Vitronectin Binds to a Specific Stretch within the Head Region of Yersinia Adhesin A and Thereby Modulates Yersinia enterocolitica Host Interaction

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Bacterial infection · Cell surface molecules · Complement · Yersinia adhesin A · Vitronectin

Abstract
Complement resistance is an important virulence trait of Yersinia enterocolitica (Ye). The predominant virulence factor expressed by Ye is Yersinia adhesin A (YadA), which enables bacterial attachment to host cells and extracellular matrix and additionally allows the acquisition of soluble serum factors. The serum glycoprotein vitronectin (Vn) acts as an inhibitory regulator of the terminal complement complex by inhibiting the lytic pore formation. Here, we show YadA-mediated direct interaction of Ye with Vn and investigated the role of this Vn binding during mouse infection in vivo. Using different Yersinia strains, we identified a short stretch in the YadA head domain of Ye O:9 E40, similar to the ‘uptake region’ of Y. pseudotuberculosis YPIII YadA, as crucial for efficient Vn binding. Using recombinant fragments of Vn, we found the C-terminal part of Vn, including heparin-binding domain 3, to be responsible for binding to YadA. Moreover, we found that Vn bound to the bacterial surface is still functionally active and thus inhibits C5b-9 formation. In a mouse infection model, we demonstrate that Vn reduces complement-mediated killing of Ye O:9 E40 and, thus, improved bacterial survival. Taken together, these findings show that YadA-mediated Vn binding influences Ye pathogenesis.

Introduction
Yersinia enterocolitica (Ye) and Yersinia pseudotuberculosis (Yps) are enteropathogens causing enteric and systemic diseases [1, 2]. Besides the chromosomally encoded adhesins invasin (Inv) and Ail [3–5], the trimeric auto-transporter adhesin (TAA) Yersinia adhesin A (YadA) is the decisive factor that determines the pathogenicity of Ye [6]. YadA forms rigid fibrous structures, which protrude approximately 23 nm from the cell surface [7, 8], and mediates adhesion to extracellular matrix (ECM) pro-
teins such as collagen, fibronectin and laminin and also to complement factors [9]. Being the prototype of the TAA family of proteins, YadA is characterized by a modular composition of several domains; the extracellularly located N-terminal head domain is followed by a connector element (also called the neck region) leading into a coiled-coil stalk. The stalk is connected to the C-terminal translocator or membrane anchor domain, consisting of 4 β-strands per monomer [9]. To form a functional adhesin on the bacterial surface, 3 YadA monomers trimerize and form the pore of the translocator domain, which is inserted into the outer membrane [10]. The translocator enables the transport of the passenger domains onto the bacterial surface, where they also form obligate trimers [9].

YadA knockout strains of Ye are avirulent and do not cause infection in a mouse model [11–13]. This striking effect has been attributed mainly to the reduced efficiency of effector protein (Yop) delivery by a dedicated type 3 secretion system (T3SS) which requires proper adhesion to host cells; loss of adherence results in the inability to resist phagocytosis [14, 15]. However, in Yps, which is more closely related to Yersinia pestis, YadA is dispensable for virulence and Yop injection [16]. YadA of Yps and Ye not only differ in their role during infection, but also in the sequence and binding repertoire of host ECM proteins and cellular receptors. YadA of Yps carries an additional stretch within its head region that enables entry into host cells [17]. This important stretch is absent in YadA of several Ye serotypes and strains. Moreover, the binding capacities of YadA differ between Ye, which binds collagen and laminin, and Yps, which binds fibronectin [18].

By interacting with several complement factors, serum resistance is an important virulence trait of Ye. It has been shown that factor H, C4b-binding protein (C4BP) and C3 bind to the YadA stalk domain and thus inhibit complement killing [19, 20]. Recently, we demonstrated a novel mechanism that contributes to serum resistance in Ye O:8 WA-314, and amended the current model of direct factor H binding to YadA0.3 and YadA0.9. We have shown that Ye binds C3b or iC3b and thereby attracts high amounts of factor H to the bacterial surface [21]. This is different from the direct binding of factor H, which was shown earlier [19, 20, 22]. Importantly, by binding these complement regulatory factors, Ye is able to interfere with complement activity by inhibiting complement-mediated killing at an early stage of the cascade.

The human glycoprotein Vn is synthesized in the liver and secreted into plasma [23], where it is present as a monomer (65 and 75 kDa) at high concentrations (200–400 μg/ml) [24]. Vn also exists as an extravascular cell-bound multimeric form in several tissues, and Vn mRNA can be detected in high concentrations in the liver, brain, heart and adipose tissue but is rare or absent in the kidney and spleen [25]. It comprises an N-terminal somatomedin-binding domain, consisting of 43 amino acid (aa) residues, followed by the host cell integrin receptor-binding motif RGD (Arg-Gly-Asp). In addition to 4 heparin-binding domains with unknown function, Vn also contains 3 heparin-binding domains (HBDs) which span aa 82–137 (HBD-1), aa 175–219 (HBD-2) and aa 348–361 (HBD-3) [26, 27]. Vn is an important regulator of complement activity at the level of terminal complement complex (TCC) formation and a component of the ECM, and it also fulfills functions in cell migration and tissue repair [27].

At the level of TCC formation, Vn regulates complement activity by directly binding to the protein complex C5b-7 or to C9 [28]. The exact mode of regulation is not fully understood. It has been postulated, however, that Vn binds the nascent precursor complex C5b-7, resulting in a Vn-C5b-7 complex that is unable to insert into the cell membrane [27, 28]. Vn can also directly bind C9 and thereby inhibit C9 polymerization. This binding takes places through HBD-3 whereas the binding site for the nascent C5b-7 is still unknown [27–29].

A wide variety of bacteria bind Vn via various surface proteins. The respiratory pathogens Moraxella catarrhalis (Mc) and Haemophilus influenzae (Hi) as well as the urogenital pathogen Haemophilus ducreyi express proteins belonging to the TAA family. These proteins are the ubiquitous surface protein A2 (UspA2) of Mc, the Haemophilus surface fibrils (Hsf) and the Haemophilus adhesin (Hia) of Hi or the H. ducreyi serum resistance protein A (DsrA) [9, 30–36]. In the invasive bacterial pathogen Neisseria meningitidis the 3 proteins Opc, Opa and Msf interact with Vn [37–40]. However, to date, no enteropathogenic bacteria have been reported to use Vn to escape complement-mediated attack and thus mediate serum resistance.

Ye has evolved a multitude of mechanisms to evade the host immune system. Amongst these, serum resistance is of uttermost importance. The significance of the complement regulator Vn in complement evasion and modulation of host cell interaction with bacterial and fungal pathogens has recently been recognized [27, 30–32, 37, 39–44]. Ye is able to bind several regulators of complement activity. The role of Vn in Ye host cell interaction and in pathogenicity has not yet been addressed in detail, but it was shown in previous studies that YadA from Ye...
O:8 does not bind Vn under stringent assay conditions [45]. In this study, we systematically investigated (1) Vn binding of different Ye strains, (2) which components of Ye might enable this binding and (3) how this interaction modulates Ye serum resistance, host cell interaction and overall pathogenicity. Importantly, we were able to demonstrate a novel mechanism that facilitates Ye serum resistance mediated by the surface adhesin YadA binding to Vn. We found that subtle differences within the YadA head domain of different Yersinia strains determine the efficacy of the Vn binding. An additional stretch in Ye YadAO:5, which is similar to the ‘uptake region’ of Yps YadA YPIII [18], was identified as a crucial region for the high-affinity binding of Vn. Moreover, we located HBD-3 within Vn as the YadA binding site. Notably, bound Vn is active on the bacterial surface and protects bacteria from complement-mediated lysis by the inhibition of C9 polymerization. This mechanism allows the enhanced survival of Ye O:9 E40 during the early phase of a mouse infection in vivo.

Materials and Methods

Mice

C57BL/6 wild-type (WT) mice were purchased from Harlan Winkelmann (Horst, The Netherlands), B6.129S2(D2)-Vtnm1Dgi/J mice (http://jaxmice.jax.org/strain/004371.html) with a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). All mice were bred under specific pathogen-free conditions in individually ventilated cages with access to water and food ad libitum. Experiments were performed with female mice (aged 6–8 weeks) according to German law with the permission of the Regierungspräsidium Tübingen (permission No. H4/15).

Plasmids

Plasmids used in this study are listed in table 1.

Bacterial Strains and Culture Conditions

All Yersinia strains were cultivated in lysogeny broth medium with supplements (antibiotics as listed in table 2) overnight at 27°C. To promote YadA expression, a 1:20 dilution of the overnight culture was made with fresh medium and incubated for 3 h at 37°C. Moraxella strains were grown overnight at 37°C in brain-heart infusion medium. All bacteria were washed twice with PBS, and the optical density at 600 nm was determined. The number of bacteria used for the individual experimental setups are indicated in the respective sections. All bacterial strains used in this study are listed in table 2.

Serum

Normal human serum (NHS) was collected from at least 4 healthy volunteers and pooled. Aliquots were stored at –80°C and thawed only once. Heat-inactivated serum (HIS) was generated by incubation at 56°C for 30 min immediately before use.

Antibodies

Antibodies used in this study are listed in table 3.

Purified Proteins Used in This Study

Purified monomeric and multimeric Vn was purchased from BD Bioscience (Heidelberg, Germany) and Millipore (Schwalbach, Germany), respectively. Vn fragments were expressed and purified as described previously [35, 54].

Table 1. Plasmids used in this study

| Plasmid name                  | Description                                                                 | Resistance | Reference |
|-------------------------------|-----------------------------------------------------------------------------|------------|-----------|
| pBla                          | expression of YopE aa 1–53 β-lactamase hybrid protein under control of the YopE promoter | kanamycin  | 46        |
| pACYC184 EGFP                 | EGFP expressed under control of a constitutive tac/lac promoter             | chloramphenicol | 47       |
| pASK-IBA4C_yadAO:8            | yada from Ye O:8 WA-314 cloned into pASK-IBA4C; expression under control of an anhydrotetracycline-inducible promoter | chloramphenicol | this study |
| pASK-IBA4C_yadAO:9            | yada from Ye O:9 E40 cloned into pASK-IBA4C; expression under control of an anhydrotetracycline-inducible promoter | chloramphenicol | this study |
| pASK-IBA4C_yadAO:9/O:8 hybrid | plasmid for inducible expression of a hybrid protein consisting of the N-terminal aa 1–89 of yada from Ye O:9 E40 fused to aa 55–422 of yada from Ye O:8 WA-314; expression under control of an anhydrotetracycline-inducible promoter | chloramphenicol | this study |
| pASK-IBA4C_yadAO:9/Auptake region | plasmid for inducible expression of yada from Ye O:9 E40 lacking aa 60–86 comprising the uptake region; expression under control of an anhydrotetracycline-inducible promoter | chloramphenicol | this study |
Table 2. Bacterial strains used in this study

| Bacterial strain | Description | Resistance | Reference |
|------------------|-------------|------------|-----------|
| Ye O:3 6471/76  | serotype O:3, fecal isolate, WT | – | 48 (GI:48607) |
| Ye O:8 8881     | serotype O:8, fecal isolate, WT | – | 49 (GI:122815846) |
| Ye O:8 WA-314 YadAwt | coding sequence of YadA WA-314 O:8 was reinserted into a YadA0 strain | Nal, Kan, Spec | 12 (GI:310923211) |
| Ye O:9 E40 pBla | Ye O:9 E40 Δasd transformed with pMK-Bla | Nal, Kan, Ars | 46 (GI:972903261) |
| Ye O:9 E40 ΔΔpVYpBla | Ye O:9 E40 Δasd without virulence plasmid transformed with pMK-Bla | Nal, Kan | 46 |
| Ye O:9 E40 ΔInv pBla | YadAwt coding sequence of YadA WA-314 O:8 was reinserted into a YadA0 strain | Nal, Kan, Ars, Strep | 47 |
| Ye O:9 E40 ΔΔpVYΔInv pBla | pVY-Δasd strain was transformed with pIJM4029 (YadA-) and with pMK-Bla | Nal, Kan, Ars, Strep | 47 |
| Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:8 | Ye O:9 E40 Δasd lacking expression of both YadA and Inv transformed with pASK-IBA4C_yadAO:8 | Nal, Kan, Ars, Strep, Cm | this study |
| Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9 | Ye O:9 E40 Δasd lacking expression of both YadA and Inv transformed with pASK-IBA4C_yadAO:9 | Nal, Kan, Ars, Strep, Cm | this study |
| Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9/O:8 hybrid | Ye O:9 E40 Δasd lacking expression of both YadA and Inv transformed with pASK-IBA4C_yadAO:9/O:8 hybrid | Nal, Kan, Ars, Strep, Cm | this study |
| Ye O:9 E40 ΔΔ + pASK-IBA4C_yadAO:9 Δuptake region | Ye O:9 E40 Δasd lacking expression of both YadA and Inv transformed with pASK-IBA4C_yadAO:9 Δuptake region | Nal, Kan, Ars, Strep, Cm | this study |
| Ye O:9 E40 pBla eGFP | Ye O:9 E40 pBla transformed with pACYC184 EGFP | Nal, Kan, Ars, Cm | 47 |
| Ye O:3 01       | clinical isolate derived from fecal sample | – | this study |
| Ye O:3 02       | clinical isolate derived from fecal sample | – | this study |
| Ye O:3 03       | clinical isolate derived from swine (tongue) | – | this study |
| Ye O:8 04       | clinical isolate derived from fecal sample | – | this study |
| Ye O:3,27 06    | clinical isolate derived from fecal sample | – | this study |
| Ye O:5,27 07    | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 08       | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 09       | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 10       | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 11       | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 12       | clinical isolate derived from fecal sample | – | this study |
| Ye O:9 13       | clinical isolate derived from blood sample | – | 50 |
| Ye O:9 14       | clinical isolate derived from fecal sample | – | this study |
| Yps YPH1        | Yps WT strain, pIB1 | – | 51 |
| Yps YP46 pB1    | yadΔA53-83 | Kan, Amp | 18 |
| Yps YP47 pB1    | yadΔ- | Kan | 17 |
| Ec omp2 + pASK-IBA4C | Ec BL21 lacking expression of ompF transformed with pASK-IBA4C | Cm | this study |
| Ec omp2 + pASK-IBA4C_yadAO:8 | Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:8 | Cm | this study |
| Ec omp2 + pASK-IBA4C_yadAO:9 | Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:9 | Cm | this study |
| Ec omp2 + pASK-IBA4C_yadAO:9/O:8 hybrid | Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:9/O:8 hybrid | Cm | this study |
| Ec omp2 + pASK-IBA4C_yadAO:9 Δuptake region | Ec BL21 lacking expression of ompF transformed with pASK-IBA4C_yadAO:9 Δuptake region | Cm | this study |
| Mc RH4 WT       | Mc WT strain | – | 52 |
| Mc RH4 ΔUspA2H  | Mc lacking expression of UspA2H | Zeo | 53 |

Amp = Ampicillin; Ars = arsenite; Cm = chloramphenicol; Kan = kanamycin; Nal = nalidixic acid; Spec = spectinomycin; Strep = streptomycin; Tet = tetracycline; Zeo = zeocine.
Table 3. Antibodies used in this study

|                | Conjugate | Clone | Manufacturer                  | Working dilutions |
|----------------|-----------|-------|-------------------------------|-------------------|
| **Primary antibodies** |           |       |                               |                   |
| Goat anti-factor H  | –         | polyclonal | Complement Technology         | 1:100             |
| Rabbit anti-Vn      | –         | polyclonal | Complement Technology         | FACS 1:100; WB 1:1,000 |
| Rabbit anti-Ye YadA | –         | polyclonal | Lab antibody; I. Autenrieth   | 1:200             |
| Rabbit anti-Yps YadA| –         | polyclonal | Lab antibody; P. Dersch       | 1:200             |
| Sheep anti-Vn       | –         | polyclonal | AbD Serotech                  | 1:100             |
| Mouse anti-human C5b-9 | – | polyclonal | aE11                          | 1:1,000           |
| Mouse anti-β subunit of E. coli RNA-polymerase | – | 8RB13 | NeoClone Biotechnology | 1:2,000 |
| **Secondary antibodies** |           |       |                               |                   |
| Donkey anti-rabbit  | APC       | Jackson ImmunoResearch       | 1:200             |
| Goat anti-rabbit    | DyLight 800 | Thermo Scientific           | 1:10,000          |
| Goat anti-rabbit    | DyLight 680 | Thermo Scientific           | 1:10,000          |
| Goat anti-mouse     | DyLight 680 | Thermo Scientific           | 1:10,000          |
| Rabbit anti-sheep   | DyLight 800 | Thermo Scientific           | 1:10,000          |
| Rabbit anti-goat    | Alexa Fluor 488 | Jackson ImmunoResearch | 1:200             |
| Goat anti-mouse     | Alexa Fluor 647 | polyclonal | Jackson ImmunoResearch | 1:2,500 |

**Binding Assay with Serum or Purified Proteins Analyzed by Flow Cytometry**

To analyze the binding of purified Vn or Vn and factor H from HIS, a total of 1 × 10⁷ bacteria per assay were incubated with 5–50% HIS or purified Vn (1–10 μg/ml) diluted with PBS (Life Technologies, Darmstadt, Germany) in a total volume of 100 μl for 30 min at 37°C. As an internal control, each strain was also treated with PBS only. Recombinant Vn fragments were used at 4 μg/ml. After washing with 1% BSA in PBS (washing buffer), bacteria were spun down and the pellet was resuspended in 200 μl 4% paraformaldehyde in PBS for 1 h at room temperature. Bacteria were washed once again and finally incubated with primary polyclonal antibodies (pAb) directed against Vn or factor H overnight at 4°C. The next day, bacteria were washed once and incubated with suitable secondary antibodies for 1 h at room temperature. After a final washing step, bacteria were transferred to FACS tubes and analyzed with a Fortessa LSR II instrument. Data analysis was carried out using WinMDI v2.8. The PBS-only control was used to determine background staining using the same primary and secondary antibodies as for all other samples. Values obtained for the control samples were subtracted from the values obtained for the corresponding samples that were incubated in serum or purified Vn. All flow cytometry figures show background subtracted values.

**Detection of Vn Binding by Western Blot**

To detect Vn binding by immunoblotting, 5 × 10⁸ bacteria (bacterial numbers were determined photometrically by measuring the optical density at 600 nm; a volume corresponding to the desired number of bacteria was harvested by centrifugation, and the bacterial pellets were then used to carry out the assay) were incubated in 100 μl of 50% HIS diluted in PBS as described above. Thereafter, bacteria were washed twice with washing buffer, once with PBS, and finally resuspended in 50 μl deionized water. For the detection of YadA, bacteria were simply washed after harvest. After the addition of 25 μl 4× Laemmli buffer (Bio-Rad Laboratories, Munich, Germany), samples were boiled for 5 min at 95°C and separated in a 10% acrylamide SDS gel (Bio-Rad Laboratories). Each lane was loaded with an equal number of bacteria. After blotting, the membranes were blocked with 3% BSA and 5% milk powder in TBS for 1 h at room temperature. They were then incubated with the desired antibodies (a complete list of antibodies and working dilutions is given in table 3) for 1 h at room temperature or at 4°C overnight, washed with 0.1% TBS-T and then incubated with the suitable secondary antibody. Fluorescence signals were recorded using a LICOR Odyssey imaging system.

**Detection of Vn Binding by Blot Overlay Assay**

Bacterial lysates were prepared as described above, separated by SDS-PAGE and blotted. After blocking with 5% milk, 3% BSA in PBS for 3 h at room temperature, the membrane was incubated with 7 μg/ml purified monomeric Vn in 3% BSA in PBS-T overnight at 4°C. After washing with 0.1% TBST, Vn was detected with rabbit anti-Vn pAb and a secondary DyLight 680-conjugated goat anti-rabbit pAb. Fluorescence signals were recorded using a LICOR Odyssey imaging system.

**Purification of DNA from Yersinia Colonies**

*Yersinia* strains were streaked on the LB agar plates without antibiotics. The next day, a single colony was used for DNA extraction using the Qiagen QIAamp DNA mini kit according to the manufacturer’s protocol. DNA was finally eluted in 100 μl of ultrapure water.
PCR Amplification of the YadA Head Region

To test *Yersinia* YadA for the presence of the additional stretch (enabling the recruitment of Vn) within its head region, we used the primers YadA_Serosq_435F (5′-gatcagtgtcctgcggcat-3′) and YadA_Serosq_453R (5′-gccctaaagtacggca-3′) that bind to highly conserved regions upstream and downstream of the uptake region (online suppl. fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000449200). According to the sequence alignment, the PCR reaction should yield a fragment of 442 bp with *Ye O*:9 E40 or 451 bp with Yps YPIII (both harboring the uptake region of approx. 90 bp) or 337 bp with *Ye O*:8 WA-314 and 346 bp with *Ye O*:3 6471/76 and *Ye O*:5.27 (all 3 lacking the uptake region) and thus allow us to discriminate between YadA with and without the uptake region. We used the following PCR program: 2 min 95°C (initial denaturation), 30 s 95°C → 1 min 55°C → 30 s 68°C (repeated 29 times), 5 min 72°C (final extension) and cooling at 4°C until further processing.

Separation of PCR Products by Capillary Gel Electrophoresis

To determine the size of the PCR products, they were analyzed using a QIAxcel capillary gel electrophoresis system according to the manufacturer’s protocol.

DNA Sequencing

PCR products were purified using the Promega Wizard® SV gel and PCR Clean-Up System according to the manufacturer’s protocol. Subsequently, Sanger sequencing was performed by GATC using the same primers as for the PCR reaction.

Heparin Inhibition Assay

Sterile glass coverslips were coated with purified Vn (10 μg/ml) at 4°C overnight and air-dried. The coverslips were then placed in a 24-well plate and either incubated with PBS or 100 μM heparin in PBS; 5 × 10⁷ bacteria (*Ye O*:9 E40 pBlA EGFP) were added to each well, spun down for 5 min at 300 g and incubated for 1 h at 37°C in a humidified atmosphere. Afterwards, the supernatant was removed, and the samples were washed 2 times and finally fixed by the addition of 4% paraformaldehyde in PBS. After washing, coverslips were mounted in Mowiol, and micrograph pictures were acquired using a Zeiss LSM 510. To quantify adhesion, the number of bacteria for a given field of view (representative for the entire coverslip) was counted.

Analysis of C5b-9 Deposition by Flow Cytometry

To analyze whether bound Vn was functionally active, bacteria were incubated with Vn (10–50 μg/ml) or C4BP (10–50 μg/ml) for 30 min at 37°C. After washing, bacteria were incubated with C5b-6 (1 μg/ml) and C7 (1 μg/ml) for 10 min, and then C8 (0.4 μg/ml) and C9 (1 μg/ml) were added for 30 min at 37°C. All complement components except for C8 were from Complement Technology (Tyler, Tex., USA). Deposited C5b-9 was detected by mouse anti-human C5b-9 mAb followed by Alexa Fluor 647-conjugated goat anti-mouse pAb. After 2 additional washes, bacteria were analyzed by flow cytometry (EPICS XL-MCL; Coulter, Hialeah, Fl., USA). All incubations were kept in a final volume of 100 μl 1% BSA in PBS, and washes were performed with the same buffer. Primary and secondary pAb were added separately as negative controls for each strain analyzed.

In vitro Serum Killing Assay

To analyze the susceptibility of *Ye* and *Yps* to complement-mediated killing in human serum, 5 × 10⁶ bacteria were incubated in 100 μl 20% NHS or HIS for 30 min at 37°C. Complement activity was stopped by adding 100 μl BHI medium and placing the samples for 5 min on ice. Afterwards, serial dilutions of the samples were prepared, plated on selective agar plates and incubated at 27°C for 48 h. The colony-forming units (CFU) were determined. The serum bactericidal effect was calculated as the survival percentage, taking the bacterial counts obtained with bacteria incubated in HIS as 100%.

In vivo Serum Killing Assay

To analyze the lytic activity of serum complement against *Ye* in C57BL/6 and B6.129S2(D2)-Vitrin(Jm128/J mice, the animals were infected intravenously with 1 × 10⁷ bacteria. After 30 min, they were sacrificed by CO₂ asphyxiation and blood was withdrawn from the heart. Heparin (100 μl at 100 μg/ml) (Sigma-Aldrich, Steinheim, Germany) was mixed with the blood to avoid coagulation. Serial dilutions of the samples were plated on selective agar and incubated at 27°C for 48 h. The CFU were determined by counting the colonies.

Bioinformatics and Statistical Analysis

The GI numbers or the references of the sequences used in this work are listed in table 2. Alignments were produced with Kalign [30, 34] and secondary pAb were added separately as negative controls for each strain analyzed.

Results

*Ye O*:9 E40 Efficiently Binds Vn

Vn plays an important role in the complement resistance of, for example, *Mc, Hi* and *Streptococcus pneumoniae* [32, 34, 44, 54, 57]. In order to test if *Ye* is able to bind Vn and if there are differences in the binding capacity of various *Ye* strains and serotypes, we incubated a set of strains in 50% HIS, washed the cells and detected Vn bound to bacteria by immunostaining with antibodies directed against Vn and subsequent flow cytometry analysis (fig. 1a). Upon incubation with HIS, we found very diverse binding properties of *Ye* strains compared to *Mc RH4* and *Yps YPIII*. *Mc RH4* served as a positive control [30, 34], whereas *Yps YPIII* was used as an additional comparator. It has been recognized that *Yps YPIII* YadA differs from the YadA sequences of other strains and that this difference coincides with a change in preferred ECM binding partners; this might possibly also affect the interaction with Vn (*Yps YPIII* YadA preferentially binds to fibronectin instead of collagen and laminin as observed.)
Fig. 1. Vn is efficiently bound by Ye O:9 E40 and Yps. a Several strains of Ye, serotype O:9 with and without virulence plasmid (O:9 E40 and O:9 E40 ΔpYV), serotype O:3 (O:3 6471/76) and serotype O:8 (O:8 8081; O:8 WA-314), and 1 Yps (Yps YP III) WT strain were incubated with HIS, washed and subsequently analyzed for the presence of Vn on the bacterial surface by flow cytometry. Mc (Mc RH4), which is known to bind Vn and Yps, which we supposed also binds Vn, were included as a positive control for Vn binding. Ye O:9 E40, cured from the virulence plasmid (plasmid of Yersinia virulence; pYV) that encodes for the Ye T3SS, effector proteins and YadA, was included as a negative control because we surmised that Vn binding is pYV dependent. YadA protein levels were analyzed by Western blot analysis in whole-cell lysates and are shown below the bar chart (1 representative Western blot is shown). RNA polymerase protein (RNA-Pol.) was used as a loading control. YadA O:3 6471/76 has a calculated molecular weight of approximately 141 kDa (455 aa), YadA O:8 8081 of 132 kDa (422 aa), YadA O:8 WA-314 of 132 kDa (422 aa), YadA O:9 E40 of 153 kDa (487 aa), YadA YPIII of 135 kDa (434 aa) and UspA2H of approximately 272 kDa (876 aa). b To test if strain-specific differences in the binding of Vn are exclusive, we compared Vn binding levels to that of factor H. In contrast to Vn, factor H is bound in comparable amounts by all Yersinia strains tested, except for the negative control strain (O:9 E40 ΔpYV). The protein levels of YadA and the RNA polymerase as a loading control were analyzed by Western blots of whole-cell lysates and are shown below the bar chart (1 representative Western blot is shown). Binding of serum-derived Vn to Ye O:9 E40 is dose dependent. Ye O:9 E40 and the pYV-cured version thereof were incubated with increasing serum concentrations. Afterwards, cell surface-associated Vn was quantified by flow cytometry. c Ye O:9 E40 and the pYV-cured version thereof were incubated with increasing amounts of purified Vn. Afterwards, cell surface-associated Vn was quantified by flow cytometry. Binding of purified Vn to Ye O:9 E40 is dose-dependent. a–d Data are means ± SD of at least 4 individual experiments. a, b The main p values were determined by one-way ANOVA. p < 0.0001. Multiple comparisons were performed by one-way ANOVA with Dunnett’s multiple-comparisons test. c, d The p values were determined by Student’s t test. The error bars denote the SD. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.
with Ye) [18]. Ye O:9 E40 was able to bind exceptionally high amounts of Vn, which led to a mean fluorescence intensity of approximately 2.8 times higher than that measured with Mc RH4 (133.9 ± 33.9 vs. 47.4 ± 19.6). Yps also bound Vn, but at concentrations comparable to that of the Mc RH4 positive control (56.7 ± 11.0 vs. 47.4 ± 19.6). Ye O:8 WA-314 and Ye O:3 6471/176 also bound Vn, though to a lesser extent than Mc RH4 (approx. 54.1 or 70.7% of Mc RH4 signal). Ye O:8 8081 bound only residual amounts of Vn (6.7 ± 0.6). Interestingly, the binding of Vn to Ye O:9 E40 depended on the presence of the plasmid of Yersinia virulence (pYV) and was dose dependent (fig. 1c, d). In a plasmid-deficient strain (Ye O:9 E40 ΔpYV), Vn binding was almost abolished (6.2 ± 2.8).

To test if the strain-specific binding pattern of Vn (O:9 E40 > YPIII > RH4 > O:3 > O:8 WA-314 > O:9 E40 ΔpYV = O:8 8081) is exclusive in comparison to other serum factors, we also tested the binding of factor H ([45]). Factor H has been shown to interact with several discontinuous stretches within the stalk domain of YadA [20–22, 58]. Our data corroborate previous findings that the binding of factor H by Yersinia strains relies on the presence of YadA, but in contrast to Vn, there is no significant difference in binding efficiency in the various serotypes tested. This indicates different mechanisms of binding of Vn and factor H. Taken together, we found that Ye O:9 E40 is able to bind high amounts of serum-derived as well as purified Vn, although only in the presence of the pYV plasmid, in a dose-dependent manner. In contrast, Ye O:8 WA-314, 8081 and Ye O:3 6471/76 are weak Vn binders, although they also carry the pYV plasmid. This partially substantiates earlier findings that YadA-dependent Vn binding is at least weak if not nonexistent for Ye O:8 WA-314 in whole-cell adhesion assays under specific flow conditions [45].

**Binding of Vn Is YadA Dependent**

In order to assess whether YadA is the determinant for the binding of Vn to Yersinia, we used flow cytometry to compare Vn binding in a Ye O:9 E40 WT strain, a mutant deficient for YadA (ΔYadA), a mutant deficient for the chromosome-encoded adhesin Inv (ΔInv), the corresponding double mutant (ΔInvΔYadA; ΔΔ) and, again, the cured strain lacking the pYV plasmid (ΔpYV) (fig. 2a, left panel). We used Mc RH4 as positive control and an Mc ΔUspA2H [32] knockout strain as a negative control (fig. 2a, right panel). Our data indicate that the presence of YadA, but not of Inv, is decisive for the binding of Vn to Ye O:9 E40. Thus, in contrast to Ye O:9 E40 WT or ΔInv, Vn did not bind to ΔYadA, the ΔInvΔYadA double mutant or the pYV-cured strain. We could corroborate these findings by blot overlay assays and Western blot (fig. 2b, c). Analysis of the influence of YadA and, more specifically, a distinct region within YadA of Yps for Vn binding revealed that in Yps, YadA is also the Vn binding determinant (fig. 2a, middle panel). Moreover, the deletion of 30 aa (Δ53–83) corresponding to the uptake region in the head domain of Yps YadA YPIII abolishes Vn binding (fig. 2a, middle panel). Thus, our data demonstrate that YadA is essential for mediating Vn binding in Ye and that a stretch of 31 aa within the head region of YadA YPIII is decisive for the binding of Vn in Yps.

**A Specific Stretch within YadA Discerns Low-Affinity Binding from High-Affinity Binding of Vn**

We found that Ye expressing YadA derived from the O:8 WA-314 strain is a relatively weak binder compared to Ye O:9 E40 (fig. 1a). Therefore, we aimed to determine if other strains also carry the uptake region and also what actually discerns YadA O:9 E40 from YadA O:8 and if this difference might be causative for the discriminative Vn binding behavior. The head domain of YadA YPIII contains a stretch of sequence (uptake region) which is crucial for cell adhesion and efficient internalization of Yersinia via YadA [18]. This motif is absent in YadA of Ye O:8 but present in the Ye O:9 E40 strain (aa 56–88) (fig. 3a). It is rich in prolines and charged residues, suggesting an undefined loop structure (fig. 3b), inserted in a shorter loop that is not resolved in the crystal structure of the Ye O:3 YadA head (PDB: 1P9H) [59].

To investigate whether this motif is present exclusively in Ye O:9 E40 or can be found also in other Yersinia strains and especially in strains isolated from clinical specimens, we carried out PCRs. We designed primers binding to rather conserved regions within the YadA sequence flanking that part of the head domain which comprises the uptake region (online suppl. fig. S1). The size of the PCR products allowed us to easily detect the presence of the uptake region. The predicted lengths of the YadA head fragments were 346 bp (Ye O:3), 337 bp (Ye O:8), 346 bp (Ye O:5, 27), 451 bp (Ye O:9) and 442 bp (Yps YPIII). Strikingly, the additional stretch present in YadA of Ye O:9 E40 and Yps YPIII was present in all tested clinical isolates of serotype O:9 but absent in all other strains (belonging to the indicated serotypes; fig. 3c) that we tested. Ye O:9 E40 ΔYadA and water control were included as negative controls (fig. 3c, all strains depicted were also tested for Vn binding). Cell surface-associated Vn after incubation in HIS was quantified by flow cytometry.

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YadA-Mediated Interaction of Ye with Vitronectin

Whereas all strains belonging to serotype O:9 (No. 08–14) and possessing the uptake region within YadA bound Vn in comparably high amounts as Ye E40 O:9, Ye strains of serotype O:3 (No. 01, 02, 03), O:8 (No. 04) and O:5,27 (No. 06, 07) turned out to be rather weak binders. Thus, we assume that the presence of the uptake region is the major determinant that allows binding of Vn and (at least in the strains we have tested) is present exclusively in the YadA of Ye strains of serotype O:9.

To test this hypothesis, we generated a YadA hybrid where we replaced the N-terminus of the head domain of YadA O:8 by that of YadA O:9 E40 (including the uptake region) and a YadA O:9 E40 deletion mutant lacking the uptake region (aa 56–88). We then compared Vn binding by flow cytometry. The strains Ye O:9 ΔYadA expressing YadA O:8 WA-314 or YadA O:9 E40 were also included in this analysis. In addition, we used Ye O:8 WA-314, Ye O:9 E40 and Ye O:9 E40 ΔYadA as controls (fig. 4b). Ectopic expression of YadA O:9 E40 was able to rescue Vn binding of Ye O:9 E40 ΔYadA. This was also true for the O:9/O:8 hybrid YadA. Additionally, deletion of the uptake region from YadA O:9 led to significantly reduced Vn binding (fig. 4b). Our data show that the uptake region of YadA O:9 E40 significantly enhances

**Fig. 2.** Vn binding to Ye is YadA dependent. a Left panel: a Ye O:9 E40 WT strain or strains carrying individual deletions for the adhesins Invasin (ΔInv) or YadA (ΔYadA) and a respective double knockout strain (ΔΔ) as well as a virulence plasmid-cured strain (ΔpYV) were incubated with serum and washed, and then Vn binding was quantified by flow cytometry. Middle panel: Yps YPIII WT and corresponding strains lacking expression of ΔYadA or expressing a YadA version lacking part of the head domain (Δ53–83) were included as controls. Right panel: an Mc WT strain known to bind Vn via the surface adhesin UspA2 and a corresponding strain lacking expression of UspA2 (ΔUspA2) were included as positive and negative controls. YadA protein levels were analyzed by Western blot analysis of whole-cell lysates and are shown below the bar chart (1 representative blot is shown). b A selection of the strains used in (a) was tested for Vn binding in a blot overlay assay. Vn and YadA were detected on the identical blot with specific antibodies and differently labeled secondary antibodies (emission maximum at 680 and 800 nm, respectively) simultaneously. Vn is bound only in the presence of YadA (Ye) or UspA2, respectively. c In a direct binding assay, essentially performed as in a, Vn can be detected at the expected molecular weight (65 and 75 kDa) by Western blot only in those Ye strains expressing YadA. Data are means ± SD of at least 4 individual experiments (a) or 1/3 representative experiments is shown (b, c). The main p value was determined by one-way ANOVA (a: p < 0.0001). Multiple comparisons were performed by one-way ANOVA with Dunnett’s multiple-comparisons test. The error bars denote the SD. ** p < 0.01, **** p > 0.0001.
recruitment of Vn. Of note, a sequence alignment of YadA from different Yersinia strains also revealed insertions in the stalk regions of Ye YadA<sup>O:9</sup> E40 and YadA<sup>O:3</sup> 6471/76 that are not found in YadA<sup>YPIII</sup> and YadA<sup>O:8</sup> WA-314 (online suppl. fig. S2). However, these regions show no clear association with Vn or factor H binding (fig. 1a). Finally, we wanted to assess whether cofactors expressed by Yersinia are necessary or if YadA containing the uptake region alone is sufficient to mediate efficient binding of Vn. We tested Vn binding of E. coli omp2 [60] which ectopically expressed the YadA version described above (online suppl. fig. S3). We found that expression of YadA<sup>O:9</sup> or the hybrid YadA<sup>O:9/O:8</sup> is sufficient to mediate the binding of Vn. Thus, we conclude that the decisive factor for Vn binding is YadA comprising the uptake region.

**Vn Interacts with YadA via Its C-Terminal HBD-3**

Previous work with Mc and Hi revealed HBD-3 as the decisive part of Vn for interaction with UspA2 or Hsf [34, 35]. Therefore, we wanted to know if this domain might also mediate the interaction of Vn with YadA. In order to test this, we first analyzed whether heparin might block the binding of Ye to Vn by occupying the HBDs. This would be a clear indicator of the involvement of one of the HBDs in the interaction with YadA. Coverslips were coated with Vn and then incubated with Ye O:9 E40, expressing enhanced green fluorescent protein for easier detection of binding, either in the presence or absence of heparin. Thereafter, coverslips were washed, fixed, mounted and analyzed by fluorescence microscopy (fig. 5a). Our results demonstrate that, in the presence of heparin, the binding of bacteria to Vn-coated coverslips is significantly reduced. Therefore, we conclude that at least one of the HBDs is involved in mediating the binding of Vn to YadA<sup>O:9</sup> E40.

To locate the sites within Vn that actually determine YadA binding, we used a set of recombinant Vn fragments (fig. 5b). These fragments essentially comprise C-terminal-truncated Vn molecules as well as deletion mutants lacking parts of HBD-3 (comprising aa 348–361) or adjacent regions. All fragments were tested for appropriate quality (online suppl. fig. S4). Our binding assay (fig. 5c) demonstrates that the fragments Vn 80–396, 80–379, 80–373 and 80–363 are efficiently bound by Ye O:9 E40. However, further C-terminal truncation, comprising either parts of or the entire HBD-3 (80–353, 80–339), led to a reduction of binding. Fragments lacking the entire HBD-3 plus the adjacent N-terminal region (80–330, 80–229) bound only weakly to Ye O:9 E40 (fig. 5c). Thus, we assume that not only HBD-3 but also the adjacent N- and especially the C-terminal approximately 10–20 aa are important for a stable interaction of Vn with Ye O:9 E40. These findings are in agreement with the fact that a Vn molecule lacking the C-terminal part of HBD-3 plus the adjacent C-terminal region (Δ352–374) is also impaired when binding to Ye O:9 E40 whereas deletion of either only part of HBD-3 (Δ352–362) or only the adjacent C-terminal region (Δ362–374) does not significantly influence binding. In conclusion, aa 331–363 are decisive for the stable interaction of Vn with Ye O:9 E40.

**Vn Is Functionally Active and Inhibits the Terminal Pathway when Bound to the Surface of Ye**

Besides modulating the adhesive properties of pathogens, Vn regulates the terminal complement pathway and blocks TCC formation. In order to test if Vn bound to Ye is functionally active and inhibits the terminal comple-

**Fig. 3.** A specific region in the YadA head domain is decisive for efficient binding of Vn. **a** Alignment of the head of various YadA variants. White letters on gray background: signal peptide. Black letters on gray background: the canonical ‘SVAIG’ head repeats of YadA. Italics: the neck region that links the head to the coiled-coil stalk of YadA. The insertion of Yps originally proposed by Heise and Dersch [18] is displayed in bold, and is slightly shifted towards the N-terminus of YadA. The dashed line on top shows the corrected position of the insertion, based on improved alignments and the structure of the YadA head from Ye O:3, where the short insertion is not resolved (underlined region). This and the unusually high number of prolines in this region suggest that it is not structured. The long version of the insertion carries a strongly positive net charge (+5 for Yps YPIII, +4 for the Ye O:9 E40), which probably plays a role in binding to fibronectin and Vn. **b** Schematic view of the differences in the YadA heads. The Yps YPIII and Ye O:9 E40 variants have long insertions in an unstructured loop region close to the N-terminus of the head. **c** PCR products comprising the YadA head region of Ye O:8 WA-314, Ye O:9 E40 with and without YadA, Yps YPIII and clinical isolates derived from fecal samples (Ye O:3, No. 01–03; Ye O:8, No. 04; Ye O:5,27, No. 06–07 and Ye O:9, No. 08–12) or blood (No. 13) were separated by capillary gel electrophoresis. The predicted length of PCR products was as follows: Ye O:3 346 bp; Ye O:8 337 bp; Ye O:9 451 bp; Ye O:5,27 346 bp, and Yps YPIII 442 bp. Water control and a YadA-deficient strain were included as negative controls. **d** The strains shown in **c** were tested for Vn binding. Cell surface-associated Vn after incubation in HIS was quantified by flow cytometry. One of 3 representative experiments is shown.

YadA-Mediated Interaction of Ye with Vitronectin
ment pathway, we assayed C5b-9 deposition in the presence of Vn bound to intact bacteria. To this end, Ye O:9 E40 was preincubated with Vn or C4BP followed by the addition of C5b-6, C7, C8 and C9. C5b-9 deposition was determined by using an anti-C5b-9 mAb and flow cytometry.

We clearly demonstrate that Vn bound to the surface of Ye O:9 E40 was functionally active and inhibited C5b-9 deposition in a dose-dependent manner (fig. 6a, b). Vn (50 μg/ml) inhibited C5b-9 deposition by 61%. C4BP, the C3 convertase inhibitor of the classical/lectin pathways, did not influence the C5b-9 deposition and thus the terminal pathway. From this, we conclude that Vn when bound to intact Ye is functionally active and inhibits the terminal complement pathway and C5b-9 deposition.

**Binding of Vn Decreases the Susceptibility to Complement-Mediated Killing by Human Serum**

YadA-mediated serum resistance is an important virulence trait of Ye [21, 61, 62]. To analyze the importance of Vn binding for preventing complement-mediated killing, we performed serum killing assays. We incubated Ye O:9 E40, the corresponding YadA-deficient mutant (ΔYadA) and Ye O:8 WA-314 in NHS (fig. 7a, 'control strains'). Their survival was calculated as the survival percentage, taking the bacterial counts obtained with samples incubated in HIS as 100%. Our data show that Ye O:9 E40 – a strong Vn binder – is resistant to complement-mediated killing (% survival in NHS compared to HIS 119.1 ± 40.39) whereas the Ye O:9 E40 ΔYadA mutant strain was highly susceptible for killing by the complement system (16.74 ± 9.83). Compared to Ye O:9 E40, the weak Vn binder Ye O:8 WA-314 was significantly more susceptible to complement-mediated killing (39.21 ± 7.11) than Ye O:9 E40 (fig. 7a). Furthermore, we also tested Ye O:9 E40 ΔΔ expressing either YadA O:9, YadA O:9/O:8, YadA O:9 Δuptake region or YadA O:9 for serum resistance. We found that the expression of YadA O:9 (103.6 ± 3.42) and also of the O:9/O:8 hybrid YadA (105.7 ± 27.56) conferred serum resistance comparable to that of the Ye O:9 E40 WT strain. In contrast, the serum survival was significantly reduced upon the expression of YadA O:8 (48.55 ± 9.36). Compared to all these strains, a strain expressing the YadA O:9 lacking the uptake region showed the greatest sensitivity towards serum treatment (16.8 ± 5.77). These data clearly indicate that the YadA-dependent binding of Vn plays an important role in preventing the lysis of Ye by the complement system.
Fig. 5. Vn interacts with YadA via its C-terminal HBD-3. a Adhesion of Ye to Vn-coated coverslips can be blocked by heparin. b Schematic representation of Vn, the C-terminal-truncated Vn molecules [54] and the Vn molecules carrying deletions within and adjacent to the HBD-3 [35] that were used for a direct binding assay. c Western blot of a binding assay of Ye O:9 E40 with full-length Vn and all fragments depicted in b. Vn fragments appear in green; YadA, which was detected simultaneously, appears in yellow bands (trimer runs at approx. 200 kDa). d Flow cytometry analysis of Vn binding to Ye O:9 E40 with full-length Vn and all fragments depicted in b. Data are means ± SD of at least 3 individual experiments (a, d), or 1/3 representative experiments is shown. The p value for the comparison with and without heparin was determined by Student’s t test. The main p value was determined by one-way ANOVA (d: p < 0.0001). Multiple comparisons were performed by one-way ANOVA with Dunnett’s multiple-comparisons test. The error bars denote the SD. * p < 0.05, *** p < 0.001.
It is known that YadA is decisive for the survival of Ye upon contact with serum [6, 12]. This is one reason why YadA-deficient strains of Ye are avirulent in the mouse model [12]. However, the contribution of the YadA-dependent recruitment of Vn to the survival of Ye in a mouse model has not been addressed so far. In order to test if the presence of Vn has an influence on the survival of Ye in vivo, we infected Vn –/– and WT mice with YeO:9 E40, sacrificed the mice 30 min after infection and determined the bacterial burden in the blood. We found that the bacterial load in the blood was significantly reduced (log 10 CFU per gram of blood = 2.7 ± 0.8) for the Vn –/– mice compared to WT mice (log 10 CFU per gram of blood = 4.2 ± 1.0) (fig. 7b). In line with the reduction of C5b-9 deposition on Ye by Vn, these data would suggest that Vn protects Ye from early killing in the bloodstream.

Compared to YadA of Ye O:9 E40, the YadA of Ye O:8 WA-314 shows a low Vn-binding capacity. Therefore, we hypothesized that due to this low Vn-binding capacity and in contrast to our findings with Ye O:9 E40, the availability of Vn should only marginally impact the outcome of an early bloodstream infection with the Ye O:8 WA-314 strain. However, since the Ye O:9 and O:8 strains exhibit additional differences with regard to sequence and also virulence mechanisms [63–66], this experiment may not solve the question of whether the uptake region actually contributes to better clearance of infection by mediating more efficient binding of Vn specifically. Therefore, we used a slightly different approach. To clearly assess the role of the uptake region and to exclude other differences between the Ye O:8 and the Ye O:9 strain tampering with the result of our experiments, we infected mice with Ye harboring pYadA O:9/O:8 hybrid or pYadA O:8 in the same strain background (Ye O:9 E40 ΔΔ). The basic sequence of the YadA of these strains is identical, with the exception of the part encoding the uptake region. Surprisingly, the infection of C57BL/6 WT or Vn –/– mice with YeO:9 E40 ΔΔ harboring pASK-IBA4c_yadAO:9/O:8 hybrid revealed a significantly reduced bacterial load in the blood for the Vn –/– mice (log 10 CFU per gram of blood = 4.9 ± 0.2) compared to WT mice (log 10 CFU per gram of blood = 5.5 ± 0.2) (online suppl. fig. S5B). This leads to the assumption that the binding of Vn to different regions of YadA may have various impli-

**Fig. 6.** Vn is functionally active and inhibits the terminal pathway when bound to the surface of Ye. **a** Histogram overlay of flow cytometry analyses of TCC formation (detected by formation of the neoepitope C5b-9) on the surface of Ye O:9 E40 WT after preincubation of bacteria with PBS or different concentrations of Vn in PBS (10, 25 and 50 μg/ml). Preincubation with Vn reduces the amount of TCC that is formed. **b** Bar chart depicting C5b-9 deposition as percent of the amount of C5b-9 that was formed on the surface of bacteria preincubated with PBS only compared to bacteria preincubated with either Vn or C4BP at different concentrations (10, 25 and 50 μg/ml). Vn, but not C4BP, is able to reduce the formation of C5b-9. Antibody (Ab) control indicates background signal that was obtained using secondary antibody only for detection. Data are means ± SD of at least 3 individual experiments. The main p value was determined by one-way ANOVA (b: p < 0.001). Multiple comparisons were performed by one-way ANOVA with Dunnett’s multiple-comparisons test. The error bars denote the SD.
YadA-Mediated Interaction of Ye with Vitronectin

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Discussions for YadA function. While binding of Vn to the uptake region seems to increase virulence, binding of Vn to other regions of YadA might also reduce virulence.

Discussion

Complement inhibitor recruitment by bacterial cell surface proteins and adhesins is an important virulence mechanism used by many pathogens. Accordingly, several complement regulators (factor H, factor H-like protein-1 and C4BP) and complement proteins (C3b and iC3b) have been identified that interact with the Gram-negative enteropathogen Ye [19–22, 61, 62, 67]. Here, we describe a novel mechanism that contributes to Ye complement resistance and the overall virulence of Ye. We show that the TAA YadA of different Yersinia species binds Vn and demonstrate that a part of the YadA head domain of YadA O:9 E40 comprising aa 56–88 binds Vn with high efficiency. Recruitment of Vn to YadA led to the reduced surface formation and deposition of C5b-9 (TCC) and thus enhanced complement resistance. Moreover, Ye O:9 E40 was completely resistant to complement-mediated killing in human serum, in contrast to the YadA-deficient strain. In addition, it turned out that, in comparison to Ye O:8 WA-314, Ye O:9 E40 is significantly more serum-resistant. Using Vn-deficient mice, we were also able to demonstrate the reduced survival of Ye O:9 E40 in the absence of Vn in an in vivo serum killing assay. Thus, the binding of Vn to the surface of Ye has a great impact on the interaction of Ye with the host.

In our experiments, we found that different strains of Ye and Yps bind Vn in a YadA-dependent manner but that different Yersinia strains exhibited divergent Vn-
binding capacities. Previous studies with different Mc
WT strains show that Mc also binds Vn with different af-
nities via UspA2 [30]. The N-terminus of the UspA2
head domain sequence displays 2 different conserved re-
regions that may explain these Vn binding differences [68].
Furthermore, we show for the first time that Ye strains of
serotype O:9 – unlike all other Ye strains we tested – ex-
hibit an additional stretch in their YadA head domain.
These strains, and to a lesser extent Yps YPIII, showed
high-affinity binding to Vn while other tested Ye strains
showed only low-affinity binding. Unfortunately, we
were not able to correlate the ability to bind Vn and the
pathogenic potential of clinical isolates due to the low fre-
quency of Ye infection (and thus available isolates) and
the fact that systemic infection with Ye happens only on
rare occasions. The stretch in YadA<sub>O:9</sub> is highly similar to
the uptake region described for Yps YPIII [18], which is
important for the ability of YadA to promote the invasion
of Ye into host cells. Yps binds preferentially to fibroen-
tin, but has low affinity for laminin or collagen type I,
which is in contrast to the ECM protein-binding capacity
of Ye which preferentially associates with collagen type I
and laminin. This indicates that the uptake region may
modulate the overall affinity to different ECM proteins.
Sequence comparison of YadA<sub>O:9 E40</sub> also revealed addi-
tional aa stretches in the YadA stalk domain, lacking in
some other Ye strains. However, comparison of the Vn-
binding capacity of different Ye and Yps strains shows no
clear indication that this region may also contribute to the
differences in Vn binding, since YadA<sub>O:3 6471/76</sub> has the
same insertion in the stalk region. In contrast to Vn bind-
ing, the interaction with factor H, which was shown to
bind to the stalk region of YadA in Ye and Yps strains,
revealed no differences [20]. This indicates that the pres-
ence or absence of the uptake region modulates affinity
to Vn.

The site of interaction between Mc and Vn was mapped
to the N-terminal residues 30–177 within UspA2 [34].
This region is located in the head domain of UspA2,
which is similar to YadA<sub>O:9 E40</sub>. Our data show that subtle
differences within the YadA protein sequence can signif-
ically influence the protein interaction repertoire of Ye.
The recruitment of such proteins to the surface of Ye may
exert a significant influence on serum resistance and host
cell interaction.

Localization of the Vn-binding domain within the
YadA protein is a crucial step when analyzing the func-
tion of YadA in complement evasion. In contrast to com-
plement regulator factor H or the complement compo-
ment C3, which bind to the stalk domain of YadA [20], we
found that Vn is bound via the YadA head domain. In Ye,
the neutrophil-binding domain is located at the N-termi-
nal part of YadA whereas the collagen-binding domain
is located at the central and C-terminal part of the YadA
head domain [59, 69–72]. The inhibition of Vn binding
with heparin was already shown for Mc and Hi. In both
species, the interaction of Vn with UspA2 or Hsf was as-
signed to HBD-3 [34, 35]. In contrast, for Ye O:9 E40, not
only HBD-3 but also the adjacent N- and C-terminal por-
tions of Vn are decisive for the efficient interaction with
YadA. We conclude that complement evasion of Ye is not
limited to interactions mediated by the stalk domain but
can involve the head domain of YadA, depending on the
strain in question. Furthermore, the uptake region in Ye
O:9 seems to provide a binding domain for Vn which
strongly amplifies the binding of Vn.

Previous studies showed that recruitment of Vn by Mc
or Hi inhibits C5b-9 formation to block pore formation
[27]. However, analyzing the TCC formation in Ye with
purified complement proteins (C5b-6, C7, C8 and C9),
we showed that bound Vn inhibits the deposition of C5b-
9 on the bacterial surface. Consequently, these data show
that Vn bound to the bacterial surface via YadA is func-
tionally active and inhibits the terminal pathway and thus
contributes to complement resistance. Indeed, in vitro
serum killing assays, we showed that Ye O:9 E40 is the
strain that sustains treatment with serum most efficiently
compared to Ye O:8 and Yps YPIII. In contrast, a YadA-
deficient strain of Ye O:9 E40 was susceptible to serum
killing. Thus YadA-mediated binding of Vn in Ye O:9 E40
is decisive for the success of serum treatment in vitro.
The situation is different in Ye O:8 WA-314. This strain is
much more sensitive to serum treatment compared to Ye
O:9. We know that in Ye O:8, serum resistance is medi-
ated by the YadA-dependent recruitment of C3b/iC3b,
factor H and C4BP [21, 33]. As all these factors bind to
YadA and, at least for C4BP, the binding site(s) within
YadA is unknown, there might be competition for bind-
ing sites, and this might lead to the binding of low levels
of Vn. Still, binding of all the other negative regulators of
complement can mediate serum resistance to a certain
extent. A decisive role of YadA for serum resistance of Yps
YPIII is rather unlikely as it has been shown that Yps se-
rum resistance occurs independently of the presence of a
virulence plasmid (that encodes YadA [73]). The known
mechanisms involved in the serum resistance of Yps are
the binding of C4BP and factor H via Ail [74, 75]. Never-
theless, we have shown that Yps also binds Vn via YadA.
We think that in this case the recruitment of Vn has a
function other than mediating serum resistance and

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Mühlenkamp et al.
speculate that it might be involved in, for example, the modulation of host cell targeting [66] and interaction [24].

Consequently, this should also improve the survival of Ye in vivo. Indeed, the short-term infection of Vn-deficient mice with Ye O:9 E40 revealed that Vn protects Ye from being killed by the immune system. A short-term infection of mice was used to avoid (as far as possible) the action of other virulence mechanisms such as those provided by the T3SS. According to ex vivo measurements, the injection of Yops should efficiently show its action at later time points. Therefore, the short-term mouse experiments should predominantly reflect the impact of Vn on complement killing, as the complement system is activated within seconds after infection. Thus, the mouse infection experiments provide evidence that the inhibition of TCC formation by Vn via binding to YadA indeed has biological relevance. These findings clearly demonstrate the importance of Vn binding to the uptake region for the pathogenicity of Ye. However, binding of Vn may also counteract YadA-mediated virulence, which is indicated by the slightly increased bacterial load after infection of Vn-deficient mice with Ye O:9 E40 ΔΔ expressing YadAO:8. We assume that the weak binding of Vn outside of the uptake region might interfere with the binding of other factors to YadA which are critical for YadA as a virulence factor. From an evolutionary point of view, the acquirement of the uptake region converts Vn from a factor protecting against infection to a factor mediating immune evasion.

Although individuals lacking terminal complement components are known to be more susceptible to N. meningitidis [76] but not especially to Ye infections, Vn binding is an important mechanism contributing to the overall serum resistance of Ye. Ye YadA interacts with a multitude of complement regulatory factors (C4bp, C3b, iC3b and factor H) that all contribute to serum resistance of Ye in a true infection situation. These interactions in sum finally determine the success of Ye within the host.

Taken together, our data present a novel mechanism of how YadA mediates immune evasion. By binding the HBD-3 domain of Vn, YadA containing the uptake region mediates the efficient inhibition of TCC formation and thus contributes to complement resistance and better survival of Ye. YadA is a multifunctional protein mediating complement resistance and also adhesion which, in turn, are critical for the subsequent injection of Yops into the host cells via the T3SS. Beyond bacteriolysis mediated by the assembly of the TCC, the even more important effect of Vn may be to modulate the interaction of Ye with immune cells [66]. Further studies will now address how Vn may influence adhesion, invasion and Yop injection during mouse infection.

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References

1 Bottone EJ: Yersinia enterocolitica: overview and epidemiologic correlates. Microbes Infect 1999;1:323–333.
2 Cover TL, Aber RC: Yersinia enterocolitica. N Engl J Med 1989;321:16–24.
3 Isberg RR: Mammalian cell adhesion functions and cellular penetration of enteropathogenic Yersinia species. Mol Microbiol 1989;3:1449–1453.
4 Isberg RR: Determinants for thermoinducible cell binding and plasmid-encoded cellular penetration detected in the absence of the Yersinia pseudotuberculosis invasin protein. Infect Immun 1989;57:1998–2005.
5 Miller VL, Falkow S: Evidence for two genetic loci in Yersinia enterocolitica that can promote invasion of epithelial cells. Infect Immun 1988;56:1242–1248.
6 El Tahir Y, Skurnik M; YadA, the multifaceted Yersinia adhesin. Int J Med Microbiol 2001;291:209–218.
7 Linke D, Riess T, Autenrieth IB, Lupas A, Kempt VA: Trimeric autotransporter adhesins: variable structure, common function. Trends Microbiol 2006;14:264–270.
8 Hoiczky E, Roggenkamp A, Reichenbecher M, Lupas A, Heesemann J: Structure and sequence analysis of Yersinia YadA and Moraxella UspAs reveal a novel class of adhesins. EMBO J 2000;19:5989–5999.
9 Mühlenkamp M, Oberhettinger P, Leo JC, Linke D, Schütz MS: Yersinia adhesin A (YadA) – Beauty and beast. Int J Med Microbiol 2015;305:252–258.
10 Szczesny P, Lupas A: Domain annotation of trimeric autotransporter adhesins – daTAA. Bioinformatics 2008;24:1251–1256.
11 Pepe JC, Wachtel MR, Wagar E, Miller VL: Pathogenesis of defined invasion mutants of Yersinia enterocolitica in a BALB/c mouse model of infection. Infect Immun 1995;63:4837–4848.
Schütz M, Weiss EM, Schindler M, Hallström T, Zipfel PF, Linke D, Autenrieth IB: Trimer stability of YadA is critical for virulence of Yersinia enterocolitica. Infect Immun 2010; 78:2677–2690.

Di Genaro MS, Waidmann M, Kramer U, Hitziger N, Bohn E, Autenrieth IB: Attenuated Yersinia enterocolitica mutant strains exhibit differential virulence in cytokine-deficient mice: implications for the development of novel live carrier vaccines. Infect Immun 2003;71:1894–1812.

Cornelis GR: Yersinia type III secretion: send in the effectors. J Cell Biol 2002;158:418–408.

Viboud GI, Bliska JB: Yersinia outer proteins: role in modulation of host cell signaling responses and pathogenesis. Ann Rev Microbiol 2005;59:69–89.

Hoyer-Hansen G, Behrendt N, Ploug M, Dano K, Preissner KT: The intact urokinase receptor is required for efficient vitronectin binding: receptor cleavage prevents ligand interaction. FEBS Lett 1997;420:79–85.

Eitel J, Dersch P: The YadA protein of Yersinia pseudotuberculosis mediates high-efficiency uptake into human cells under environmental conditions in which invasion is repressed. Infect Immun 2002;70:4880–4891.

Heise T, Dersch P: Identification of a domain in Yersinia virulence factor YadA that is crucial for extracellular matrix-specific cell adhesion and uptake. Proc Natl Acad Sci USA 2006;103:3375–3380.

Biedzka-Sarek M, Jarva H, Hyttiainen H, Meri S, Skurnik M: Characterization of complement factor H binding to Yersinia enterocolitica serotype O3. Infect Immun 2008;76:4100–4109.

Biedzka-Sarek M, Salmenlinna S, Gruber M, Lupas AN, Meri S, Skurnik M: Functional mapping of YadA and Aim-mediated binding of human factor H to Yersinia enterocolitica serotype O3. Infect Immun 2008;76:5016–5027.

Schindler MK, Schütz M, Mülenkamp MC, Rooijakkers SH, Hallström T, Zipfel PF, Autenrieth IB: Yersinia enterocolitica YadA mediates complement evasion by recruitment and inactivation of C3 products. J Immunol 2012;189:4900–4908.

China B, Sory MP, N’Guyen BT, De Bruyere M, Cornelis GR: Role of the YadA protein in prevention of opsonization of Yersinia enterocolitica by C3b molecules. Infect Immun 1995;63:3129–3136.

Preißner KT: Structure and biological role of vitronectin. Annu Rev Cell Biol 1991;7:275–310.

Boyd NA, Bradwell AR, Thompson RA: Quantitation of vitronectin in serum: evaluation of its usefulness in routine clinical practice. J Clin Pathol 1993;46:1042–1045.

Seifert D, Crain K, Wagner NV, Loskutoff DJ: Vitronectin gene expression in vivo. Evidence for extrahepatic synthesis and acute phase regulation. J Biol Chem 1994;269:19836–19842.
YadA-Mediated Interaction of Ye with Vitronectin

DOI: 10.1159/000449200

Strobel E, Heesemann J, Mayer G, Peters J, Müller-Wehrich S, Emmerling P: Bacteriological and serological findings in a further case of transfection-mediated Yersinia enterocolitica sepsis. J Clin Microbiol 2000;38:2788–2790.

Bohin I, Norlander L, Wolf-Watz H: Temperature-inducible outer membrane protein of Yersinia pseudotuberculosis and Yersinia enterocolitica is associated with the virulence plasmid. Infect Immun 1982;37:506–512.

Mollenkrist A, Nordström T, Hollden C, Christensen JI, Forsgren A, Riesbeck K: The Moraxella catarrhalis immunoglobulin D-binding protein MID has conserved sequences and is regulated by a mechanism corresponding to phase variation. J Bacteriol 2003;185:2285–2295.

Nordström T, Blom AM, Tan TT, Forsgren A, Riesbeck K: Ionic binding of C3 to the human pathogen Moraxella catarrhalis is a unique mechanism for combating innate immunity. J Immunol 2005;175:3628–3636.

Singh B, Jalalvand F, Mörgelin M, Zipfel P, Blom AM, Riesbeck K: Haemophilus influenzae protein E recognizes the C-terminal domain of vitronectin and modulates the membrane attack complex. Mol Microbiol 2011;81:80–98.

Edgar RC: MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 2004;32:1792–1797.

Lassmann T, Sonnhammer EL: Kalign – an accurate and fast multiple sequence alignment algorithm. BMC Bioinformatics 2005;6:298.

Hallström T, Singh B, Resman F, Blom AM, Mörgelin M, Riesbeck K: Haemophilus influenzae protein E binds to the extracellular matrix by concurrently interacting with laminin and vitronectin. J Infect Dis 2011;204:1065–1074.

Flügel A, Schulze-Koops H, Heesemann J, Kuhn K, Sorokin L, Burkhart H, von der Mark K, Emmrich F: Interaction of enteropathogenic Yersinia enterocolitica with complex basement membranes and the extracellular matrix proteins collagen type IV, laminin-1 and -2, and nidogen-entactin. J Biol Chem 1994;269:29732–29738.

Nummelin H, Merckel MC, Leo JC, Lankinen H, Skurnik M, Goldman A: The YadA adhesin YadA collagen-binding domain structure is a novel left-handed parallel beta-roll. EMBO J 2004;23:701–711.

Pilipovic A, Phala PS, Van Gelder P, Rosenbusch JP, Koebnik R: Coupling site-directed mutagenesis with high-level expression: large scale production of mutant porins from E. coli. FEMS Microbiol Lett 1998;163:65–72.

Pilz D, Vocke T, Heesemann J, Brade V: Mechanism of YadA-mediated serum resistance of Yersinia enterocolitica serotype O3. Infect Immun 1992;60:189–195.

Biedzka-Sarek M, Venho R, Skurnik M: Role of YadA, Ail, and lipopolysaccharide in serum resistance of Yersinia enterocolitica serotype O:3. Infect Immun 2005;73:2232–2244.

Dersch P, Isberg RR: An immunoglobulin superfamily-like domain unique to the Yersinia pseudotuberculosis invasin protein is required for stimulation of bacterial uptake via integrin receptors. Infect Immun 2000;68:2930–2938.

Deutsche E, Keller B, Siegfried A, Manncke B, Späth T, Köberle M, Drechsler-Hake D, Reber J, Böttcher RT, Autenrieth SE, Autenrieth IB, Bohn E, Schütz M: Role of beta1 integrins and bacterial adhesins for Yop injection into leukocytes in Yersinia enterocolitica systemic mouse infection. Int J Med Microbiol 2016;306:77–88.

Duan R, Liang J, Shi G, Cui Z, Hai R, Wang P, Xiao Y, Li K, Qiu H, Gu W, Du X, Jing H, Wang X: Homology analysis of pathogenic Yersinia species Yersinia enterocolitica, Yersinia pseudotuberculosis, and Yersinia pestis based on multilocus sequence typing. J Clin Microbiol 2014;52:20–29.

Perry RD, Brubaker RR: Vwa+ phenotype of Yersinia enterocolitica. Infect Immun 1983;40:166–171.

Ho DK, Riva R, Kirjavainen V, Jarva H, Ginsstrom E, Blom AM, Skurnik M, Meri S: Functional recruitment of the human complement inhibitor C4BP to Yersinia pseudotuberculosis outer membrane protein Ail. J Immunol 2012;188:4450–4459.

Ho DK, Riva R, Skurnik M, Meri S: The Yersinia pseudotuberculosis outer membrane protein Ail recruits the human complement regulatory protein factor H. J Immunol 2012;189:3593–3599.

Ram S, Lewis LA, Rice PA: Infections of people with complement deficiencies and patients who have undergone splenectomy. Clin Microbiol Rev 2010;23:740–780.