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Yersinia enterocolitica YadA Mediates Complement Evasion by Recruitment and Inactivation of C3 Products

Magnus K. H. Schindler,*† Monika S. Schütz,*‡ Melanie C. Mühlenkamp,* Suzan H. M. Rooijakkers,‡ Teresia Hallström,‡ Peter F. Zipfel,‡§ and Ingo B. Autenrieth*

Yersinia adhesin A (YadA) is a major virulence factor of Yersinia enterocolitica. YadA mediates host cell binding and autoaggregation and protects the pathogen from killing by the complement system. Previous studies demonstrated that YadA is the most important single factor mediating serum resistance of Y. enterocolitica, presumably by binding C4b binding protein (C4BP) and factor H, which are both complement inhibitors. Factor H acts as a cofactor for factor I-mediated cleavage of C3b into the inactive form iC3b and thus prevents formation of inflammatory effector compounds and the terminal complement complex. In this study, we challenged the current direct binding model of factor H to YadA and show that Y. enterocolitica YadA recruits C3b and iC3b directly, without the need of an active complement cascade or additional serum factors. Enhanced binding of C3b does not decrease survival of YadA-expressing Yersinia because C3b becomes readily inactivated by factor H and factor I. Binding of factor H to YadA is greatly reduced in the absence of C3. Experiments using Yersinia lacking YadA or expressing YadA with reduced trimeric stability clearly demonstrate that both the presence and full trimeric stability of YadA are essential for complement resistance. A novel mechanism of factor H binding is presented in which YadA exploits recruitment of C3b or iC3b to attract large amounts of factor H. As a consequence, formation of the terminal complement complex is limited and bacterial survival is enhanced. These findings add a new aspect of how Y. enterocolitica effectively evades the host complement system.


Yersinia enterocolitica is a facultative anaerobic pleomorphic gram-negative rod that belongs to the family Enterobacteriaceae. Infections are caused by ingestion of contaminated food or drinking water and can cause severe diarrhea, enterocolitis, and mesenteric lymphadenitis (1, 2). The pathogenicity of Y. enterocolitica is associated with the presence of a 70-kb virulence plasmid (pYY) that encodes a type three secretion system, translocated effector proteins, and the trimeric autotransporter Yersinia adhesin A (YadA) (3).

YadA has manifold functions associated with the pathogenicity of Y. enterocolitica. YadA mediates adhesion to host cells, promotes autoaggregation, and protects from complement-mediated killing (4). Y. enterocolitica virulence in vivo is associated with the presence of YadA, and it is known that the trimeric stability of YadA correlates with pathogenicity in a mouse model of infection (5). Being the prototype of trimeric autotransporter adhesins, YadA consists of three 50-kDa monomers that trimerize upon insertion into the bacterial outer membrane (6). The exchange of a highly conserved glycine within the C-terminal β-barrel membrane anchor domain by amino acids with larger side chain size (such as threonine, asparagine, histidine) reduces YadA trimer stability and eventually impairs passenger domain translocation. YadA mutants that carried the small amino acids Alanine or Serine instead of Glycine at position 389 resembled wild type expression levels and phenotype except that they were sensitive to complement-dependent killing and displayed reduced trimer stability in Western blot (5, 7).

Complement resistance is a common mechanism of human and animal pathogens to establish an infection in an immune competent host. Several pathogens have evolved a plethora of mechanisms that enable their evasion from complement-mediated killing (8–11). The complement system is an important arm of the innate immune system. It consists of >30 soluble and membrane-bound proteins that initiate a cascade of catalytic cleavage reactions upon contact with foreign surfaces and structures. Three pathways of complement activation are distinguished: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP). All three pathways are activated by recognition of non–self structures on target surface (e.g., bacterial sugars) and result in the formation of C3 convertases, which catalyze an important step in the complement cascade: the cleavage of C3 into C3b. The C3 protein contains a reactive thioester group that is hidden within the C3 molecule (12). Upon cleavage of C3, the thioester group becomes exposed and reacts with the target surface to deposit covalently bound C3b. Most of the generated C3b never attaches to the bacterial surface because its thioester reacts with water forming fluid phase C3b (C3bH2O), which is rapidly inactivated by host regulators.

Surface-bound C3b molecules initiate a powerful amplification loop to deposit more C3b on the surface. Bacteria covered with C3b are effectively taken up by phagocytes, because C3b and its
cleavage product iC3b are recognized by phagocyte receptors (CR1, CR3, CR4) (13). Finally, the binding of C3b to a C3 convertase changes this enzyme into a C5 convertase. This step is the first toward the formation of the multiprotein complex termed terminal complement complex (TCC; also known as the membrane attack complex). These complexes are pores with a diameter of up to 10 nm that are stably inserted into the bacterial outer membrane; they induce a loss of membrane integrity and lysis of the pathogen (14).

To prevent complement-mediated self-destruction of host cells, the entire complement system has to be tightly regulated at several levels of the cascade. Key regulators of the complement cascade are C4BP, factor H, factor H-like protein 1 (FHL1) and factor H-related proteins (CFHR). C4BP accelerates the decay of the classical and lectin C3 convertase and mediates degradation of C4b and also of C3b (15, 16). Factor H, FHL-1, and CFHR5 accelerate the decay of the inherently instable C3 convertases and act as cofactors for factor I-mediated cleavage of C3b into iC3b and C3d (17, 18). A number of pathogens express specific surface molecules to recruit complement regulators like C4BP or factor H to the bacterial surface. As a result, they can protect themselves from complement attack (9, 10, 19).

The role of YadA for interaction with complement factors has been investigated over the last few years. Skurnik and coworkers compared the roles of Yersinia proteins YadA, Ail, and the LPS O-antigen for complement resistance and concluded that YadA is the most potent individual factor mediating resistance against complement-mediated killing. In addition, they demonstrated that surface-bound C3b levels do not correlate with a specific resistance phenotype, but that most strains resistant to complement-mediated lysis accumulated iC3b on their surface (20–23). It was shown that the stalk domain of the YadA trimer is involved in preventing TCC killing (24). As a follow-up, Biedzka-Sarek et al. (21) generated a large set of YadA stalk and neck deletion mutants to identify the exact binding domain for factor H. Their data revealed that factor H targets several conformations of YadA and discontinuous sites of the stalk. However, the consequences of deletions in the coiled-coil stalk of YadA are not easily interpreted as distortions of the stalk may influence the structure of distant parts of YadA.

In this study, we show that stable YadA trimer of Y. enterocolitica recruits C3bH2O and iC3b to the bacterial surface. Binding of C3b then supports further recruitment of factor H from serum, proteins, and Abs which assists in the subsequent factor I-mediated conversion of C3bH into C3bi and iC3b to the bacterial surface. As a result, they can protect themselves from complement attack (9, 10, 19).

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Materials and Methods

Bacterial strains and culture conditions

Y. enterocolitica YadA0 (25) was grown overnight in Luria–Bertani broth at 27°C supplemented with nalidixic acid (10 μg/ml) and kanamycin (50 μg/ml). Y. enterocolitica strains YadAw, G389A, and G389S (5) were grown in the presence of nalidixic acid, kanamycin, and spectinomycin (50 μg/ml). A 1:20 dilution of the overnight Y. enterocolitica culture was incubated for 1 h at 27°C and an additional 2 h at 37°C to induce expression of YadA. The bacteria were washed once with PBS (Invitrogen), and the OD at 600 nm was determined.

Sera, proteins, and Abs

Pooled normal human serum (NHS) from healthy donors was purchased from the transfusion medicine department of the university hospital Tübingen. C6-depleted serum (ΔC6S) and C3-depleted serum (ΔC3S) was purchased from Complement Technology. The sera were aliquoted and stored at −80°C. Treatment at 56°C for 30 min results in inactivation of the complement cascade. The efficient depletion of ΔC6 and ΔC3 sera was verified by specific antibody application and quantification and testing their ability to kill bacteria. Heat-treated sera were used in several experiments and were treated heat-inactivated serum (HIS), HILAC3S, or HILAC6S throughout the article. Purified proteins C3, C3b, and iC3b were purchased from Complement Technology. As a result of unspecific background staining of Yersinia, factor H ( goat–anti-human; Complement Technology), and C5b-9 (rabbit–anti-human; Complement Technology), these Abs were preabsorbed before usage. Therefore, 1 × 10^7 Y. enterocolitica YadAw were incubated in 1 ml of 50-fold diluted factor H or SC5b-9 Ab at 4°C for 1 h. Bacteria were spun down, and the supernatant was used for detection of factor H at a final dilution of 1:300 or 1:100 for detection of complement flow cytometry, respectively. For Western blot analysis, the factor H Ab was used at a final dilution of 1:1000. Peroxidase conjugated F(ab)2 C3 Ab (goat–anti-human; 1:300) and FITC-conjugated F(ab)2 C3 Ab (goat–anti-human; 1:100) were obtained from Protons Immunoresearch. The C3 Ab is described to recognize the C3 α-chain (∼120 kDa) and β-chain (∼75 kDa), the C3b α’-chain (∼100 kDa), and the iC3b α’-chain fragments (∼62 and ∼40 kDa). Purified IgG fraction was prepared from polyclonal rabbit YadA antiserum (dilution 1:2000) that was collected from immunized rabbits (7). Peroxidase-conjugated secondary anti-goat Ab (1:5000) was obtained from Santa Cruz Biotechnology. DyLight488-conjugated rabbit–anti-goat Ab (1:400) and APC-conjugated donkey–anti-rabbit Ab (1:200) were purchased from Jackson ImmunoResearch.

Sample preparation for Western blot analysis

Bacterial pellets were lysed in SDS sample buffer (1 × 10^7 Y. enterocolitica cells were loaded per lane) and incubated for 5 min at 95°C prior to loading if not indicated otherwise.

Western blot analysis

After SDS-PAGE proteins were transferred onto nitrocellulose membranes, the membranes were blocked for 1 h with PBS-5% milk powder at room temperature. For the detection of C3 peroxidase conjugated goat F(ab)_2 anti-human C3 Ab was used. Factor H was detected by a polyclonal rabbit factor H antiserum and a peroxidase conjugated secondary anti-goat Ab. Detection of bound Abs was performed using an enhanced chemiluminescence detection kit (Amersham Biosciences).

Complement deposition investigated by Western blot

To detect the binding of the complement factor C3 and C3 products Y. enterocolitica YadA0, Y. enterocolitica YadAw, Y. enterocolitica G389A, and Y. enterocolitica G389S (1 × 10^7) were incubated at 37°C in 200 μl of 50% HIS diluted with PBS. After 5 and 20 min, 100 μl of the samples were removed and bacteria were separated from the supernatant by centrifugation (12,000rpm for 1 min). Pellets were washed twice with PBS 1% BSA and once with PBS. As an internal Ab specificity control, one sample of each bacterial strain was incubated in PBS only. To analyze the binding of purified C3b to Y. enterocolitica YadAw, Y. enterocolitica YadAwt, Y. enterocolitica G389A, and Y. enterocolitica G389S (5 × 10^9) bacteria were incubated at 37°C in 100 μl of 40-μg/ml C3b diluted in PBS for 30 min. Afterward, the bacteria were washed two times in PBS 1% BSA and once in PBS. Finally, bacterial pellets were transferred into a new tube and resuspended in sample buffer and suspected to SDS-PAGE and Western blot.

Complement deposition analyzed by flow cytometry

Binding of serum-derived C3 was investigated by incubation of Y. enterocolitica YadA0, Y. enterocolitica YadAw, Y. enterocolitica G389A, and Y. enterocolitica G389S (1 × 10^7) at 37°C in 100 μl NHS (20%, 10%, 5%, 2.5%, 1.25%) or HIS diluted with PBS containing Mg^{2+} and Ca^{2+} (5%, 10%, 2.5%, 1.25%) for 30 min. Binding of purified proteins was analyzed by incubation of bacteria in 100 μl of complement proteins C3, C3b, or iC3b at several concentrations (20, 10, 5 μg/ml). As a negative control, each strain was treated with PBS only. After a washing step, the samples were incubated with FITC-conjugated goat F(ab)_2, anti-human C3 Ab. Following the Ab incubation, samples were washed once and finally fixed in 1% PFA in PBS for flow cytometry analysis (BD Biosciences LSRII). To detect the binding of serum-derived complement regulator factor H, Y. enterocolitica YadA0, Y. enterocolitica YadAw, Y. enterocolitica G389A, and Y. enterocolitica G389S (1 × 10^7) were incubated at 37°C in 100 μl HIS or HILAC3S (20%, 10%, 5%) diluted with PBS for 30 min. The negative controls were samples incubated in PBS only. Samples were washed and then incubated with polyclonal goat anti-human factor H Ab.
After a washing step, the samples were incubated with DyLight-888-conjugated rabbit-anti-goat Ab. After a washing step, all samples were resuspended and fixed in 1% PFA in PBS and then analyzed in a flow cytometer. To test how purified proteins C3b/iC3b might influence binding of purified factor H, bacteria were preincubated with 300 µg/ml purified C3b or iC3b for 30 min at room temperature. After a washing step, bacteria were incubated with 100 µg/ml purified factor H for 30 min at room temperature. Bacteria were washed again, and factor H that had bound to the bacterial surface was subsequently stained as described above for detection by flow cytometry.

**Serum killing assay**

*Y. enterocolitica* were grown to exponential phase at 37°C, washed once with PBS and OD600 was determined; 3 x 10^8 bacteria were incubated in 25% ΔC5S for 45 min at 37°C. After a washing step, the bacteria were transferred into a new tube and were incubated in ΔC3S for 45 min at 37°C. As controls, bacteria were incubated in heat-inactivated ΔC6S (HIΔC6S) followed by heat-inactivated ΔC3S (HIΔC3S). Complement activity was stopped by placing the samples on ice and by the addition of 1 volume of brain heart infusion medium. Serial dilutions (10^2–10^-2) of the bacteria were plated on selective agar and incubated at 27°C for 24 h. The serum bactericidal effect was calculated as the survival percentage, taking the bacterial counts obtained with bacteria incubated in HIΔC6S and HIΔC3S as 100%. The killing experiment was repeated for each strain at least three times, starting from independent cultures.

**TCC formation investigated by flow cytometry**

Formation of the TCC was investigated after incubation of *Y. enterocolitica* YadA0, *Y. enterocolitica* YadAwt, (5 x 10^7) for 5, 10, and 15 min at 37°C in 100 µl of 25% or 50% NHS diluted with PBS. As a negative control, each strain was treated with PBS only. After a washing step, the samples were incubated with monoclonal rabbit-anti-human SC5b-9 Ab. The samples were washed once and incubated with APC-conjugated goat-anti-rabbit Ab. After a washing step, all samples were resuspended and fixed in 1% PFA in PBS and then analyzed in a flow cytometer (BD Biosciences LSR II).

**Results**

**YadA mediates binding of C3 products to *Y. enterocolitica***

*Y. enterocolitica* strains expressing YadA are resistant to complement-dependent killing (26–28), and YadA was shown to bind human complement inhibitors C4BP and factor H (20–23). However, the aim of this study was to find out how YadA controls C3b deposition and whether YadA trimer stability may influence this process. For this purpose, we used *Y. enterocolitica* strains expressing wild type YadA (YadAwt), YadA with reduced trimer stability (Yada G389A and YadA G389S) (5, 7), or a strain deficient for YadA (YadA0). The strains were cultivated at 37°C to enforce YadA expression and surface levels of YadA were analyzed using specific Abs and flow cytometry (Fig. 1A). As expected, YadA0 strains did not express YadA while the YadA surface levels of *Y. enterocolitica* G389A are comparable to those of *Y. enterocolitica* YadAwt. *Y. enterocolitica* YadA G389S exhibits slightly reduced YadA surface levels (5). To study the role of YadA in complement modulation, *Y. enterocolitica* strains were incubated with NHS allowing complement activation on the surface, after which surface-bound C3 products was detected using a polyclonal Ab against C3. In NHS, we found no significantly different levels of surface-bound C3 products on all tested strains (Fig. 1B). As a control, we incubated the strains with HIS in which heat-labile complement factors like factor B and C2 are rendered inactive (29). Because these factors are essential for convertase formation, C3 activation and subsequent covalent C3b deposition will not occur in the presence of HIS. Surprisingly, YadA expressing *Y. enterocolitica* strains showed high levels of surface-bound C3 products after incubation with HIS, whereas no C3 products were found on the surface of a YadA-negative strain (Fig. 1C, 1D). We observed that this binding was dose dependent and that trimer stability was important for binding, because binding was lower for *Y. enterocolitica* YadA G389A and YadA G389S. Taken together, YadA expression fosters the binding of fluid-phase C3 products from HIS to *Y. enterocolitica*, and trimeric stability of YadA is associated with increased binding.

**YadA binds to C3b and iC3b**

To unravel which form of fluid-phase C3 (C3b, C3f, iC3b) in HIS is recruited by *Y. enterocolitica* YadA, we analyzed surface-bound C3 by Western blotting (Fig. 2). To this end, *Y. enterocolitica* YadAwt, *Y. enterocolitica* G389A, *Y. enterocolitica* G389S, and *Y. enterocolitica* YadA0 were incubated in HIS and washed, and the proteins of whole cell lysates were separated by SDS-PAGE.

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**FIGURE 1.** Binding of C3 products to *Y. enterocolitica*. *Y. enterocolitica* strains were grown for 2 h at 37°C to induce YadA expression. (A) Histogram overlays of the YadA surface levels of all bacterial strains used for the C3 binding assay. (B–C) Aliquots of the same bacterial cultures were used for incubations with different concentrations of human serum. Bacteria were washed and C3 fragments bound to the bacterial surface were stained with Abs specific for C3 (detecting all C3 products) and analyzed by flow cytometry. *Yersinia* strains expressing wild type YadA (YadAwt), the point mutated versions (Yada G389A or YadA G389S) or a strain devoid of YadA (YadA0). (B) Bacteria were incubated with NHS containing active complement. (C) Bacteria were incubated with heat-inactivated serum (HIS) in which the complement activation cascade is not active. (D) Representative histogram overlays of (C) at 10% HIS. Data are means of at least three individual experiments (B, C), or a representative experiment out of three is shown (A, D).
bound proteins were analyzed with flow cytometry. Data are means of at least three individual experiments (C, open triangles), or deficient for YadA YadA0 (open diamond) were incubated with the indicated concentrations of purified C3, C3b, or iC3b. Afterward, the three is shown (B chain of C3b or the 40-kDa chain of iC3b. (C antiserum reacting with the C3a chain of C3b.) Y. enterocolitica YadAwt (incubated in HIS, washed and then resuspended in Laemmli buffer to obtain whole cell lysates containing all surface-bound complement proteins), and subjected to SDS-PAGE and Western blotting. From left to right, the following samples were loaded: heat-inactivated serum (HIS), Y. enterocolitica YadAwt. Furthermore, we studied binding of purified C3d and YadA0 in a dose-dependent matter. The above data indicate that YadA directly binds C3b; therefore, we were prompted to investigate whether YadA itself could catalyze the cleavage of C3b into iC3b. This would be beneficial for the bacterium because conversion of C3b into iC3b would stop both the amplification loop and downstream formation of the TCC. To test this hypothesis, bacteria grown as described were incubated with purified C3b. Afterward, samples were subjected to Western blot analysis with C3-specific antiserum recognizing both the α- and β-chains of C3b and iC3b (Fig. C). C. enterocolitica strains expressing YadAwt (filled squares), YadA G389A (filled triangles), YadA G389S (open triangles), or deficient for YadA YadA0 (open diamond) were incubated with the indicated concentrations of purified C3b, C3, or iC3b. Afterward, the bound proteins were analyzed with flow cytometry. Data are means of at least three individual experiments (C), or one representative experiment out of three is shown (B).

The above data indicate that YadA does not cleave C3b into iC3b. The data suggest that YadA specifically binds C3b and iC3b, but not C3.
factor H to *Y. enterocolitica* and may result in formation of a tripartite YadA-factor H-C3b complex. To this end, factor H binding from HIS and HIΔC3S to *Y. enterocolitica* was compared using flow cytometry. All strains (*Y. enterocolitica* YadAwt, *Y. enterocolitica* G389A, *Y. enterocolitica* G389S, and *Y. enterocolitica* YadA0) bound factor H dose dependently from 5%, 2%, and 1% HIS (Fig. 4A). There was no significant difference in the overall amount of surface-associated factor H at one discrete serum concentration in *Y. enterocolitica* YadAwt compared with *Y. enterocolitica* G389A and *Y. enterocolitica* G389S. Only *Y. enterocolitica* YadA0 showed significantly less factor H binding.

Performing the same assay with HIΔC3S, binding of factor H was significantly reduced even at the highest serum concentration (i.e., 5%) compared with full serum and was even lower than the levels detected with the lowest serum concentration of HIS (i.e., 1%). To rule out that this was due to reduced levels of factor H in the HIΔC3S, a Western blot (Fig. 4B) with the same Ab that was used for flow cytometry was performed. The blot clearly demonstrates that the total amount of factor H (and also the related CFHR-1) present in HIS and HIΔC3S is comparable. These data suggest that factor H binding to YadA depends on the presence of C3 products. To test this hypothesis, we analyzed binding of purified factor H to *Y. enterocolitica* YadAwt that were preincubated with purified C3b or iC3b compared with bacteria that were treated with buffer alone before incubation with purified factor H. We found that preincubation with iC3b could enhance binding of factor H (Fig. 4C). The addition of C3b did not increase the binding of factor H, which is in concordance with our previous findings suggesting that YadA binds more strongly to iC3b than C3b.

Inactivation of C3b by *Y. enterocolitica* YadAwt results in diminished activation of the terminal pathway

To decipher whether the YadA-mediated capture and inactivation of C3b interfered with the terminal complement pathway, TCC formation on the bacterial surface was quantified by flow cytometry (Fig. 5). Bacteria were incubated with serum and stained with an Ab recognizing a neoepitope of C9 only present in the polymeric C5b-9 complex. These data show that the formation of the TCC on the surface of *Y. enterocolitica* YadAwt is significantly lower compared with *Y. enterocolitica* YadA0. This finding is consistent with the higher susceptibility of *Y. enterocolitica* YadA0 to serum killing. Finally, we developed an assay to determine whether YadA recruitment of C3b/iC3b actually contributes to inhibition of serum killing (Fig. 6). Bacteria were first incubated in human serum de-
sufficient for C6 (ΔC6S). Here, binding of C3 products may occur in the same way as in NHS, but the TCC cannot be assembled because of the lack of C6. After a washing step, bacteria were transferred to human serum deficient for C3 (ΔC3S). As a result, only C3 molecules that were bound in the first incubation step can contribute to TCC formation. TCC-mediated killing of the bacteria was monitored by plating serial dilutions (Fig. 6A). Neither of the individually depleted sera induced bacterial lysis and thus both sera were suitable for our purpose. However, sequential incubation of bacteria in ΔC6S followed by ΔC3S serum triggered bacterial lysis (Fig. 6B).

Y. enterocolitica YadAwt, the strain that recruited the greatest amount of C3b/iC3b (Fig. 2B), was most resistant in this assay. Y. enterocolitica YadA G389A, G389S, and Y. enterocolitica YadA0 exhibited increased susceptibility to serum killing. To conclude, this experiment suggests that YadA-dependent recruitment of C3b/iC3b is important for serum resistance. In addition, these data show that YadA trimer stability is important for efficient complement inactivation.

Discussion

YadA, a major virulence determinant of Y. enterocolitica, was previously shown to be the most important single factor mediating complement resistance. Our study demonstrates a novel mechanism for complement resistance mediated by YadA (schematic overview in Fig. 7), amending the known mechanism through direct binding of factor H. Instead of directly binding to factor H, we show that YadA recruits C3b or iC3b to attract large amounts of factor H. Furthermore, we show that trimeric stability of YadA is essential for complement resistance.

In our experiments using purified C3 products, we observed that YadA specifically binds C3b and iC3b but not C3 or C3d. This differential binding can be explained by the different conformations of these molecules (Fig. 8). When native C3 is cleaved into C3b, its thioester-containing domain (C3d) becomes exposed to react with the bacterial surface. This is accompanied by a large conformational change in the C3 molecule, also exposing new binding sites for complement receptors and other complement proteins like factor B. The fact that we observe binding of purified C3b and iC3b to YadA indicates that YadA noncovalently associates with these proteins. During the purification process of C3b/iC3b, the thioester bond reacts with water and is therefore no longer active. Because YadA does not bind C3, we believe that it specifically binds the C3b/iC3b conformation. Owing to the difficulties to purify trimeric YadA in its native structure, it will be challenging to map the decisive region for interaction with C3b/iC3b. Even minor changes in the protein sequence will disrupt the trimer or tertiary protein structure.

Analyzing the deposition of C3 products with our laboratory strain Y. enterocolitica WA-314 serotype O:8, we found that C3 binding upon incubation in active NHS was independent from YadA expression and stability. This is in contrast to previous findings in which less C3b deposition was observed in a YadA-positive strain compared with YadA-negative bacteria (26). Our finding might be contributable to the usage of diverse serotypes.
(26, 27, 30), individual serum activity, and strain-specific expression levels of factors involved in serum resistance (YadA, Ail LPS), and also to subtle serotype-specific differences in the YadA protein sequence. Recently, Ho et al. (31) showed that the outer membrane protein Ail of *Yersinia pseudotuberculosis* binds to the complement regulator C4BP regardless of serotype. However, serum resistance was also independent of YadA in two strains of *Y. pseudotuberculosis*, whereas in another strain serum resistance involved factors in addition to YadA and Ail. These data demonstrate that a complex interplay between bacterial proteins involved in serum resistance defines the resistance phenotype and that YadA in contrast to Ail in some cases seems dispensable for serum resistance.

Our finding that YadA binds to C3b was initially triggered by experiments in which we incubated YadA-expressing bacteria with HIS. The purpose of heat-treatment of serum at 56˚C is to inactivate the most heat-labile complement components being C2, C1, and factor B (29, 32). As a consequence, the complement cascade cannot be initiated and C3 will not be cleaved into C3b. Although we expected that HIS only has C3 and none of its activation products, we observed that our HIS sample contained high amounts of C3b and iC3b (Fig. 2B). These C3 products were probably generated during heat inactivation. Of course, bacterial pathogens will not encounter HIS in vivo, but the finding is still physiologically relevant because C3 products such as C3b and iC3b are continuously generated during complement activation on the bacterial surface. The advantage of using heat-inactivated serum is that it allowed us to examine the recruitment of C3 products in the absence of covalent C3b deposition.

Our study challenges the current model that YadA directly binds to factor H. With YadA being expressed on the bacterial surface, we did not observe direct binding of factor H to YadA. Factor H is known to bind to C3b and iC3b, but not to C3 (33), and it therefore seems likely that it is bound to YadA via C3b/iC3b. Unfortunately,
it was not possible to restore the binding of factor H by reconstituting the C3-depleted serum with purified C3, C3b, or iC3b. The conformation of the proteins is possibly altered during the purification process in a way that abrogates efficient interaction with factor H. In addition, our finding raises the question about the biologic relevance of enhanced factor H binding. It has been shown that factor H, which binds directly to YadA, acts as a cofactor for factor I-mediated degradation of C3b. However, these data also revealed that only a very small amount of C3b present in the experiments was degraded into iC3b (5, 20, 21). The amount of C3b typically used in these experiments was considerably lower than that of C3 in human serum. Thus, it can be speculated that, especially in vivo, the amount of factor H that binds directly to YadA is not sufficient to degrade all bound C3b-molecules and Y. enterocolitica rely on additional factor H binding mediated by C3b/iC3b.

Active binding and inactivation of C3b is a two-edged sword. On the one hand, the rapid inactivation of C3b on the bacterial surface is advantageous for Y. enterocolitica. The binding and inactivation of C3b by factor H and factor I might lower the local amount of C3 in the fluid phase and may form a kind of “protective shell” that sterically hinders further deposition of C3b. In addition, iC3b stabilizes the complex of C3b and factor H (34), which in turn enhances inactivation of C3b into iC3b. Taken together, these effects might inhibit the complement cascade at the stage of C3 and prevent later steps of the complement cascade. Indeed, a reduced number of TCC on Y. enterocolitica YadAwt compared with YadA-deficient bacteria could be observed as it was described earlier (27). On the other hand, iC3b is a very potent opsonin, with high affinity and avidity to complement receptors expressed on phagocytes, induces a strong oxidative burst, and is thus detrimental for the pathogen (35). However, Y. enterocolitica encodes for a type 3 secretion system that injects host cells with several effector proteins that induce the rearrangement of the host cell cytoskeleton and thus prevent the internalization of Y. enterocolitica (36). One could envision a scenario in which Y. enterocolitica exploits opsonins to tightly adhere to target cells. Because adherence to host cells is the prerequisite for efficient effector injection, it might be enhanced by surface deposited C3b/iC3b and its interaction with complement receptors CR1, 3, and 4 (37). However, the interaction of Y. enterocolitica with its target cells is a highly complex and multifactorial process, and further investigation has to reveal the effects of complement interaction on the pathogenesis of Y. enterocolitica.

The mechanism presented in this article for YadA shows strong similarities with the Staphylococcus aureus protein Bti that forms a tripartite complex with C3b and the complement regulator factor H. Moraxella catharralis, which expresses the trimeric autotransporter adhesin UspA1 and 2 (that share structural homologies to YadA), can noncovalently attach to C3 and methylamine-treated C3 (this does not offer a thioester bond for attachment, but otherwise resembles C3b) (38).

Our studies strongly indicated that the trimeric stability of YadA is important for its interaction with C3b/iC3b. The significant differences observed in C3b/iC3b binding between Y. enterocolitica YadAwt and Y. enterocolitica G389A and Y. enterocolitica G389S might be explained by the fact that even point mutations within the membrane-embedded C-terminal β-barrel-domain can lead to distortions at very distant parts of the coiled-coil stalk region. These distortions might then adversely affect the interplay between YadA and complement regulatory factors. Indeed, it has been reported earlier that the exchange of the entire YadA membrane anchor domain against the C-terminal domains of other trimeric autotransporter adhesin family members such as EibA, Hia, or UspA1 (39), or small deletions within the YadA stalk domain (21) significantly reduce the serum resistance of Y. enterocolitica.

In the current study, we demonstrated that Y. enterocolitica recruits C3b/iC3b via YadA to attract large amounts of factor H and by this mechanism significantly contributes to Y. enterocolitica complement resistance. Our work emphasizes the importance of YadA as a pathogenicity factor that does not only mediate adherence but also efficient immune evasion. As a result, YadA is an attractive target for the development of anti-infectives or the creation of vaccines. Given that YadA represents a large family of proteins that share common structural characteristics, it remains to be elucidated whether our findings are assignable to other pathogenic species. Future studies might succeed in mapping the exact interaction sites of YadA and C3b/iC3b to enable interference with this interaction. In vivo infection models will then reveal the physiologic relevance of YadA-mediated recruitment of C3b/iC3b.

Disclosures
The authors have no financial conflicts of interest.

References


