infected mice were determined by ELISA. Sera were obtained from mice that were killed one day post intravenous infection with $10^5$ Ye. Values are means of at least five animal sera $\pm$ standard deviation. (C) Bacterial load (expressed as log$_{10}$ CFU per gram of tissue) in Peyer’s patches, mesenteric lymph nodes and spleen after orogastric infection of BALB/c (dosis $10^9$, 3 dpi) mice. (*** $p < 0.001$). The horizontal lines indicate the limit of detectable CFU per gram of tissue.

**Figure 8**

**Immunohistochemistry of the spleen of i.v. infected mice.** The spleens of Ye infected mice were embedded in TissueTek and shock frozen. 7 $\mu$m cryosections were stained with an antibody against Ye and a secondary antibody coupled to peroxidase. DAB was used as a chromogenic substrate which forms a brown precipitate. Bacteria and abscesses appear in brown colour. Cells were counterstained with hematoxyline. LF = lymph follicle, A = abscess. Abscesses are demarkated by dashed lines. Arrows point at polymorphonuclear
leukocytes found in high numbers in spleen of mice infected with Ye G389S despite abscess formation could not be detected.


Table 1. Bacteria used in this work.

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<th>Bacterial strain</th>
<th>Description</th>
<th>Reference</th>
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<td><em>Yersinia enterocolitica</em> WA-314 serotype O:8 clinical isolate, virulent wild-type strain; pYV⁺ (YadA⁺)</td>
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Table 2. Summary of the phenotypes of the various mutants.

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<th>Trimer stability (WB)</th>
<th>Surface display (IF)</th>
<th>Auto-aggl.</th>
<th>Adhesion (collagen/Hela cells)</th>
<th>Induction of IL-8 and Invasion</th>
<th>Serum resistance</th>
<th>Binding of factor H/C4bp</th>
<th>Virulence i.v./oral</th>
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<td>G389T</td>
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<td>n.a.</td>
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++ very high
+ high
± intermediate
- low
o below detection limit
WB western blot
IF immunofluorescence
n.a. not available