Functional Mapping of YadA- and Ail-Mediated Binding of Human Factor H to *Yersinia enterocolitica* Serotype O:3[∇]

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***Yersinia enterocolitica* is an enteric pathogen that exploits diverse means to survive in the human host. Upon *Y. enterocolitica* entry into the human host, bacteria sense and respond to variety of signals, one of which is the temperature. Temperature in particular has a profound impact on *Y. enterocolitica* gene expression, as most of its virulence factors are expressed exclusively at 37°C. These include two outer membrane proteins, YadA and Ail, that function as adhesins and complement resistance (CR) factors. Both YadA and Ail bind the functionally active complement alternative pathway regulator factor H (FH). In this study, we characterized regions on both proteins involved in CR and the interaction with FH. Twenty-eight mutants having short (7 to 41 amino acids) internal deletions within the neck and stalk of YadA and two complement-sensitive site-directed Ail mutants were constructed to map the CR and FH binding regions of YadA and Ail. Functional analysis of the YadA mutants revealed that the stalk of YadA is required for both CR and FH binding and that FH appears to target several conformational and discontinuous sites of the YadA stalk. On the other hand, the complement-sensitive Ail mutants were not affected in FH binding. Our results also suggested that Ail- and YadA-mediated CR does not depend solely on FH binding.**

The complement system is the first line of immune defense against invading pathogens that directly activate the lectin pathway or the alternative pathway (AP) cascades in the human host. To survive, the pathogens have developed strategies to prevent deleterious consequences of complement activation. One of these strategies involves the acquisition of the host AP regulator factor H (FH). FH consists of 20 repetitive units, named short consensus repeats (SCRs), of ca. 60 amino acids each (46). The binding of FH is beneficial for microbes, as FH acts as a cofactor for the factor I (FI)-mediated cleavage of C3b, interferes with the association of factor B with C3b, and contributes to the dissociation of preformed AP C3 convertase C3bBb (23, 38, 55). Several pathogens were previously reported to take advantage of FH protective properties, including *Streptococcus pyogenes* (8, 18), group B streptococci (1), *Borrelia* sp. (16, 45), and *Neisseria gonorrhoeae* (35, 42). Also, *Yersinia enterocolitica*, an enteropathogen resistant to complement (2, 15, 26, 27, 39, 41, 48), has been shown to bind FH from human serum (3, 9, 44).

The *Y. enterocolitica* outer membrane proteins YadA and Ail confer serum resistance (4, 5, 9, 40, 52) and mediate the binding of FH and the classical and lectin pathway inhibitor C4b-binding protein (C4bp) to bacteria (3, 21). YadA functions as the major FH and C4bp receptor, while Ail can bind only the regulators when not blocked by the lipopolysaccharide O antigen and outer core (3, 21). Furthermore, our results showed that YadA appears to bind throughout the entire polypeptide chain of FH, while Ail targets SCRs 6 and 7. Both YadA-bound FH and Ail-bound FH, however, were found fully functional as cofactors in the FI-mediated cleavage of C3b (3).

YadA is encoded on a 70-kb virulence plasmid (pYV) (14, 53, 57) and expressed exclusively at 37°C (24, 50). YadA is a homotrimeric autotransporter protein with a monomer size of about 44 kDa that forms a lollipop-shaped structure on the bacterial surface (see Fig. 2A). From the N terminus to the C terminus, YadA contains a head, a neck, and a coiled-coil stalk domain, followed by a translocation and membrane-anchoring unit (17, 22). It was previously suggested that YadA serum resistance determinants are located within the stalk (43).

The membrane anchor of YadA is a 12-stranded β-barrel with the pore closed by the C-terminal part of the stalk, a left-handed coiled-coil segment of four heptads (residues 368 to 395 of the *Y. enterocolitica* O:3 YadA sequence [GenBank accession no. CAA32086]) (22). The stalk continues into a right-handed coiled coil (residues 214 to 367) formed of nine 15-residue repeats (pentadecads) and a 19-residue segment separating the right- and left-handed parts (17, 22). The neck region (residues 192 to 213) comprises three safety pin-like elements (36) and connects the stalk to the head domain (residues 26 to 191). The head domain is a tight cylindrical trimeric structure formed by left-handed β-rolls (36); it binds to extracellular matrix proteins and mediates bacterial autoagglutination (reviewed in reference 11). The neck and head are dispensable for the YadA-mediated complement resistance (CR) (43).

Ail is a 17-kDa protein encoded chromosomally (33, 34). It belongs to a family of proteins predicted to form eight outer membrane-spanning amphipatic β-strands and four short ex-

TABLE 1. Bacteria and plasmids used in this work

Bacterial strain or plasmid	Description	Source or reference
Strains		
Y. enterocolitica		
6471/76-c (YeO3-c)	Virulence plasmid cured derivative of YeO3	47
YeO3-c-Ail	Δ*ail*::Km-GenBlock; derivative of YeO3-c; Km^r	4
YeO3-c-Ail-OCR	Spontaneous OC mutant derivative of YeO3-c-Ail-R; Km^r	4
E. coli		
JM109	*reaA1 Δlac-pro endA1 gyrA96 thi-1 hsdR17 supE44 relA1* F′ *traD36 proAB*^+ *lacI*^q*ZΔM15*	56
DH10B	F^− *mcrA Δ(mrr-hsd* RMS-*mcrBC*), ϕ80*lacZΔM15 ΔlacX74 deoR recA1 endA1 araΔ139Δ(ara leu)7697 galU galK* λ^− *rpsL nupG* λ^− *tonA*	Life Technologies
HB101/pRK2013	Triparental conjugation helper strain; Km^r	12
Plasmids		
pYMS4505	*yadA* gene of YeO3 cloned as a 1,735-bp PCR fragment (nucleotides 478–2213)^a into the BamHI site of pTM100; Clm^r; the *yadA* gene is transcribed by the constitutive *tet* promoter of pTM100	10
pYMS4505:ΔNECK	Deletion of bp 1194–1271 of the *yadA* gene in pYMS 4505; expresses YadA with a 26-aa deletion (aa 193–218)	This work
pYMS4505:A	Deletion of bp 1257–1301 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 214–228)	This work
pYMS4505:S	Deletion of bp 1281–1325 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 222–236)	This work
pYMS4505:Δ#1	Deletion of bp 1311–1355 of the *yadA* gene in pYMS 4505; expresses YadA with a 16-aa deletion (aa 231–246)	This work
pYMS4505:B	Deletion of bp 1347–1391 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 244–258)	This work
pYMS4505:Δ#2	Deletion of bp 1356–1403 of the *yadA* gene in pYMS 4505; expresses YadA with a 16-aa deletion (aa 247–262)	This work
pYMS4505:C	Deletion of bp 1359–1403 of the *yadA* gene in pYMS 4505; does not express YadA due to a frameshift mutation introduced during construction	This work
pYMS4505:D	Deletion of bp 1371–1415 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 252–266)	This work
pYMS4505:E	Deletion of bp 1383–1427 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 256–270)	This work
pYMS4505:Δ#3+6	Deletion of bp 1398–1448 of the *yadA* gene in pYMS 4505; expresses YadA with a 17-aa deletion (aa 261–277)	This work
pYMS4505:F	Deletion of bp 1392–1436 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 259–273)	This work
pYMS4505:Δ#3	Deletion of bp 1404–1448 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 263–277)	This work
pYMS4505:H	Deletion of bp 1416–1460 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 267–281)	This work
pYMS4505:I	Deletion of bp 1428–1472 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 271–285)	This work
pYMS4505:Δ#4	Deletion of bp 1449–1493 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 278–292)	This work
pYMS4505:J	Deletion of bp 1482–1571 of the *yadA* gene in pYMS 4505; expresses YadA with a 30-aa deletion (aa 289–318)	This work
pYMS4505:Δ#5	Deletion of bp 1494–1538 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 293–307)	This work
pYMS4505:Δ#5-6	Deletion of bp 1494–1583 of the *yadA* gene in pYMS 4505; expresses YadA with a 30-aa deletion (aa 293–322)	This work
pYMS4505:K	Deletion of bp 1506–1595 of the *yadA* gene in pYMS 4505; expresses YadA with a 30-aa deletion (aa 297–326)	This work
pYMS4505:L	Deletion of bp 1518–1607 of the *yadA* gene in pYMS 4505; expresses YadA with a 30-aa deletion (aa 301–330)	This work
pYMS4505:Δ#6	Deletion of bp 1539–1583 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 308–322)	This work
pYMS4505:Δ#6+3	Deletion of bp 1539–1586 of the *yadA* gene in pYMS 4505; expresses YadA with a 16-aa deletion (aa 308–323)	This work
pYMS4505:M	Deletion of bp 1572–1616 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 319–333)	This work
pYMS4505:Δ#7-9	Deletion of bp 1584–1706 of the *yadA* gene in pYMS 4505; expresses YadA with a 41-aa deletion (aa 323–363)	This work
pYMS4505:N	Deletion of bp 1617–1661 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 334–348)	This work

TABLE 1—*Continued*

Bacterial strain or plasmid	Description	Source or reference
pYMS4505:O	Deletion of bp 1662–1706 of the *yadA* gene in pYMS 4505; expresses YadA with a 15-aa deletion (aa 349–363)	This work
pYMS4505:P	Deletion of bp 1707–1727 of the *yadA* gene in pYMS 4505; expresses YadA with a 7-aa deletion (aa 364–370)	This work
pYMS4505:Δ1-3	Deletion of bp 1707–1772 of the *yadA* gene in pYMS 4505; does not express surface-located YadA; 22-aa deletion (aa 364–385)	This work
pYMS4505:Q	Deletion of bp 1728–1748 of the *yadA* gene in pYMS 4505; expresses YadA with a 7-aa deletion (aa 371–377)	This work
pYMS4505:R	Deletion of bp 1749–1769 of the *yadA* gene in pYMS 4505; expresses YadA with a 7-aa deletion (aa 378–384)	This work
pTM100	Mobilizable derivative of pACYC184	31
pTM100-ail	*ail* gene cloned in a 1,711-bp PCR fragment (nucleotides 1490–3201)[b] into EcoRV site of pTM100; Clm^r	This work
pTM100-ail1	CAC at bp 2459–2461 substituted for GCA in pTM100-ail; expresses Ail with His65Ala substitution (loop 2)	This work
pTM100-ail2	GATCTT at bp 2465–2470 substituted for GGAGGT in pTM100-ail; expresses very low levels of Ail with Asp67Gly and Leu68Gly substitutions (loop 2)	This work
pTM100-ail3	GATCTT at bp 2465–2470 substituted for GCACGA in pTM100-ail; expresses very low levels of Ail with Asp67Ala and Leu68Arg substitutions (loop 2)	This work
pTM100-ail4	TCA at bp 2564–2566 substituted for GAT in pTM100-ail; expresses Ail with Ser100 substituted for Asp (loop 3)	This work

[a] In all *yadA* mutants, nucleotide positions refer to the numbering of the 2,551-bp sequence containing the *yadA* gene of *Y. enterocolitica* O:3 under GenBank accession no. X13882.

[b] In all *ail* mutants, nucleotide positions refer to the numbering of the 3,865-bp sequence containing the *ail* gene of *Y. enterocolitica* O:3 under GenBank accession no. AJ605740.

tracellular loops (51, 54). Critical regions for CR of serotype O:8 Ail have been mapped to the C-terminal end of loop 2 and the N-terminal end of loop 3 (32).

In this report, we aimed to characterize YadA and Ail regions mediating serum resistance and FH binding. We show that FH targets several structural motifs of the YadA stalk and that this is biologically significant. The Ail region(s) involved in FH binding, however, could not be precisely mapped.

MATERIALS AND METHODS

Bacteria, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. For bactericidal assays, collagen affinity blotting, verification of expression of YadA deletion mutants, and adsorption of goat anti-human FH antiserum, bacteria were grown to stationary phase overnight in 5 ml of MedECa (4) at 37°C without shaking. For the examination of bacterial ability to bind serum FH, bacterial cultures grown overnight were diluted 1:20 in fresh medium and incubated for 3 h at 37°C without shaking to obtain bacteria in exponential phase of growth. When appropriate, antibiotics were added to the growth medium at the following concentrations: kanamycin at 100 μg/ml in agar plates and 20 μg/ml in broth and chloramphenicol at 20 μg/ml.

Cloning of ail. The *ail* gene was PCR amplified using genomic DNA from YeO3-c as a template and primers ail-9 and ail-12 (Table 2). A PCR product of 1,711 bp was gel purified, ligated into EcoRV-digested pTM100, and transformed into *Escherichia coli* JM109 cells. The transformant carrying pTM100 with the *ail* gene cloned in the correct orientation was named pTM100-ail. The expression of Ail by JM109/pTM100-ail was verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (33).

Mutagenesis of yadA and ail. Site-directed mutagenesis of the *yadA* and *ail* genes was carried out by inverse PCR with pYMS4505 and pTM100-ail as PCR templates, respectively (7). The primers used are listed in Table 2. PCR fragments were DpnI digested, phosphorylated, ligated, and electroporated into *E. coli* DH10B (*yadA* mutagenesis) and JM109 (*ail* mutagenesis) cells. The recovered plasmids were analyzed by PCR and sequencing to confirm the desired mutations. Triparental conjugation with the helper strain *E. coli* HB101/pRK2013 was used to mobilize the plasmids carrying (i) the mutated *yadA* of *Y. enterocolitica* strains YeO3-c and YeO3-c-Ail and (ii) *ail* genes of *Y. enterocolitica* strain YeO3-c-Ail-OCR, as described previously (4).

Antibodies and antisera. The antibodies used were horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin G (IgG) (catalog no.

P260; Dako), HRP-conjugated swine anti-rabbit IgG (catalog no. P217; Dako), HRP-conjugated rabbit anti-goat IgG (catalog no. P449; Dako), mouse anti-collagen type I (catalog no. C2456; Sigma), rabbit anti-human C3c (catalog no. A0062; Dako), rabbit anti-human C3d (catalog no. A0063; Dako), and goat antiserum against human FH (catalog no. A312; Quidel). The latter antiserum cross-reacted with *Y. enterocolitica* O:3 proteins, including YadA. The anti-YadA antibodies were efficiently removed by 1 h of adsorption on ice with YadA-expressing bacteria, YeO3 and YeO3-R2, as shown elsewhere previously (3). The preadsorbed antiserum was used for the FH detection in all the experiments performed unless otherwise indicated. The YadA-specific monoclonal antibodies (MAbs) 2A9, 2G12, and 3G12 raised against Tx-114-isolated YadA were described previously (49).

SDS-PAGE, immunoblotting, dot blotting, and affinity blotting. Bacteria were grown overnight at 37°C in 5 ml of MedECa. Whole-cell lysates were prepared from 5 ml of bacterial suspension with the optical density at 600 nm adjusted to 0.5. The pelleted bacteria were resuspended into 50 μl of phosphate-buffered saline (PBS), 200 μl of Laemmli buffer was then added, and the samples were heated at 95°C for 5 to 10 min. The samples (10 μl) were subjected to electrophoresis in 8 or 10% polyacrylamide gels and transferred onto nitrocellulose membranes, or 1 μl was spotted directly onto the membrane for dot blotting. After blocking with 5% skim milk–PBS, the membranes were incubated overnight at +4°C with appropriate primary antibody diluted in blocking solution. The YadA protein was visualized using Coomassie staining or detected with MAbs 3G12-15, 2A9, and 2G12 or with 1:2,000-diluted nonadsorbed polyclonal goat anti-FH antiserum. After washes with PBS, primary antibodies were detected with HRP-conjugated rabbit anti-mouse (1:2,000) or rabbit anti-goat (1:2,000) Ig. Peroxidase activity was detected using an electrochemiluminescence system. Quantitative analysis of YadA expression by the mutants was performed using Coomassie-stained gels and NIH ImageJ software. The loading differences between the lanes were corrected using the intensities of a distinct nonvariable bacterial protein band.

The same samples (10 μl) were used for collagen affinity blotting as described previously (10). Membranes were incubated with 20 μg/ml of type I collagen (catalog no. C7774; Sigma) overnight at +4°C, and bound collagen was detected with anti-collagen type I MAb.

Serum bactericidal assay. Normal human serum (NHS) was obtained on two occasions from the same healthy human donors who were devoid of anti-*Yersinia* antibodies, and the sera were pooled (pools 1 and 2). The ability of *Y. enterocolitica* O:3 strains to survive in the pooled 66.7% NHS–EGTA-Mg serum or in heat-inactivated (56°C for 30 min) serum (HIS) was performed as described previously (4). The survival percentage was calculated by taking the bacterial

TABLE 2. Oligonucleotide primers used in inverse PCR with template plasmids pYMS4505 and pTM100-ail to mutagenize the *yadA* and *ail* genes, respectively[a]

Primer	Sequence (5′–3′)[b]	5′–3′ positions	Purpose
yadA-1193r	AAGGCTTTCATGACCAATGG	1193–1974	Deletion of aa 193–218 of YadA
yadA-1272f	GAAAAAACACAGGAAAATACAAATAAAAG	1272–1300	
yadA-1310r	CTCAGCTGATCTTTTATTTGTATTTTCCTG	1310–1281	Deletion of aa 231–246 of YadA
yadA-1356f	GTGCTAGGGATCGCAAATAACTATAC	1356–1381	
yadA-1355r	GCTAGAAGACTTGTTGTCTGCATACG	1355–1330	Deletion of aa 247–262 of YadA
yadA-1404f	TTGGAAAATGCGCGTAAAGAGGC	1404–1426	
yadA-1403r	TGTTTCAGCACTTTTACTATCAGT	1403–1380	Deletion of aa 263–277 of YadA
yadA-1449f	TTGAATATGGCAAAAGCACACTC	1449–1471	
yadA-1448r	AACATCTTTAGACTGAGCAAAAGC	1448–1425	Deletion of aa 278–292 or 293–307 of YadA
yadA-1494f	TTAGAAACTGCTGAAGAACATGC	1494–1516	
yadA-1493r	AGTTGTTCTAGCAACACTATTTGAG	1493–1469	
yadA-1538r	AGTTGTTCTGGCAACACTATTTGC	1538–1515	Deletion of aa 308–322 of YadA
yadA-1584f	TTAGCAAGCGCTAATGTGTATGC	1584–1606	
yadA-1583r	CGCCTCAGCTGATTTTTTATTTGC	1583–1560	Deletion of aa 323–363 of YadA
yadA-1707f	ACAGATCATAAATTCCGTCAACTTGAC	1707–1733	
yadA-1256r	TTGCGCGACATTCACTG	1256–1240	Deletion of aa 214–228 of YadA
yadA-1302f	TCAGCTGAGCTGTTAGCA	1302–1319	
yadA-1346r	CTTGTTGTCTGCATACGCAT	1346–1327	Deletion of aa 244–258 of YadA
yadA-1392f	AGTGCTGAAACATTGGAAAAT	1392–1412	
yadA-1358r	CACGCTAGAAGACTTGTTGTCTGC	1358–1335	Deletion of aa 248–262 of YadA
yadA-1404f	TTGGAAAATGCGCGTAAAGAGGC	1404–1426	
yadA-1370r	TGCGATCCCTAGCACGCT	1370–1353	Deletion of aa 252–266 of YadA
yadA-1416f	CGTAAAGAGGCTTTTGCTCAGTCTAAA	1416–1442	
yadA-1382r	AGTATAGTTATTTGCGATCCCTA	1382–1360	Deletion of aa 256–270 of YadA
yadA-1428f	TTTGCTCAGTCTAAAGATGTT	1428–1448	
yadA-1391r	TTTACTATCAGTATAGTTATTTGCGAT	1391–1365	Deletion of aa 259–273 of YadA
yadA-1437f	TCTAAAGATGTTTTGAATATGGCA	1437–1460	
yadA-1415r	CGCATTTTCCAATGTTTCAGCA	1415–1394	Deletion of aa 267–281 of YadA
yadA-1461f	AAAGCACACTCAAATAGTGTTGC	1461–1483	
yadA-1427r	AGCCTCTTTACGCGCAT	1427–1411	Deletion of aa 271–285 of YadA
yadA-1473f	AATAGTGTTGCTAGAACAACTTTAGAAAC	1473–1501	
yadA-1481r	AACACTATTTGAGTGTGCTTTTGC	1481–1458	Deletion of aa 289–318 of YadA
yadA-1572f	TCAGCTGAGGCGTTAGC	1572–1588	
yadA-1505r	AGCAGTTTCTAAAGTTGTTCTAGCA	1505–1481	Deletion of aa 297–326 of YadA
yadA-1596f	AATGTGTATGCAGACAGCAAGT	1596–1617	
yadA-1517r	TGCATGTTCTTCAGCAGTTTCTAA	1517–1494	Deletion of aa 301–330 of YadA
yadA-1608f	GACAGCAAGTCTTCTCACACAC	1608–1629	
yadA-1571r	TTTTTTATTTGCATGTTCTTCAGCAGT	1571–1545	Deletion of aa 319–333 of YadA
yadA-1617f	TCTTCTCACACACTAAAAACTGCA	1617–1640	
yadA-1616r	CTTGCTGTCTGCATACACATTAG	1616–1594	Deletion of aa 334–348 of YadA
yadA-1662f	GTAAGTAATTCGACTAAGAAAGCAATCC	1662–1689	
yadA-1661r	AGTCACATCGGTATAGCTATTTGCA	1661–1637	Deletion of aa 349–363 of YadA
yadA-1707f	ACAGATCATAAATTCCGTCAACTTGAC	1707–1733	
yadA-1706r	GTATTGATTCGATTCACGGATTGCTTTC	1706–1679	Deletion of aa 364–370 of YadA
yadA-1728f	CTTGACAACCGGTTAGATAAACTTGAC	1728–1754	

Continued on following page

TABLE 2—*Continued*

Primer	Sequence (5′–3′)[b]	5′–3′ positions	Purpose
yadA-1727r	TTGACGGAATTTATGATCTGTGTATTGA	1727–1700	Deletion of aa 371–377 of YadA
yadA-1749f	CTTGACACACGAGTTGACAAAG	1749–1770	
yadA-1748r	TTTATCTAACCGGTTGTCAAGTTGA	1748–1724	Deletion of aa 378–384 of YadA
yadA-1770f	GGTTTAGCCAGTTCAGCC	1770–1787	
yadA-1280r	TGTTTTTTCAATTTCTTTCTTTAATTGC	1280–1253	Deletion of aa 222–236 of YadA
yadA-1326f	AATGCGTATGCAGACAAC	1326–1343	
yadA-1706r	GTATTGATTCGATTCACGGATTGCTTTC	1706–1679	Deletion of aa 364–385 of YadA
yadA-1773f	TTAGCCAGTTCAGCCGCTTTAAAC	1773–1796	
ail-9	AAGCGCTTGAGGTCAAAGCACAA	1490–1512	Cloning of *ail* gene
ail-12	TTTTCCGGCAGCGGCCCGG	3201–3183	
H65Ar	ACCAAACTTATTACTGCCATAGAAGAAATCGTAT	2458–2424	His65Ala substitution in loop 2 of Ail
H65Af	<u>GCA</u>GGTGATCTTGATTACTATTCAGTAACAATGGGGC	2459–2494	
D67G L68Gr	ACCGTGACCAAACTTATTACTGCCATAGAAG	2464–2433	Asp67Gly, Leu68Gly, Asp67Ala, and Leu68Arg substitutions in loop 2 of Ail
D67G L68Gf	<u>GGAGGT</u>GATTACTATTCAGTAACAATGGGGCCATCTTTC	2465–2503	
D67A L68Rf	<u>GCACGA</u>GATTACTATTCAGTAACAATGGGGCCATCTTTC	2465–2503	
S100r	CTTAACCTTTCCGTGAGCAGCAC	2563–2540	Ser100Asp substitution in loop 3 of Ail
S100Df	<u>GAT</u>TCTGTATTTGATGGGTCAGTCAGTACAAGTAAG	2564–2599	

[a] Nucleotide positions refer to the numbering of the sequences containing the *yadA* and *ail* genes of *Y. enterocolitica* O:3 under GenBank accession no. X13882 and AJ605740, respectively.

[b] The nucleotide substitutions in the mutagenic primers are underlined.

counts obtained with bacteria incubated in HIS as 100%. The killing experiment was repeated for each strain at least three times, each time in duplicate, starting from independent cultures. For the calculation of statistical significances between the deletion mutants and wild-type YadA, the percentages of survival values for each experiment were used. To allow comparison between separate experiments, the results of the tested strains were adjusted to the wild-type survival percentage, which was set to 100.

Binding of serum FH to bacteria and cofactor assay for C3b inactivation. The FH binding experiments by immunoblotting and enzyme-linked immunosorbent assay (ELISA) as well as the cofactor assay performed to analyze FI-mediated cleavage of C3b to iC3b were performed as described in detail elsewhere previously (3). The bacterial pellets from each cofactor assay sample were additionally subjected to 8% SDS-PAGE and immunoblotting using preadsorbed goat antiserum against human FH for detection (1:2,000).

Binding of truncated recombinant FH constructs to bacteria. Bacteria were grown to logarithmic phase and washed three times with Veronal-buffered saline (VBS). The bacteria (10^9) were incubated with shaking (850 rpm) at 37°C for 30 min in 200 μl of VBS alone or containing SCRs 1 to 5, 1 to 6, 1 to 7, 8 to 11, 11 to 15, or 8 to 20 in equimolar (0.3 μM) concentrations. Following incubation, bacteria were washed three times with VBS, and bacterial pellets were suspended in 30 μl of VBS and mixed with Laemmli buffer. As all the recombinant FH constructs are recognized by the goat anti-FH antiserum (3), the samples were subjected to 12% SDS-PAGE and immunoblotting using goat antiserum against human FH for the detection of the truncated FH constructs.

Modeling of the stalk coiled-coil structure. The stalk models for the YadA deletion mutants were generated with a modified version of BeammotifcCC (37), in which the supercoil radius can be specified individually for each residue (22). The basic periodicity used to model the entire stalk (residues 214 to 396) was 15-15-15-15-15-15-15-15-15-19-7-7-7-7; from this, we subtracted the deletion to be modeled and adjusted the local periodicity. The adjustments were based on the observation that insertions and deletions can be accommodated into coiled coils without disrupting the helices (25) as long as the local sequence can be resolved into combinations of 3- and 4-residue segments, each of which starts

with a hydrophobic residue or, when multiple segments of 4 succeed each other, with a small residue (Ala, Ser, Thr, and Val). In all 28 deletion mutants that could be expressed, the effect of the deletions could be resolved in this way. We were particularly intrigued by deletion at residues 323 to 363 (Δ323-363), which we found could be modeled best by assuming that a sequence element of 23 residues (4-3-4-4-4-4) would form at the deletion site. While this may seem unusual, stalks that include this periodicity are found in other trimeric autotransporter adhesins, and, in the present case, all the resulting segments of 4 residues started with Ala, Ser, or Thr, while the single segment of 3 started with Leu. Thus, even though some deletions seemed incompatible with coiled-coil periodicity at first glance, they actually yielded models with favorable core interactions. Coordinates for the models can be obtained upon request.

Statistical methods. Statistical analyses were performed using the two-sample *t* test; a *P* value of <0.05 was considered to be statistically significant.

RESULTS

Deletion mapping of YadA functions and MAb epitopes. Since YadA appeared to be the major FH binding determinant of *Y. enterocolitica* O:3 (3), we aimed to identify YadA regions involved in CR and FH binding. To this end, we engineered 30 overlapping in-frame deletions to the *yadA* gene of plasmid pYMS4505. The deletions mapped within the neck and stalk regions of YadA (Fig. 1). The head domain was not mutated since it was previously reported not to play a role in CR (43). Two of the constructed mutants did not express YadA. One of them carried a deletion of 22 residues overlapping the junction between the right-handed and left-handed stalks (Δ364-385), while the other (pYMS4505:C) had an out-of-frame deletion
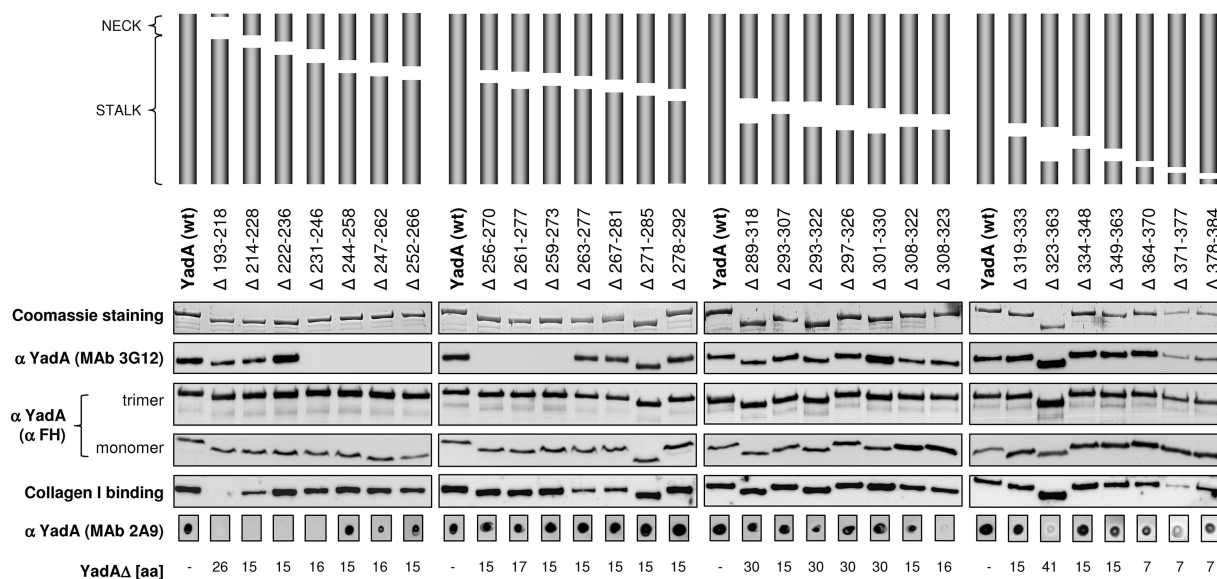
FIG. 1. Characteristics of YadA deletion mutants. A schematic representation of the in-frame deletions generated within the YadA neck and stalk is shown at the top. Whole-cell lysates of the strains were subjected to SDS-PAGE, and the gels were either stained with Coomassie blue (top row) or processed for immunoblotting or affinity blotting (four middle rows). Parts of the gels or immunoblots with the trimeric YadA bands and, in the case of nonadsorbed polyclonal anti-FH antiserum (that cross-reacts with YadA) (see Materials and Methods), also the monomeric band are shown. An aliquot of the lysates was analyzed for MAb 2A9 reactivity by dot blotting (bottom row). Affinity blotting was used to test the ability of YadA deletion mutants to bind type I collagen. The bound collagen was detected using type I collagen-specific MAb. wt, wild type.

within the stalk-coding region. The latter served as a control in all the experiments. The other 28 mutants expressed trimeric YadA as shown by Coomassie-stained SDS-PAGE and by immunoblottings and affinity blottings (Fig. 1). Quantitative analyses revealed no significant differences between the YadA expression levels of these mutants (data not shown).

To elucidate the impact of the generated deletions on the stalk structure, we modeled the stalks of the 28 YadA deletion mutants (for details, see Materials and Methods). According to the modeling, most deletions would not alter the structure of the rest of the stalk, while a few would change the local periodicity and thus the sense and degree of supercoiling (Fig. 2).

Several structure-related observations could be made based on the analyses of these YadA mutants. Firstly, the epitopes of MAbs 3G12-15, 2A9, and 2G12 could be mapped to amino acids 236 to 263, 193 to 244, and 193 to 214 of YadA, respectively (Fig. 1 and data not shown). All the epitopes were distinct, in close proximity to each other at the N-terminal end of the YadA stalk. Interestingly, the reduced signal for MAb 2A9, observed with the C-terminal deletions Δ308-323, Δ323-363, and Δ371-377 (Fig. 1), suggested a possible long-distance effect on the conformation of the N-terminal epitope. Secondly, all the oligomerized YadA forms as well as YadA monomers were detected by the cross-reacting YadA-specific antibodies present in the unadsorbed polyclonal anti-FH antiserum (see Materials and Methods) (Fig. 1). Thirdly, all the mutants were tested for the ability to bind collagen type I to demonstrate that the introduced mutations did not affect the integrity of the head domain. All but the neck deletion mutant (Δ193-218) were able to bind collagen as expected (Fig. 1), confirming that the constructed mutants had an intact head domain. The neck deletion covers the neck region and a few N-terminal amino acids of the stalk; this result corroborates the previously re-

ported observation that the neck region is also necessary for the collagen binding ability of YadA (43).

Serum resistance profiles of the YadA deletion mutants. In order to map the YadA regions involved in CR, the YadA deletion mutants were all expressed in a virulence plasmid-cured strain, YeO3-c, and tested for the ability to survive under conditions of normal and EGTA-Mg-treated serum and classical pathway (CP)/AP- and AP-mediated killing, respectively (Fig. 3). For a number of mutants, the deletions did not apparently affect the CR. However, deletions within the neck domain (Δ193-218) or within the N-terminal end of the stalk (Δ214-228, Δ222-236, Δ231-246, Δ244-258, Δ247-262, and Δ252-266) resulted in increased CR (Fig. 3). The differences in the survival in EGTA-Mg-treated serum between these strains and YeO3-c/pYMS4505 were statistically significant (P values of between 0.0001 and 0.037). The only exception was Δ247-262, for which the difference did not reach significance (P = 0.066). On the other hand, several deletions within the rest of the right-handed part of the stalk (Δ261-277, Δ259-273, Δ263-277, Δ271-285, Δ278-292, Δ289-318, Δ293-322, Δ301-330, Δ308-323, and Δ323-363) resulted in reduced CR. The differences in survival in EGTA-Mg-treated serum between these strains and YeO3-c/pYMS4505 were also statistically significant (P values of between 0.0001 and 0.042). Finally, the deletion of the middle heptad in the left-handed part of the stalk (Δ371-377), but not the flanking heptads (Δ364-370 and Δ378-384), strongly affected CR (P = 0.0014 for EGTA-Mg serum).

Serum resistance versus FH binding phenotype of the YadA deletion mutants. To examine if the decrease in resistance to AP killing was due to reduced FH binding, the mutants were further analyzed by immunoblotting and ELISA for the ability to bind this AP regulator from HIS (Fig. 3). Of note, the heat treatment of serum does not denature FH, as even after 20 min
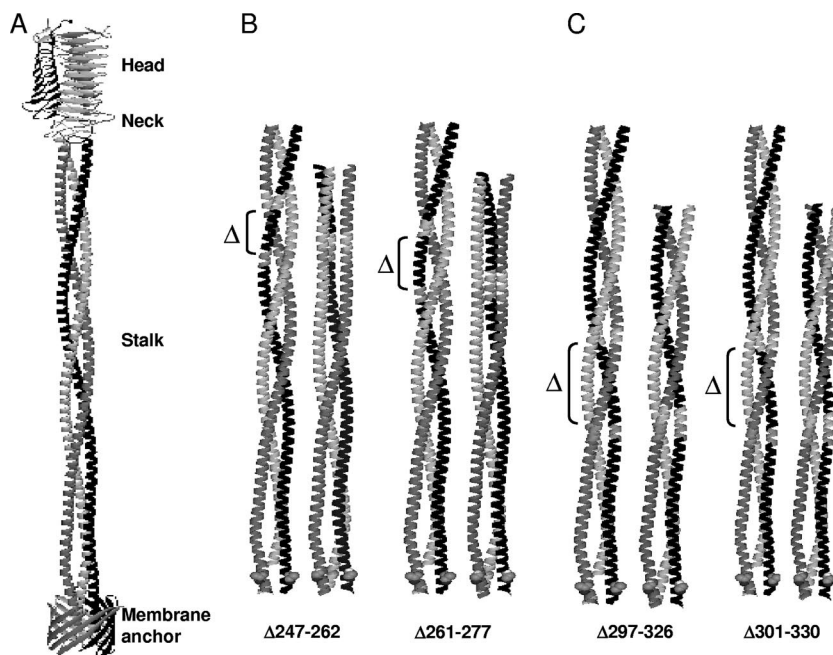
FIG. 2. Effect of deletions on the structural model of the YadA stalk. (A) Ribbon presentation of the full-length wild-type YadA structure. (Modified from reference 22 with permission of the publisher.) (B and C) Comparison of the trimeric coiled-coil stalks of the wild type and four deletion mutants. Two of the mutants shown were predicted to alter the periodicity of the coiled-coil (B), and the other two were predicted to retain the wild-type periodicity of the stalk (C). The deleted regions in each stalk pair are indicated for the wild-type stalks.

of incubation at 95°C, FH displays cofactor activity for the FI-mediated cleavage of C3b (20). Not surprisingly, all the AP-resistant mutants were able to bind FH (Fig. 3).

On the other hand, the FH binding ability had not been lost by most of the AP-sensitive mutants; instead, four of them displayed wild-type levels of FH binding (Δ293-322, Δ308-323, and Δ323-363) or even significantly higher levels ($P = 0.013$ for Δ371-377 by ELISA). Furthermore, when tested for the ability to bind FH from NHS or EGTA-Mg-treated serum during a short 5-min incubation, these four sensitive strains again displayed FH binding at wild-type levels or higher (Fig. 4). Interestingly, in this 5-min experiment, bacteria acquired much more FH from NHS or EGTA-Mg-treated serum than from HIS, indicating that the binding is enhanced by complement activation (Fig. 4).

In order to understand the AP-sensitive phenotype of the YadA deletion mutants that bind serum FH, their ability to interact with purified FH or with truncated FH fragments was analyzed. All these YadA deletion mutants were introduced into YadA- and Ail-negative *Y. enterocolitica* strain YeO3-c-Ail to exclude the FH binding influence of Ail. Interestingly, the mutants bound purified FH at the wild-type YadA level or greater (Fig. 5). On the other hand, we could see some reduction in the binding of Δ308-323, Δ323-363, and Δ371-377 to FH recombinant fragments comprising SCRs 1 to 7 or 8 to 11 (Fig. 6). However, the importance of this reduction of binding remains elusive, as the purified FH bound to bacteria expressing these YadA mutants retained the cofactor activity for FI-mediated C3b cleavage (Fig. 5).

The significant decrease in levels of FH binding by the YadA deletion mutants Δ247-262, Δ256-270, and Δ293-307 ($P < 0.009$ by ELISA) did not greatly affect CR. On the other hand,

the decreased levels of FH binding of mutants Δ261-277 and Δ301-330 ($P < 0.0027$ by ELISA), although not as dramatic as that of the YadA-negative control pYMS4505:C, was reflected in decreased CR.

Collectively, these results showed that several deletions in the central part of the stalk and a deletion in the C-terminal part decreased CR, whereas deletions in the neck domain or in the N-terminal part of the stalk increased CR. Furthermore, the deletion mapping of YadA suggested that FH targets several regions of the YadA stalk and that the FH binding site on YadA is discontinuous and conformational. Surprisingly, FH binding by these YadA deletion derivatives did not always correlate with CR, suggesting that CR is a complex phenomenon. This is supported by our recent finding that YadA and Ail also bind C4BP, another complement regulator, from serum (21).

Serum resistance and FH binding phenotypes of the Ail mutants. Similar to YadA, Ail was also shown to be a receptor for FH but solely in the absence of blocking O antigen and outer core (3). As lipopolysaccharide composition is known to be regulated by environmental cues, it is possible that Ail could be unveiled at some stages of infection. Therefore, to identify Ail regions responsible for FH binding, four point mutations were introduced into *ail*. The choice of the constructed point mutations was based on the mapping of the CR-associated residues of the Ail of *Y. enterocolitica* serotype O:8 (32). We engineered single-amino-acid substitutions in loop 2 (His65Ala expressed by pTM100-ail1) or in loop 3 (Ser100Asp expressed by pTM100-ail4) and substituted two amino acids critical for CR in loop 2 (Asp67Gly and Leu68Gly expressed by pTM100-ail2 and Asp67Ala and Leu68Arg expressed by pTM100-ail3). Unfortunately, the latter double-substitution *ail* mutants ex-
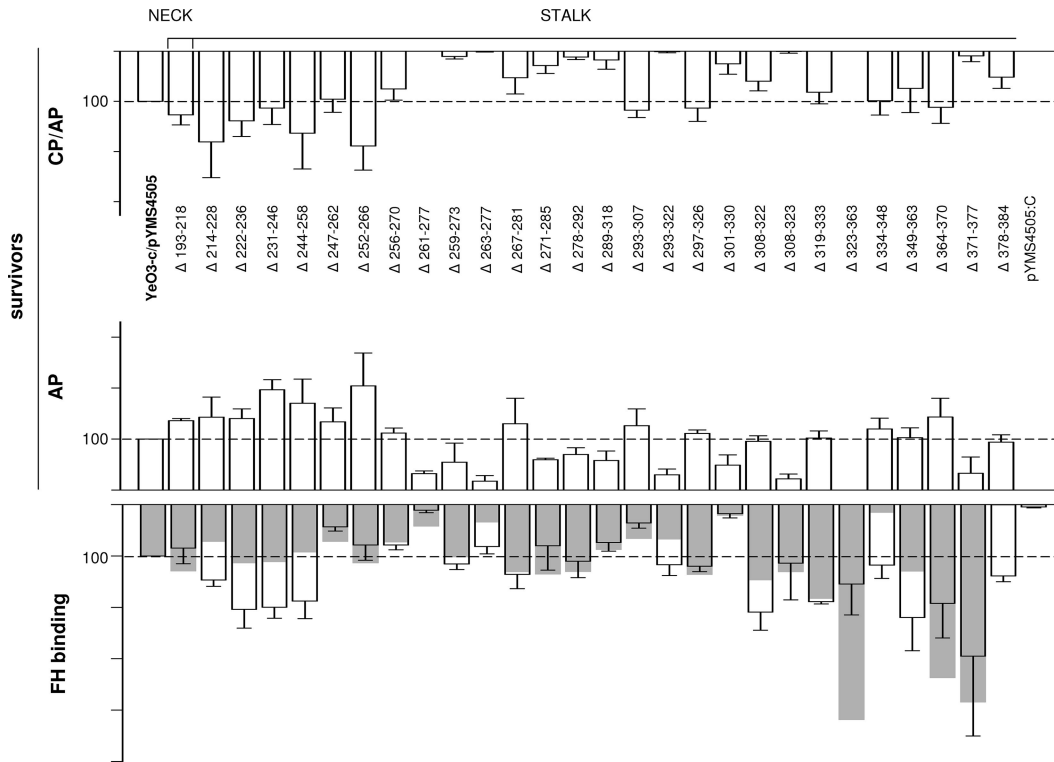
FIG. 3. Deletion mapping of YadA for CR determinants. The resistance of the mutants to CP/AP- and AP-mediated killing is presented for the 2-h time point. For the same strains, the FH binding from HIS was determined by ELISA (for each strain, the transparent columns with standard deviation bars represent the average FH binding for three independent duplicate experiments) and by immunoblotting (superimposed gray columns) (quantitation of the FH band intensities was performed with the Typhoon 9400 Image Quant analyzer). Survival and FH binding values of the strain expressing wild-type YadA (YeO3-c/pYMS4505) were set to 100, and the survival percentage and FH binding of the mutants are expressed relative to those of the wild type. For the calculation of statistical significances between the deletion mutants and wild-type YadA, the actual survival percentages for each experiment were used. The average YeO3-c/pYMS4505 survival value for AP killing in serum pool 1 was 76.1 ± 22.1, and that in pool 2 was 103.7 ± 27.7; the value for the CP/AP killing in serum pool 1 was 49.4 ± 17.4, and that in pool 2 was 73.3 ± 7.2. In ELISA, the average optical density at 492 nm value for strain YeO3-c/pYMS4505 was 0.47 ± 0.19 (range, 0.27 to 0.92). The strain with a frameshift mutation in the *yadA* gene (YeO3-c/pYMS4505:C), unable to express YadA, was included as a negative control.

pressed very low levels of the mutated protein and were not studied further. Plasmid pTM100-ail, carrying wild-type *ail*, as well as plasmids pTM100-ail1 and pTM100-ail4, carrying mutated *ail*, were transferred into YeO3-c-Ail-OCR. YeO3-c-Ail-OCR lacks YadA, Ail, and, importantly, also the O antigen and outer core, which are known to block FH binding to Ail (3). YeO3-c-Ail-OCR expressing wild-type or mutated Ail from the plasmids were examined for resistance to CP/AP- and
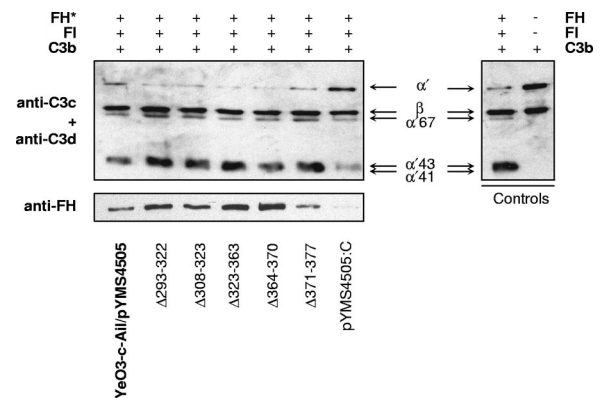

FIG. 5. Cofactor activity of FH bound to bacteria expressing wild-type or mutated YadA. Bacteria were preincubated with FH (30-μg/ml final concentration) (left) and washed. Subsequently, the bacteria were exposed to factor I and C3b. The bacterial surface-bound FH is indicated by the asterisk, while in the control panel (right), reactions were carried out without bacteria. C3b and its cleavage products from the supernatants were detected using a mixture of rabbit anti-C3c and anti-C3d antisera. Inactivation of C3b can be seen by the reduced intensity of the C3b α′-chain and the appearance of α′-chain cleavage fragments of 67, 43, and 41 kDa. FH bound to the bacteria was detected from pellets by immunoblotting using preadsorbed goat anti-FH antiserum.
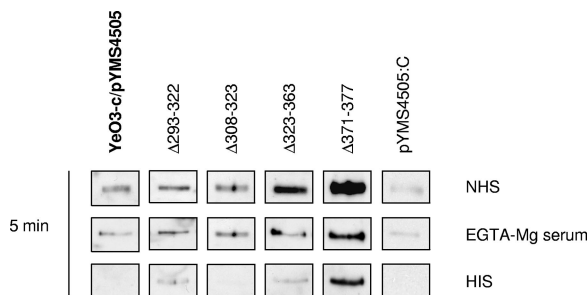

FIG. 4. Comparison of FH binding to bacteria expressing wild-type YadA to that of bacteria expressing mutated YadA during a 5-min incubation at 37°C in NHS, EGTA-Mg-treated serum, or HIS.
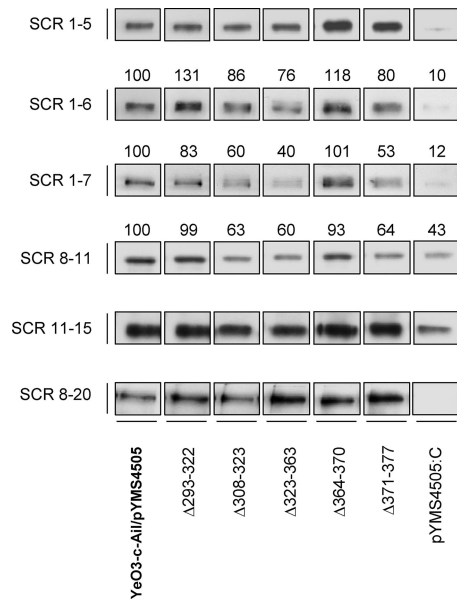
FIG. 6. Binding of recombinant FH fragments to bacteria expressing wild-type or mutated YadA. Bacteria were incubated with truncated recombinant FH constructs representing SCRs 1 to 5, 1 to 6, 1 to 7, 8 to 11, 11 to 15, and 8 to 20. Following incubation, bacteria were washed, and whole-cell lysates were run on 12% SDS-PAGE gels and analyzed by immunoblotting using goat anti-FH and HRP-conjugated rabbit anti-goat antibodies for detection. The intensities of the bands for SCRs 1 to 6, 1 to 7, and 8 to 11 were measured. The intensity of the wild-type band was adjusted to 100, and those of the mutants are relative to that of the wild type. The relative intensity values are indicated above the bands.
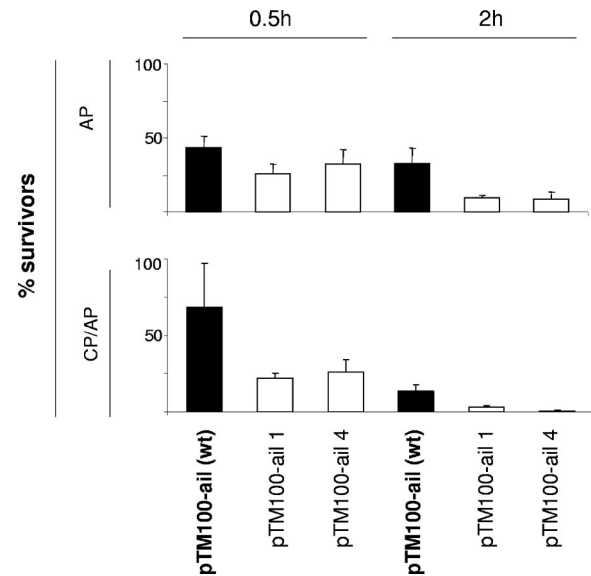

FIG. 7. Effect of loop 2 and loop 3 substitutions of Ail on CR. Wild-type (wt) and mutated Ail proteins were expressed in strain YeO3-c-Ail-OCR. The survival percentages of the Ail mutants to CP/AP- and AP-mediated killing are presented for the 0.5-h and 2-h time points. The columns with standard deviation bars show the averages of data for three independent duplicate experiments. pTM100-ail1, His65Ala substitution in loop 2; pTM100-ail4, Ser100Asp substitution in loop 3.

AP-mediated killing. YeO3-c-Ail-OCR carrying the vector alone was used as a negative control and was completely killed already at the early 0.5-h time point. Both mutants were more sensitive to NHS and EGTA-Mg-treated sera than the wild-type Ail-expressing strain (Fig. 7). The differences in CR between the mutants and the control strain expressing wild-type Ail were statistically significant ($P < 0.0031$), with the exception of YeO3-c-Ail-OCR/pTM100-ail4, for which the difference did not reach significance at 0.5 h of incubation in EGTA-Mg serum ($P = 0.13$). However, despite decreased serum resistance, the mutants bound amounts of FH similar to those of the wild type (data not shown), indicating that the introduced mutations did not affect the FH binding ability of Ail.

DISCUSSION

YadA and Ail mediate the binding of FH to *Y. enterocolitica* (3). In this study, we further characterized regions on YadA and Ail involved in the serum resistance and interaction with FH.

YadA is the main FH receptor on the surface of *Y. enterocolitica*, and YadA-bound FH retains its biological function as an FI cofactor for C3b cleavage (3). To identify YadA regions involved in serum resistance and FH binding, short deletions were introduced into the neck and stalk, taking into consideration the proposed coiled-coil structure of the trimeric stalk (22). The YadA head domain was previously shown not to

confer resistance against complement-mediated killing and was not mutagenized for this reason (43). None of the in-frame deletions within the stalk interfered with the surface exposure of the mutated YadA, except for a deletion of 22 residues overlapping the junction between the right-handed and left-handed parts of the stalk. YadA was not detected in this mutant, consistent with the work by Roggenkamp et al. demonstrating that this part of the stalk is indispensable for YadA translocation across the outer membrane (43). Additionally, all the stalk mutants bound collagen type I, indicating that the deletions did not affect the head and neck domains involved in collagen binding.

Functional analysis employing the YadA deletion mutants revealed that deletions in the N-terminal part of the stalk resulted in increased FH binding and CR. The reason for this is not clear. It is possible that in these deletions, FH gained better access to its binding site; however, since our stalk deletions were designed such that they left the collagen-binding head domain of YadA intact, the removal of the head was not the reason. A more likely possibility remains that the N-terminal part of the stalk contains the target site(s) for other serum proteins that could slow down or compete with FH binding. The deletion of these sites would then result in the facilitated access of FH to its binding site(s) on the YadA stalk. On the other hand, some deletions within the middle and C-terminal 15- and 7-mer regions of the stalk reduced the CR. In general, all serum-resistant YadA deletion mutants bound FH. The binding of FH to YadA seemed to depend on the recognition of several complementing and discontinuous structural YadA motifs rather than on one segment of the stalk, and the interaction seemed to be sensitive to both the conformation and the

spacing of these motifs. This reasoning is based on the following observations.

Firstly, although most AP-sensitive mutants had not lost the FH binding activity, decreased FH binding was associated with decreased CR for some mutants. Since Δ261-277 disturbs both the pitch and the angle of the supercoil in the stalk, one could suggest that the reduction the FH binding of this mutant was due to a change in the protein conformation. Deletions Δ263-277 and Δ301-330, however, being in a pentadecad register, do not distort the supercoil. We can only speculate that the reduced ability to bind FH by these mutants is due to a change of a distance between the putative FH binding subsites on YadA. In addition, these three deletions were partially included in some other mutants (Fig. 1) whose ability to bind FH was not affected. Thus, the reduced FH binding to these three mutants was most probably not due to a deletion of the actual FH binding subsites of YadA.

Secondly, deletions Δ308-323, Δ323-363, and Δ371-377, which reduced CR but not FH binding, affected the MAb 2A9 epitope (see above), showing that the deletions introduced close to the C terminus could affect the N-terminal conformation of the YadA stalk. Even though FH bound strongly to these mutants, it was not protective. The cofactor assay, however, showed that FH bound to these mutants retained its cofactor activity (Fig. 5). Moreover, these mutants, similarly to wild-type YadA, bound FH fragments representing the SCRs of the entire polypeptide chain of FH (Fig. 6). We are forced to speculate that although FH bound to these mutants displays the cofactor activity, its ability to interfere with the formation of the alternative pathway convertases and/or to accelerate their decay must be affected. In addition, these mutants could be more susceptible to complement-mediated killing due to the defect(s) in the binding of other serum factors crucial for CR, e.g., C4bp. C4bp, the classical and lectin pathway regulator, has recently been shown to bind to YadA (21). The reduced binding of this regulator to bacteria could have an impact on CR, especially since C4bp is also able to stimulate the FI-mediated cleavage of C3b (6). Alternatively, the excessive binding of FH by the mutants could lead to the gradual depletion of FH from serum and, as a result, provoke an uncontrolled activation of the complement system.

Thirdly, although the YadA stalk has been shown to mediate serum resistance (43) and deletions generated in this study covered the whole stalk, none of these deletions, even those with broken periodicity (Fig. 2), totally abolished FH binding. This suggests that there is no single FH-binding site on the YadA stalk but instead that the binding is mediated by several conformational and discontinuous sites.

The notion of multiple FH binding sites on YadA may not be surprising given the very large size and extended nature of the FH protein. FH contains 20 short consensus repeats of different activities, and we have shown that SCRs throughout the length of FH are involved in the FH-*Y. enterocolitica* interaction (3). Thus, the abolishment of FH binding to one of the YadA sites involved in the interaction does not necessarily affect the other FH regions to find their complementary structural motifs elsewhere on YadA. We also note that the right-handed part of the YadA stalk is formed of 15-residue repeats and has a clear internal sequence symmetry so that a binding site could occur in similar forms several times along the stalk.

YadA deletion mutants proved to be useful for the epitope mapping of three MAbs specific for YadA (3G12-15, 2A9, and 2G12). Previous attempts to map the epitopes of these MAbs using overlapping 16-mer YadA peptides were not successful (10). The deletion mutants generated in this study allowed us to localize the epitopes within the N terminus of the stalk. This suggested that the epitopes are conformational, as several overlapping deletions destroyed the epitopes, and the MAb 2A9 epitope seemed to be affected by deletions several nanometers apart. This long-distance effect was shown by a reduced signal in the dot blotting of the C-terminal deletions Δ308-323, Δ323-363, and Δ371-377 (Fig. 1). In the case of Δ371-377, this could be due to problems of protein folding and/or trimerization, also reflected as a lower level of collagen binding (Fig. 1). Our modeling analyses suggested that deletions Δ308-323 and Δ323-363, however, would affect the periodicity of the stalk and thus the supercoil structure. Interestingly, MAb 2A9 binding was much less influenced by two other deletions, Δ247-262 and Δ261-277, which also perturb the supercoil and are located even closer to the actual MAb A9 epitope than Δ308-323 and Δ323-363. The reason for this observation is unclear to us at present.

The conformational and discontinuous nature of FH binding was reported previously for OspE and BBA68 of *Borrelia burgdorferi* and FhbA of *Borrelia hermsii* (19, 28–30). All three proteins were predicted to form coiled coils, and these structural motifs were shown to be involved in forming the binding site for FH. The distortion of one of the three widely spaced coiled coils of OspE resulted in the attenuation or complete abolishment of FH binding (29, 30), while the destabilization of the BBA68 coiled coils resulted in a reduced FH binding capacity (28). Interestingly, FhbA shares some similarity with YadA, as a deletion of the N-terminal coiled-coil segment of FhbA did not affect FH binding. The FhbA region responsible for the interaction with FH was mapped to the C-terminal coiled coils and the loop that they flank (19). Although the loop might be a contact point for FH, the proper presentation of this region by the flanking coiled coils seems to be critical for the interaction to occur (19). The binding of FH to streptococcal M protein was also shown to involve coiled-coil conformation (13); hence, several FH binding proteins appear to present the conformational and discontinuous epitope for FH.

Ail acts as a receptor for FH on *Y. enterocolitica* provided that it is well surface exposed. Moreover, FH bound to Ail showed cofactor activity for FI (3). An attempt to locate Ail regions involved in FH binding was undertaken in this work. Amino acids shown to be crucial for serotype O:8 Ail-mediated serum resistance (32) were substituted in loop 2 or loop 3 of serotype O:3 Ail, and the mutants showed decreased CR in both NHS and EGTA-Mg serum. Despite this, their FH binding was not affected. This suggests (i) that these Ail mutants would bind FH in a way that prevents the biological activity of FH, resulting in reduced serum resistance, or, more likely, (ii) that these Ail mutants fail to interact with another serum factor(s) important for CR of *Y. enterocolitica*. In support of the latter hypothesis, we have evidence that, similar to YadA, Ail is also able to bind C4bp (21). However, a more comprehensive set of Ail mutants is needed to elucidate the structural requirements of Ail-mediated FH and C4bp binding and the relative importance of these binding activities to Ail-mediated

CR. On the other hand, the biological significance of FH binding by Ail, a factor promoting *Yersinia* entry into tissue culture cells in vitro (34), could also be related to bacterial adherence to the host cells as FH binds to glycosaminoglycans and sialic acids.

In conclusion, we have demonstrated that FH binding seems to involve multiple higher-order structural motifs on the YadA stalk. As a rule, YadA deletion mutants that bound FH survived well in serum. The FH binding-deficient deletion mutants identified in this study can be used to study the biological significance of this interaction in vivo. On the other hand, our results demonstrated that the two serum-sensitive Ail mutants were not affected in their abilities to bind FH. Thus, it appears that FH binding might not be the main mechanism of Ail-mediated CR.

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Editor: J. B. Bliska