Molecular Details of Serum Resistance of \textit{Yersinia enterocolitica}

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ACADEMIC DISSERTATION

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To my family
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BIBLIOGRAPHY
LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original publications, which are referred to in the text by their Roman numerals.


V. Kirjavainen V, Jarva H, Biedzka-Sarek M, Blom A, Skurnik M, and Meri S. *Yersinia enterocolitica* serum resistance proteins YadA and Ail bind the complement regulator C4b-binding protein. PLoS Pathogens 2008 Aug; 4(8), *these authors contributed equally*

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ABBREVIATIONS

aa           amino acid(s)
aHUS         atypical hemolytic uremic syndrom
Ail          attachment and invasion locus
AP           alternative pathway
bp           base pair(s)
BSA          bovine serum albumin
C1-INH       C1 inhibitor
C4bp         C4b-binding protein
CCP          complement control protein
Clm          chloramphenicol
CP           classical pathway
CR1          complement receptor type 1
DAF          decay-accelerating factor
DNA          deoxyribonucleic acid
EGTA         ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'- tetraacetic acid
ELISA        enzyme-linked immunosorbent assay
FH           factor H
FHL-1        FH-like protein 1
FHR          FH-related protein
FI           factor I
hms          hemin storage locus
HPI          high-pathogenicity island
IC           inner core of LPS
kb           kilo base (1000 base pairs)
kDa          kilodalton = 1.660 × 10⁻²¹ g
Km           kanamycin
LP           lectin pathway
LPS          lipopolysaccharide
mAb          monoclonal antibody
MAC          membrane attack complex
MASP         mannose-binding lectin-associated serine protease
MBL          mannose-binding lectin
MCP          membrane cofactor protein
MPGN         membranoproliferative glomerulonephritis
NDP          nucleotide diphosphate
NHS          normal human serum
O-ag         O-antigen
OC           outer core of LPS
PAGE         polyacrylamide gel electrophoresis
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pYV</td>
<td>Yersinia virulence plasmid</td>
</tr>
<tr>
<td>RCA</td>
<td>regulators of complement activation</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeat</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Tet</td>
<td>tetracyclin</td>
</tr>
<tr>
<td>Tx-114</td>
<td>Triton X-114</td>
</tr>
<tr>
<td>Und-P</td>
<td>undecaprenyl phosphate</td>
</tr>
<tr>
<td>YadA</td>
<td>Yersinia adhesin A</td>
</tr>
<tr>
<td>Yops</td>
<td>Yersinia outer proteins</td>
</tr>
<tr>
<td>Yst</td>
<td>Yersinia heat-stable toxin</td>
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</table>
ABSTRACT

The complement system, consisting of a set of plasma and membrane-bound proteins, serves as an essential part of the human immune defense. Its activation (through the classical, lectin, and alternative pathways) tags microbes for destruction by phagocytic cells or even leads to microbial lysis. In addition, anaphylatoxins generated during complement activation can trigger a range of pro-inflammatory responses. This antimicrobial action of the complement system can, however, be evaded by many microbes. Pathogens have developed a number of complement-evasion strategies, one of which is the recruitment of serum proteins called complement regulators. These regulators protect host tissues against excessive complement-mediated damage and are present in plasma in high concentrations. This, however, makes them an easy target for pathogens. Acquisition of proteins such as factor H (FH) and C4b-binding protein (C4bp) provides microbes with the means to inhibit complement activation on their surfaces.

*Yersinia enterocolitica* is a Gram-negative enteropathogen that resists bactericidal action in human serum. This bacterium causes gastroenteritis and as post-infection sequelae reactive arthritis, myocarditis, erythema nodosum, and glomerulonephritis. The aim of the present study was to characterize complement evasion strategies of *Y. enterocolitica* and to identify bacterial surface factors associated with this phenomenon.

This thesis reveals that the *Y. enterocolitica* serotype O:3 adhesins, YadA and Ail, are the most crucial serum resistance factors (I). Although the lipopolysaccharide (LPS) of this serotype was not found to participate in serum resistance, serotype O:9 LPS strengthened YadA-mediated resistance (I, II). Differences in serum resistance between different *Y. enterocolitica* serotypes can thus be expected.

*Y. enterocolitica* was found to bind FH, a fluid-phase inhibitor of the alternative complement pathway (III). The receptors for FH on the *Y. enterocolitica* surface were mapped to YadA and Ail (III). Ail was found to acquire FH only in the absence of the LPS O-antigen (O-ag) and the outer core (OC), whereas YadA binds the regulators also in the presence of LPS (III). In addition, YadA appears to bind to short consensus repeats (SCRs) of the entire polypeptide chain of FH, whereas Ail targets SCRs 6 and
Importantly, both YadA- and Ail-bound FH is fully functional and displays cofactor activity for factor I (FI)-mediated cleavage of C3b (III). YadA is a lollipop-shaped outer membrane protein that consists of a head, a neck, and a coiled-coil stalk domain. FH targets multiple higher structural and discontinuous motifs of the YadA stalk (IV).

Another complement regulator shown to be acquired by *Y. enterocolitica* is C4bp (V). C4bp retains its function when bound to the bacterial surface, and YadA and Ail function as C4bp receptors (V). Similar to FH binding, however, Ail acquires C4bp only in the absence of the LPS O-ag and OC (V). C4bp binding to Ail was likely hydrophobic, but that to YadA, ionic in nature (V).

Taken together, these data provide evidence that *Y. enterocolitica* recruits the complement regulators FH and C4bp. Thus, *Y. enterocolitica* belongs to a constantly growing group of pathogens utilizing the most widely disseminated mechanism of complement evasion.
1. REVIEW OF THE LITERATURE

1.1 Introduction

Human beings provide a home for a myriad of microbes. Many microbes produce vitamins and nutrients, ferment food, break down toxic chemicals, and protect us from pathogenic microbes which we encounter every day. The latter, however, often find ways to cause infection, exploiting a wide range of strategies to penetrate physical barriers such as skin or mucous membranes and to survive and persist in the host. The first obstacle they must combat is the action of the non-specific innate immune system. One of its essential arms is the complement system, the first line of defense activated immediately upon pathogen entry. The importance of the complement system in host defense against invading pathogens is reflected by increased susceptibility to microbial infection of individuals deficient in certain complement components. Complement is bactericidal against Gram-negative bacteria, it acts as an opsonin, and its cleavage products contribute to induction of inflammation.

That pathogens adapt quickly to environmental changes makes them tenacious opponents. They express surface factors to manipulate the host complement system and avoid complement-mediated recognition and eradication. Studying microbial factors conferring complement-resistance at molecular level may provide means for their neutralization, and this could contribute to the prevention and treatment of microbial infections.
1.2 Serum bactericidal activity – discovery and overview of the complement system

The complement story, as reviewed by Lachmann (Lachmann, 2006), began in the late 1880’s when von Fodor, Nuttall, and Buchner independently observed the bactericidal activity of normal serum. Discovery of the complement was, however, carried out by Bordet (1870-1961), who received the Nobel Prize in Medicine in 1919. In 1895 Bordet showed the lysis of *Vibrio cholerae* by a mixture of normal and heated immune sera. Thereby he demonstrated the importance of both for bacterial lysis: the heat-labile “activity” of serum (termed by Ehrlich complement), and the heat-stable antibody. Six years later, together with Gengou, Bordet published the first and seminal report on complement fixation, in which he described the ability of antigen-antibody complexes to remove complement activity from serum. Complement was regarded as a single substance until 1907 when Ehrlich and Brand fractionated serum and showed that the heat-labile complement proteins C1 and C2 act sequentially and are required for complement activity. The first heat-stable complement component identified was C3, successfully removed from serum by use of cobra venom by Ritz in 1912, and yeast cells by Coca in 1914. Although C4 was described by Gordon in 1926, the correct sequence in which these four complement components reacted (C1→C4→C2→C3) was only established 30 years later by Ecker’s group. Until the late 1950’s complement function was connected exclusively to complementing the ability of the antibodies to cause a target lysis. Then Louis Pillemer suggested a properdin-dependent but antibody-independent pathway of C activation. This “alternative pathway” and its distinct protein constituents were fully elucidated and accepted by the scientific community only in the 1970’s. At that time, the lytic activity of C was shown to involve at least 11 different proteins. In recent years, a “lectin pathway” of C activation, functioning through recognition of mannan polysaccharides on microbial pathogens has been identified [the entire history is reviewed in (Lachmann, 2006)].

These initial studies regarding complement were, therefore, dealing with bactericidal activity against antibody-coated Gram-negative bacteria. Today, in addition to this function, complement is known to act as an opsonin and an inducer of inflammation. Its opsonin activity results in enhanced phagocytosis of the complement-tagged microbes whereas its inflammatory activity is related to the attraction of immune cells.
along the gradient of complement cleavage products. The complement system comprises approximately 35 fluid-phase (Table 1) and membrane-bound proteins. It is activated via the classical, the alternative, and the lectin pathways, which sense microbes by means of different recognition molecules. Once the complement is activated, a cascade of proteolytic reactions leads to the cleavage of C3, which is a converging stage for all three pathways. Subsequently, C3 cleavage is followed by the lytic pathway and the generation of the membrane attack complex (MAC). MAC forms a pore in the target cell and in consequence disrupts the membrane, causing the cell death.

Table 1. Components of the complement system.

<table>
<thead>
<tr>
<th>Component</th>
<th>Structure</th>
<th>Plasma conc. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Classical pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>3 subunits: C1q (460kDa), C1r (80kDa), C1s (80kDa) in a complex (C1qr2s2)</td>
<td>180</td>
</tr>
<tr>
<td>C4</td>
<td>3 chains (α, 97 kDa; β, 75 kDa; γ, 33 kDa)</td>
<td>600</td>
</tr>
<tr>
<td>C2</td>
<td>single chain, 102 kDa</td>
<td>20</td>
</tr>
<tr>
<td><strong>Alternative pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factor B</td>
<td>single chain, 93 kDa</td>
<td>210</td>
</tr>
<tr>
<td>Factor D</td>
<td>single chain, 24 kDa</td>
<td>2</td>
</tr>
<tr>
<td>Properdin</td>
<td>oligomers of 53-kDa chains</td>
<td>5</td>
</tr>
<tr>
<td><strong>Lectin pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBL</td>
<td>polymer of 32-kDa chains</td>
<td>0-5</td>
</tr>
<tr>
<td>MASP-1</td>
<td>single chain, 100 kDa</td>
<td>1.5-12</td>
</tr>
<tr>
<td>MASP-2</td>
<td>single chain, 76 kDa</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Common:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3</td>
<td>2 chains: α, 110 kDa; β, 75 kDa</td>
<td>1300</td>
</tr>
<tr>
<td><strong>Terminal pathway</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C5</td>
<td>2 chains: 115 kDa, 75 kDa</td>
<td>70</td>
</tr>
<tr>
<td>C6</td>
<td>single chain, 120 kDa</td>
<td>65</td>
</tr>
<tr>
<td>C7</td>
<td>single chain, 110 kDa</td>
<td>55</td>
</tr>
<tr>
<td>C8</td>
<td>3 chains: α, 65 kDa; β, 65 kDa; γ, 22 kDa</td>
<td>55</td>
</tr>
<tr>
<td>C9</td>
<td>single chain, 69 kDa</td>
<td>60</td>
</tr>
</tbody>
</table>

MBL, mannose-binding lectin; MASP, mannose-binding lectin-associated serine protease; ND, non-detectable
1.2.1 Classical pathway (CP)

Binding of antibody to its specific antigen on the surface of a microbe opens up the binding sites in the bound antibody for the classical pathway pattern recognition molecule C1q. C1q, a 460-kDa multimeric protein, resembles in structure a bouquet of six tulips with globular “head” domains and collagen-like triple-helical “stems” (Calcott and Müller-Eberhard, 1972; Reid and Porter, 1976; Schumaker et al., 1982; Strang et al., 1982). In the presence of Ca\(^{2+}\), C1q, together with two C1r and two C1s serine protease molecules (C1qr\(_2\)s\(_2\)), forms the C1 complex (Busby and Ingham, 1990; Loos et al., 1980; Thielens et al., 1990). Six globular heads of the C1q subunits recognize Fc regions of IgM or multiple IgG molecules only when they are deposited on a microbial surface (Ishizaka et al., 1966; Schumaker et al., 1986). C1q can also activate the classical pathway via binding to C-reactive protein (CRP), nucleic acids, LPS and immune complexes (Jiang et al., 1992a, 1992b; Loos, 1982; Nauta et al., 2002).

C1q binding to a target causes a mechanical stress transmitted from the C1q stems to C1r molecules (Gaboriaud et al., 2004), which in turn activates two molecules of C1s. C1s first targets the C4 molecule (Patrick et al., 1970). C4 comprises three chains linked together by disulfide bridges and an internal thioester group which upon disruption mediates a covalent attachment to a microbial surface (Fig. 2) (Dodds et al., 1996; Janatova and Tack, 1981; Schreiber and Müller-Eberhard, 1974). C1s-mediated cleavage of C4 generates two fragments: a small C4a acting as an anaphylatoxin (Gorski et al., 1979) and a larger C4b. C4b binds to the microbial surface, since the exposed thioester group is prone to hydrolysis (Law and Dodds, 1990, 1997), and acts as the C2 receptor in the presence of Mg\(^{2+}\) (Fig. 1). C2 becomes susceptible to C1s and is cleaved into C2a and C2b (Nagasawa and Stroud, 1977). C2a remains complexed with C4b, whereas C2b is released in the microenvironment. C4b2a complex is known as the classical pathway C3 convertase (Kerr, 1980), in which the catalytic subunit C2a cleaves C3 (Fig. 1).
C3 is the most abundant complement protein in serum. It consists of a 119-kDa α-chain and a 75-kDa β-chain linked together by a single disulfide bond and non-covalent forces (Fig. 2) (Lambris, 1988). Similarly to C4, C3 possesses an internal thioester bond hidden within a hydrophobic pocket in native C3 (Janssen et al., 2006; Law and Dodds, 1990, 1997). Upon C3 cleavage at a single site on the α-chain, C3a, called anaphylatoxin, is released into the environment (Hugli, 1975), while C3b binds to nearby surfaces via an exposed thioester (Law and Levine, 1977; Law and Dodds, 1997; Sim et al., 1981). Some of the C3b binds to CP C3 convertase (C4b2a) to form C4b2a3b complex, which is termed the CP C5 convertase (Fig. 1) (Takata et al., 1987).

**Figure 1.** Schematic overview of the complement activation cascade (modified from Miwa and Song, 2001).
1.2.2 Alternative pathway (AP)

The activation of the alternative complement pathway begins with the activation of C3 and it requires factors B and D, properdin, and Mg\(^{2+}\), all of which are present in normal serum (Fig. 1). The classical pathway is not the only source of the C3b participating in activation of the alternative pathway. C3b is also generated spontaneously by hydrolysis of the internal thioester bond in the native fluid phase C3 (tick-over mechanism) (Lachmann and Hughes-Jones, 1984). Native C3 is hydrolyzed to an active intermediate known as C3(H\(_2\)O) (Pangburn et al., 1981). C3(H\(_2\)O) binds factor B, making the latter susceptible to cleavage by factor D (Lesavre and Müller-Eberhard, 1978). Factor D is a serine protease and cleaves Factor B into Ba and Bb. Generated C3(H\(_2\)O)Bb complex acts as the fluid phase alternative pathway C3 convertase. Fluid phase C3b, generated by the convertase, is deposited on a bacterial surface via its thioester group exposed on that surface (Law and Dodds, 1990, 1997). In the presence of Mg\(^{2+}\) it forms a complex with factor B (Fig. 1). The resulting C3bB is activated to become AP C3 convertase, C3bBb, by factor D and is stabilized by properdin (Fig. 1).

AP convertases are crucial for the C3b amplification loop (Fig. 3). The serine protease Bb of the AP convertase very efficiently degrades C3, generating large amounts of C3b, which in turn can become deposited on nearby surfaces or contribute to C5 convertase formation via binding to C3 convertases of AP and CP. In consequence, the AP amplifies activation of the CP (Lachmann and Hughes-Jones, 1984).

1.2.3 Lectin pathway (LP)

The C4 activation can also be achieved without the participation of the antibodies and C1 via the lectin pathway. This pathway is initiated by MBL and the MBL-associated serine proteases: MASP-1 and MASP-2 (Sato et al., 1994; Thiel et al., 1997). MBL binds to certain mannose residues on many bacteria and subsequently interacts with MASP-1 and MASP-2. The MBL-MASP1-MASP2 complex is analogous to the C1 complex of the classical pathway. A major difference is, however, that C1 binds solely to a target tagged with specific antibodies, while MBL binds directly to mannose- or N-acetyl-glucosamine-rich polysaccharides commonly presented by a wide range of microorganisms (Jack et al., 2001). Upon activation, the MASPs cleave the downstream complement components C4 and C2 (Matsushita and Fujita, 1992; Thiel
et al., 1997). From this point onwards, the lectin pathway is identical to the classical pathway (Fig. 1).

1.2.4. Lytic pathway

The classical, lectin, and alternative pathways end with the formation of C5 convertase that then leads to the assembly of MAC via the lytic pathway. C5 convertases (C3b4b2a, C3bBbC3b) generated by one of the pathways cleave C5 (Fig. 1) (Cooper and Müller-Eberhard, 1970; DiScipio et al., 1983). C5 comprises two chains linked together with one disulfide bond. C5, however, lacks the internal thioester group characteristic of C3 and C4 (DiScipio et al., 1983). C5 cleavage generates two biologically active fragments: C5a, a potent chemokine, and anaphylatoxin (Cochrane and Müller-Eberhard, 1968; Ward and Newman, 1969), and C5b, an initial constituent in MAC assembly (Fig. 1).

C5b is very labile and needs to bind to C6 very rapidly (DiScipio et al., 1983). C5b6 subsequently binds C7 to yield the hydrophobic C5b67 complex (DiScipio et al., 1988; DiScipio, 1992). The C5b67 complex attaches to nearby membranes without harming the cells. Upon C8 binding, the resulting C5b678 complex inserts deeply into the membrane (Kolb and Müller-Eberhard, 1973; Tamura et al., 1972) and subsequently binds C9, causing its nonenzymatic polymerization (Tschopp et al., 1982b). In consequence, C9 undergoes hydrophilic-amphiphilic and conformational transition to a tubular structure (Tschopp et al., 1982a). Acquisition of C9 molecules leads to the formation of a pore-like structure of MAC which passes through the membrane (Tschopp et al., 1982a). The pore is approximately 110 Å wide (Podack and Tschopp, 1982) and has a hydrophilic lumen through which, according to the “doughnut theory”, cell contents leak out and water is drawn inside, generating the osmotic pressure which causes rupture of cellular membranes (Lachmann, 2006). In addition, leakage through the pore may be assisted by passage of the solutes and water through the “leaky patches” formed in the membrane upon MAC insertion (Lachmann, 2006).
Table 2. Fluid-phase regulators of the complement system

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Size (kDa)</th>
<th>Serum conc. (μg/ml)</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1 inhibitor</td>
<td>105</td>
<td>200</td>
<td>blocks active sites on C1s ans C1r or removes them from the C1 complex</td>
<td>Harrison, 1983; Harpel and Cooper, 1975; Ziccardi and Cooper, 1978; Ziccardi, 1982, 1983; Jiang et al., 2001</td>
</tr>
<tr>
<td>(C1-INH)</td>
<td></td>
<td></td>
<td>prevents C1 autoactivation</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>inhibits formation of AP C3 convertase</td>
<td></td>
</tr>
<tr>
<td>FH</td>
<td>150</td>
<td>500</td>
<td>accelerates decay of AP C3 convertase</td>
<td>Nilsson and Müller-Eberhard, 1965; Pangburn et al., 1977; Weiler et al., 1976; Whaley and Ruddy, 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>inhibits formation of AP C3 convertase</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>cofactor for FI</td>
<td></td>
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<tr>
<td>C4bp</td>
<td>570</td>
<td>200</td>
<td>accelerates decay of CP/LP C3 convertase</td>
<td>Barnum, 1991; Gigli et al., 1979; Dahlbäck, 1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>inhibits formation of CP/LP C3 convertase</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>cofactor for FI</td>
<td></td>
</tr>
<tr>
<td>FI</td>
<td>90</td>
<td>30</td>
<td>cleaves C3b and C4b in the presence of cofactor</td>
<td>Ruddy and Austen, 1969; Ahearn and Fearon, 1989</td>
</tr>
<tr>
<td>Properdin</td>
<td>210</td>
<td>26</td>
<td>stabilizes AP C3 convertase</td>
<td>Fearon and Austen, 1975; Medicus et al., 1976; Smith et al., 1984</td>
</tr>
<tr>
<td>Clusterin</td>
<td>70</td>
<td>50</td>
<td>prevents MAC insertion by binding to terminal complement complexes C5b-7, C5b-8, and C5b-9</td>
<td>Tsuruta et al., 1990; Choi et al., 1989; Murphy et al., 1989; Tschopp et al., 1993</td>
</tr>
<tr>
<td>(SP40,40)</td>
<td></td>
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<tr>
<td>Vitronectin</td>
<td>75</td>
<td>500</td>
<td>prevents MAC insertion by binding to terminal complement complexes C5b-7</td>
<td>Podack and Müller-Eberhard, 1979</td>
</tr>
<tr>
<td>(S-protein)</td>
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</table>
1.3 Complement regulation

1.3.1 Fluid-phase complement regulators

Tight regulation of the complement system crucial in preventing bystander injury of host cells and systemic depletion of complement, is provided by fluid phase complement regulators (Table 2). These regulate complement activation at C1 (C1 inhibitor), C3 (factor H, C4b-binding protein, properdin) or MAC (clusterin, vitronectin) levels. The complement inhibitors that act at the C3 level function as cofactors for serine protease FI-mediated cleavage of C3b and C4b. In this way, they prevent formation of C3 convertases or downregulate their activity. The latter function, termed decay-accelerating activity, relies on displacing the C3 convertase enzymatic subunits C2a or Bb.

1.3.1.1 Factor I

FI is a 90-kDa (heavy chain, 50 kDa; light chain, 38 kDa) plasma protease which circulates in its active form at a concentration of \(30 \mu g/ml\) (Ruddy and Austen, 1969). It cleaves arginyl bonds of its substrates C3b and C4b only when these are complexed to one of the complement regulators. Membrane cofactor protein (MCP), complement receptor type 1 (CR1), and C4bp contribute to the cleavage of both C3b and C4b, whereas FH exclusively participates in the cleavage of C3b (Ahearn and Fearon, 1989; Blom et al., 2003; Fujita and Nussenzweig, 1979; Pangburn et al., 1977; Seya et al., 1986).

Inactivation of C3b by FI can occur at three sites of the \(\alpha\)'-chain and results in the generation of iC3b1, iC3b2, C3c or C3dg (Fig. 2A) (Davis and Harrison, 1982; Lambris et al., 1996; Medicus et al., 1983; Ross et al., 1982). The latter two products are generated when CR1 serves as a co-factor (Ahearn and Fearon, 1989). FI-mediated inactivation of C4b targets two sites of the \(\alpha\)-chain and results in the generation of C4c and C4d fragments (Fig. 2B).
Figure 2. Schematic representation of C3 (A) and C4 (B) cleavage sites (modified from Morgan, 2000; Sahu and Lambris, 2001)
1.3.1.2 Factor H protein family

1.3.1.2.1 Factor H

FH was identified as a β1H globulin by Nilsson and Müller-Eberhard (Nilsson and Müller-Eberhard, 1965). It is a soluble single-chain glycoprotein of 150 kDa present in plasma at a concentration of ~500 μg/ml. FH accelerates the decay of the AP C3 convertase, competes with FB for C3b binding, and acts as a cofactor for FI-mediated C3b inactivation (Fig. 3) (Pangburn et al., 1977; Weiler et al., 1976; Whaley and Ruddy, 1976). In this way, FH downregulates complement in the fluid phase and on cell surfaces.

Figure 3. FH and C4bp control steps. Solid lines, complement activation; dashed lines, complement inhibition

FH distinction of self from non-self (AP activators) surfaces is based on recognition of polyanionic substances such as sialic acids and glycosaminoglycans (Fearon, 1978; Meri and Pangburn, 1990). These coat the host cells but, in general, do not cover foreign cell membranes. High-affinity interaction between FH and C3b occurs when the latter is accompanied by polyanions and results in the inhibition of AP. In the
absence of polyanions, however, FH affinity for C3b dramatically decreases, and in consequence AP activation proceeds (Kazatchkine et al., 1979; Pangburn et al., 1983). FH is composed of 20 SCRs joined by short linker regions of 3-8 amino acids. SCRs are arranged in a continuous fashion and resemble a string of beads (Discipio, 1992; Ripoche et al., 1988). SCRs 1-4, SCRs 12-14, and SCRs 19-20 provide the binding sites for C3b, whereas SCR7, SCR 13, and SCRs 19-20 bind heparin and polyanions (Alsenz et al., 1985; Blackmore et al., 1996, 1998b; Gordon et al., 1995; Jokiranta et al., 1996, 2000; Kühn et al., 1995; Pangburn et al., 1991; Prodingler et al., 1998; Sharma and Pangburn, 1996). Decay-accelerating and cofactor functions have been ascribed to SCRs 1-4 (Gordon et al., 1995; Kühn et al., 1995; Kühn and Zipfel, 1996) (Fig. 4).

![ FH protein family (modified from Jozsi et al., 2005; Zipfel et al., 2002). SCRs are aligned according to regions of homology. Binding sites for C3b and heparin are shown for FH and FHL-1.](image)

Deficiency of FH in humans has been associated with membranoproliferative glomerulonephritis (MPGN) and atypical hemolytic uremic syndrom (aHUS). Complete FH deficiency is, however, a rare phenomenon (Reis et al., 2006). Missense mutations clustered within SCRs 19-20 have been exclusively linked to aHUS (Ault, 2000; Dragon-Durey et al., 2004; Jokiranta et al., 2006; Manuelian et al., 2003;
Neumann et al., 2003; Zipfel, 2001), a rapidly progressing renal disease that manifests as an endothelial cell damage, hemolysis, massive AP activation, and kidney failure (Ruggenenti et al., 2001). As the C terminus is crucial for binding to C3b, C3d, heparin, and polyanions, mutation within the C-terminal SCRs abolishes critical FH functions for complement regulation.

1.3.1.2.2 Factor H-like protein (FHL-1)

FHL-1 (reconectin) is an alternatively spliced product of the FH-encoding gene (Estaller et al., 1991). It comprises seven SCRs, being identical to the first seven N-terminal SCRs of FH and the unique C-terminal sequence of four amino acids (Fig. 4). Thus, FHL-1, similar to FH, displays cofactor and decay-accelerating activities and possibly contributes to complement regulation in vivo (Kühn and Zipfel, 1996; Zipfel and Skerka, 1999). However, the plasma concentration of this 42 kDa protein is approximately one-tenth that of FH.

1.3.1.2.3 Factor H-related proteins (FHR)

FHRs are encoded by separate genes located within the regulators of complement activation (RCA) gene cluster on chromosome 1 (Zipfel et al., 1999). To date, five human FHRs have been identified, and these share structural similarities with FH (Fig. 4). FHR-1 consists of five SCRs and is found in the 37-kDa (FHR1-α) or 43-kDa (FHR1-β) forms with one or two carbohydrate-side residues, respectively (Estaller et al., 1991; Skerka et al., 1991). FHR-2 is composed of four SCRs and exists in a glycosylated (29-kDa) or in a non-glycosylated (24-kDa) form (Skerka et al., 1992). FHR-3 and FHR-4B are similar to each other and are organized into five SCRs. FHR-3 occurs in several glycosylated forms in human plasma (ranging from 35 kDa to 56 kDa), whereas FHR-4B, a 42-kDa protein, is one of the two FHR-4 gene products derived by means of alternative splicing (Jozsi et al., 2005; Skerka et al., 1993, 1997). The other product of the FHR-4 gene is FHR-4A. This glycosylated protein of 86 kDa consists of nine SCRs, due to duplication of four SCRs (Fig. 4) (Jozsi et al., 2005). FHR 5 contains nine SCRs homologous to those of FH (McRae et al., 2001).

The structural similarity between FHR proteins and FH suggests that FHRs share some of the FH activities. Indeed, FHR-3, FHR-4, and FHR-5 have been shown to bind C3b while FHR-1, FHR-3, and FHR-5 bind heparin (Hellwage et al., 1999; McRae et al.,...
2001, 2005; Prodinger et al., 1998). In addition, FHR-3 and FHR-4 have been suggested to modify the conformation of C3b to enhance FH cofactor activity (Hellwage et al., 1999). FHR-5 is a relatively weak cofactor for FI; it is able, however, to inhibit the C3 convertase activity (McRae et al., 2005).

1.3.1.3 C4b-binding protein

C4bp is a large (570 kDa), spider-like glycoprotein (Chung et al., 1985; Dahlbäck, 1983; Dahlbäck et al., 1983), present in plasma at a concentration of 200 μg/ml. It circulates complexed with the vitamin K-dependent protein S. Protein S, bound to SCR1 of the C4bp β-chain (Härdig et al., 1993; Härdig and Dahlbäck, 1996), does not display its anticoagulant function (Dahlbäck, 1986). It can, however, direct C4bp to negatively charged surfaces and apoptotic cells (Schwalbe et al., 1990; Webb et al., 2002).

![Figure 5. Schematic structure of C4bp (modified from Blom et al., 2004). Binding sites for C4b indicated.](image)

C4bp consists of seven identical α-chains (70 kDa) and one β-chain (45 kDa) (Hillarp and Dahlbäck, 1990). The α- and β-chains are composed of eight and three SCRs, respectively (Fig. 5). These chains are linked together by disulfide bonds at their most C-terminal parts (Kask et al., 2002).
C4bp down-regulates complement activity (Fig. 3), acting as a fluid-phase inhibitor of the classical and lectin pathway activation steps that involve C4b (Barnum, 1991). High C4bp concentrations, however, can also force decay of AP C3 convertase (Blom et al., 2003). N-terminal SCRs 1-3 of C4bp α-chains bind C4b (Kask et al., 2002), which renders C4b susceptible to FI-mediated cleavage (Fig. 2). In addition, C4bp prevents the assembly of the CP C3-convertase (C4b2a) and accelerates a natural decay of the preformed convertase by forcing C2a dissociation from the complex (Gigli et al., 1979). Heparin can disturb the interaction between C4bp and C4b because it also binds SCRs 1-3 of C4bp α-chains (Blom et al., 2001; Hessing et al., 1990).

1.3.2 Membrane-bound complement regulators

In addition to fluid phase regulators, cells are equipped with membrane-bound complement inhibitors that block complement activation at C3 (DAF, MCP, CR1) or MAC level (CD59) (Morgan and Harris, 1999). Some pathogens, however, use these membrane-bound regulators for adhesion or invasion, or both, because they are widely expressed by human cells (Table 3).

1.4 Functions of the complement system

1.4.1 Opsonin-mediated clearance of microbes and host debris

A crucial complement function is to facilitate the phagocytosis of pathogens and the clean-up of host cell debris. Phagocytic cells are known to bear complement receptors such as CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18). CR1 recognizes the targets coated with C1q, C3b, C4b, and iC3b, whereas the latter two are iC3b-specific (Klickstein et al., 1988, 1997; Krych et al., 1992; Krych-Goldberg and Atkinson, 2001; Newman et al., 1984). These opsonins immobilize microbes on the phagocytic cell surface, which ensures their efficient uptake and destruction.
Table 3. Membrane-bound regulators of the complement system and their interaction with pathogens

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Function</th>
<th>Structure</th>
<th>Regulatory activity</th>
<th>Pathogens</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCP (CD46)</td>
<td>Cofactor for FI-mediated cleavage of C3b and C4b</td>
<td>51-68 kDa transmembrane glycoprotein&lt;br&gt;4 SCR domains&lt;br&gt;transmembrane region and cytosolic tail</td>
<td>SCR 3-4</td>
<td>Measles virus, Neisseria gonorrhoeae, Neisseria meningitidis, Streptococcus pyogenes</td>
<td>Liszewski et al., 1991; Naniche et al., 1993; Dörg et al., 1993; Källström et al., 1997; Okada et al., 1995</td>
</tr>
<tr>
<td>DAF (CD55)</td>
<td>Accelerates decay of CP and AP C3 convertases Ligand for leukocyte-activating antigen CD97</td>
<td>70 kDa GPI-anchored glycoprotein&lt;br&gt;4 SCR domains</td>
<td>SCR 2-4</td>
<td>picornaviruses, echoviruses, coxackieviruses&lt;br&gt;Escherichia coli</td>
<td>Lublin and Atkinson, 1989; Hamann et al., 1996; Evans and Almond, 1998; Nowicki et al., 1993</td>
</tr>
<tr>
<td>CR1 (CD35)</td>
<td>Cofactor for FI-mediated cleavage of C3b and C4b Accelerates decay of CP and AP C3 convertases Cofactor in iC3b cleavage to C3c and C3d Immune complexes processing and clearance</td>
<td>200 kDa transmembrane glycoprotein&lt;br&gt;30 SCR</td>
<td>SCR 1-2&lt;br&gt;SCR 8-9&lt;br&gt;SCR 15-16</td>
<td>Plasmodium falciparum</td>
<td>Ahearn and Fearon, 1989; Rowe et al., 1997</td>
</tr>
<tr>
<td>CD59 (protectin)</td>
<td>Inhibits MAC formation by association with C5b-8 and C5b-9</td>
<td>18-23 kDa GPI-anchored glycoprotein&lt;br&gt;4Y, 47F, 61Y, 62Y</td>
<td></td>
<td>Escherichia coli, Helicobacter pylori</td>
<td>Davies and Lachmann, 1993; Kieffer et al., 1994; Zhou et al., 1996; Rautemaa et al., 1998, 2001</td>
</tr>
</tbody>
</table>

MCP, membrane cofactor protein; DAF, decay-accelerating factor; CR1, complement receptor type 1
Immune complexes, small and soluble antigens derived from antigen-antibody complexes, are found in the circulation after most infections. C1q binds to immune complexes or to blebs of apoptotic cells (Korb and Ahearn, 1997; Navratil et al., 2001). C1q initiates the complement activation on their surfaces and that results in the deposition of C3b and C4b (Nauta et al., 2002). Apoptotic cells are subsequently recognized by tissue macrophages and phagocytosed. Ligation of C3b and C4b deposited on the immune complexes with CR1 on erythrocytes initiates removal of the complexes from the circulation (Schifferli et al., 1986). Erythrocytes carry this cargo to the liver and spleen to finally pass it on to macrophages.

1.4.2 Direct lysis of pathogens

Activation of the complement system leads to the generation of MAC on the surface of target cells, thus causing their death. The complement system by this means plays an important role in host defense against Gram-negative bacteria. Gram-positive bacteria are, however, resistant to direct killing mediated by complement. A thick and impenetrable peptidoglycan layer protects the cytoplasmic membrane of Gram-positive bacteria from such MAC-induced damage. Although the complement system does not directly kill Gram-positive bacteria, it still controls the course of Gram-positive infections by its ability to opsonize and tag the microbes for phagocytic destruction (Moffitt and Frank, 1994).

1.4.3 Induction of inflammation

Complement cleavage fragments such as C3a, C4a, and C5a are able to induce a local inflammatory response (Hugli, 1981). Of the three, C5a has the highest inflammation-inducing activity. These anaphylatoxins act as stimulators and chemoattractants for those myeloid cells bearing anaphylatoxin-specific receptors. In addition, they act on blood vessels, increase vascular permeability, and induce endothelial cells for expression of adhesin molecules (DiScipio et al., 1999; Foreman et al., 1996; Gerard and Gerard, 1994). This all contributes to a rapid recruitment of phagocytic cells to the site of inflammation and promotes clearance of pathogens.

1.4.4 Shaping the specific immune response

Complement-derived fragments influence the antibody response of adaptive immunity. CD19 and CD21 (CR2) are constituents of the B cell co-receptor complex. Binding of
C3d to CD21 triggers CD19-mediated signaling, and this leads to B cell activation (Dempsey et al., 1996; Dempsey and Fearon, 1996). In addition, the α-chain of C4bp can activate B cells via the CD40 receptor in a manner similar to that of CD40L (Brodeur et al., 2003).

1.5 Bacterial mechanisms of complement evasion

Pathogenic microbes have developed a range of strategies to evade complement-mediated recognition and destruction. To achieve this goal, they have equipped themselves with surface factors which either directly interfere with the complement activation or bind host complement regulatory proteins. Microbial complement-evasion strategies include interference with the initial complement activation, with C3 convertase functions, or with MAC insertion.

1.5.1 Interference with initial complement activation

1.5.1.1 Binding of immunoglobulins via Fc portion

Some microbes interfere with classical pathway activation via binding of the immunoglobulin Fc portion, e.g., protein G of streptococci or protein A of Staphylococcus aureus (Björck and Kronvall, 1984; Forsgren and Sjöquist, 1966). Because the ligation of the Fc receptor on phagocytic cells with the Fc region of target-bound IgG leads to efficient phagocytosis, blocking of the Fc portion by protein A inhibits this process (Foster, 2005). In addition, it interferes with C1q binding to IgG, thereby inhibiting complement activation (Laky et al., 1985).
1.5.1.2 Immunoglobulin and C3/C3b degradation

Several microbes directly or by use of host factors degrade immunoglobulins and C3/C3b and block the complement activation. Cysteine proteinases, IdeS (IgG-degrading enzyme of *Streptococcus pyogenes*) and SpeB (streptococcal pyrogenic exotoxin B), of group A streptococci remove the Fc portion of bacterium-bound IgG (von Pawel-Rammingen and Björck, 2003). *Porphyromonas gingivalis prtH*-encoded protease degrades both IgG and C3 (Schenkein *et al.*, 1995), whereas *S. aureus* degrades these complement-activation initiators indirectly. *S. aureus* first binds host plasminogen and converts it to plasmin with the help of staphylokinase. In consequence, plasmin attached to the bacterial surface cleaves both: C3b and IgG (Rooijakkers *et al.*, 2005b). Yeast, *Candida albicans*, proteinases can also degrade C3 and IgG and prevent complement activation (Kaminishi *et al.*, 1995).

1.5.1.3 Binding of C1 inhibitor or C1q inhibition

The binding and utilization of C1 inhibitor (C1INH) occurs for several bacteria. Enterohemorrhagic *E. coli* O157:H7 secretes StcE, a protease of C1-INH. StcE recruits C1-INH to the cell surface and cleaves it to potentiate its ability to inhibit CP (Lathem *et al.*, 2004). This prevents complement activation and in consequence may restrict an inflammatory response at the infection site. In addition, *Bordetella pertussis*, the etiological agent for whooping cough, recruits C1-INH from human serum to prevent its own destruction (Marr *et al.*, 2007). Another mechanism to block the CP activation at C1 level has been reported to take place in helminthic parasites. Paramyosins from *Taenia solium* and *Schistosoma mansoni* bind C1q, block its activity, and prevent complement activation at its very early stage (Laclette *et al.*, 1992).

1.5.2 Inhibition of C3 convertases

1.5.2.1 Microbial factors directly interfering with C3 convertases

Some pathogens directly interfere with C3 convertase function. Staphylococcal complement inhibitor (SCIN) is a secreted protein that binds to AP- and CP/LP-C3 convertases, impairing their enzymatic activity. Because such binding stabilizes C3 convertases, it also prevents the formation of new convertases (Rooijakkers *et al.*, 2005a). Another mechanism is exploited by group A streptococci. SpeB degrades host
serum properdin to inhibit the formation of functional AP C3-convertase and resist opsonophagocytosis (Tsao et al., 2006). Some microbes also disassemble C3 convertases. *Herpes simplex* virus (HSV) type 1 expresses the glycoprotein gC-1 which accelerates decay of the pre-formed C3 convertase. In addition, gC-1 binds C3b and blocks its interaction with properdin, thereby inhibiting formation of AP C3 convertase (Fries et al., 1986; Kostavasili et al., 1997).

### 1.5.2.2 Microbial factors mimicking FI-cofactors to accelerate decay of the C3 convertases

Some microorganisms mimic the host cofactors for FI-mediated cleavage of C3b and prevent their own destruction by using a mechanism similar to that exploited by mammalian cells. *Trypanosoma cruzi*, a causative agent of Chagas disease, expresses a glycoprotein gp160 that is functionally and genetically similar to DAF; gp160 is GPI-anchored, accelerates decay of C3 convertase, and has a coding sequence displaying ~60% similarity to the DAF-encoding gene (Norris et al., 1991). On the other hand, the amino acid sequence of the vaccinia virus C-control protein (VCP) shows homology to the C4bp sequence. This accounts for a functional similarity between the two proteins; SCR-composed VCP binds C4b, prevents formation of CP C3 convertase, and in addition accelerates decay of pre-formed convertases (Kotwal and Moss, 1988; Kotwal et al., 1990).

### 1.5.2.3 Microbial factors binding host complement regulators

Pathogens that do not express surface factors that mimic host regulatory proteins often interact with host complement regulators to protect their own surfaces. Several microorganisms bind specific fluid-phase regulators, such as FH, FHL-1 and C4bp that trigger C3 convertase decay and C3b/C4b inactivation.

Pathogens that acquire FH or FHL-1 gain an ability to disassemble AP C3 convertases and to degrade any C3b deposited on their surfaces. The interaction of FH with the sialic acid-covered host cells is known to block AP activation (Fearon, 1978). To bind FH, pathogens such as group B streptococci (Marques et al., 1992) and *N. gonorrhoeae* (Ram et al., 1998b) cover their surfaces with sialic acid whereas
trypomastigotes of *T. cruzi* express an unusual cell-surface trans-sialidase that enables the parasite to sialylate its surface rapidly (Tomlinson *et al.*, 1994).

Bacterial surface proteins also exist that are able to bind to FH, to FHL-1, or both. BbCRASP-3 to -5 of *Borrelia burgdorferi* (Kraiczy *et al.*, 2001), the β protein of group B streptococcus (Areschoug *et al.*, 2002), Por1A of *N. gonorrhoea* (Ram *et al.*, 1998a), or Hic and PspC of *Streptococcus pneumoniae* (Dave *et al.*, 2001; Janulczyk *et al.*, 2000) bind FH, whereas BaCRASP-3 of *Borrelia afzelii* can interact with FHL-1 (Kraiczy *et al.*, 2001). Some microbial proteins bind both FH and FHL-1. These include M protein and fibronectin-binding protein (Fba) of *S. pyogenes* (Horstmann *et al.*, 1988; Kotarsky *et al.*, 2001; Pandiripally *et al.*, 2002) and BbCRASP-1 and BbCRASP-2 of *B. burgdorferi* (Kraiczy *et al.*, 2001).

FH-binding sites have been identified for several of these proteins (Fig. 6): BbCRASP-1, -3 and -5 engage the C terminus; β protein, PspC, and Hic bind to the middle part; and M protein and Fba interact with the N-terminal region of FH (Blackmore *et al.*, 1998a; Dave *et al.*, 2004; Duthy *et al.*, 2002; Jarva *et al.*, 2002, 2004; Kotarsky *et al.*, 1998; Kraiczy *et al.*, 2001; Pandiripally *et al.*, 2003).

![Figure 6](image)

**Figure 6.** Schematic representation of factor H domains interacting with microbial surface proteins (modified from Zipfel *et al.*, 2002).

Several microbes express surface proteins snatching the host CP/LP regulator, C4bp, to accelerate decay of CP C3 convertase and degrade C4b deposited on the microbial surface. These proteins include the M protein of *S. pyogenes*, Por1A and Por1B of *N. gonorrhoeae*, OmpA of *E. coli* K1, UspA1 and UspA2 of *Moraxella catarrhalis*, and FHA of *Bordetella pertussis* (Berggård *et al.*, 1997; Johnsson *et al.*, 1996; Nordström
et al., 2004; Prasadarao et al., 2002; Ram et al., 2001). Most of the C4bp-binding proteins interact with the very N-terminal SCRs of C4bp α-chains, whereas UspA-1 and UspA-2, in addition to N-terminal SCR2, bind C-terminal SCR5 and SCR7 (Fig. 7) (Accardo et al., 1996; Berggård et al., 2001; Nordström et al., 2004; Prasadarao et al., 2002; Ram et al., 2001).

**Figure 7.** Schematic representation of C4bp domains interacting with microbial surface proteins (modified from Blom et al., 2004). Binding sites for bacterial factors on C4bp α-chains indicated.

### 1.5.3 Interference with MAC formation (terminal pathway)

**1.5.3.1 Physical barriers**

Gram-positive bacteria are resistant to complement-mediated killing due to the presence of thick peptidoglycan layer that prevents access of C5b-9 to the cytoplasmic membrane (Joiner et al., 1983). Also long O-polysaccharide side chains of *Salmonella minnesota* interfere with MAC insertion into the outer bacterial membrane. C5b-9 is generated on the distal parts of LPS molecules which sterically hinder access of MAC to the outer membrane (Joiner et al., 1986).
1.5.3.2 Microbial proteins interfering with membrane attack complex

Resistance to the final step of the complement cascade is a prerequisite for the resistance of Gram-negative bacteria to complement-mediated cytolysis. One example of protein strongly inhibiting the terminal complement pathway is the *E. coli* outer membrane protein TraT. It probably inhibits the formation of C5b-6 complex or causes its structural alteration to render it nonfunctional (Pramoonjago *et al.*, 1992). Another example is *B. burgdorferi* CD59-like protein, which like human membrane-bound CD59, prevents C9 polymerization and formation of MAC (Pausa *et al.*, 2003).

1.5.3.3 Acquisition of host terminal pathway regulators

To date, few reports describe recruitment of normally membrane-bound terminal pathway regulators from serum; for instance, *E. coli* and *H. pylori* bind CD59 from the fluid phase to resist MAC-induced lysis (Rautemaa *et al.*, 1998, 2001). On the other hand, *M. catarrhalis* UspA2 binds a fluid-phase regulator, vitronectin, to block MAC insertion into the cell membrane (Attia *et al.*, 2006).

1.5.4 Interference with generation and function of complement-derived chemoattractants

Gram-positive bacteria, though resistant to MAC-induced cytolysis, inhibit the terminal pathway to prevent the release of complement chemoattractants such as C5a. *S. aureus*, for example, produces a superantigen-related protein SSL7 that binds to C5 and in consequence blocks its cleavage into C5a and C5b (Langley *et al.*, 2005). Group A and Group B streptococci do not prevent C5 cleavage but inactivate the C5a formed. Cell wall-anchored C5a peptidase expressed by these bacteria, though unable to cleave the whole C5, efficiently cleaves C5a (Chmouryguina *et al.*, 1996; Cleary *et al.*, 1992; Wexler *et al.*, 1985; Wexler and Cleary, 1985). Another strategy to avoid C5a action has been developed by *S. aureus*. It secretes CHIPS, an inhibitory protein, that binds to receptors for C5a and formylated peptides (C5aR and FPR, respectively) on phagocytic cells. This prevents the phagocytic cells from sensing the early signs of bacterial infection such as C5a (de Haas *et al.*, 2004).
1.6 Genus *Yersinia*

*Yersinia* are Gram-negative rods belonging to the family *Enterobacteriaceae*. The genus *Yersinia* comprises fourteen species. Three of them are human pathogens: *Y. pestis* and the enteropathogens *Y. pseudotuberculosis* and *Y. enterocolitica* (Brubaker, 1991). All three species harbor a 70-kb virulence plasmid (pYV) essential for immune evasion and survival in mammalian host (Cornelis *et al.*, 1998) and commonly display a tropism for lymphoid tissue (Brubaker, 1991). Enteropathogenic yersiniae are motile at 25°C and non-motile at 37°C due to repression of *fliA*, the positive regulator of flagellin structural genes. *Y. pestis* is non-motile regardless of the growth temperature due to several mutations in flagellar genes (reviewed in (Minnich and Rohde, 2007). In addition to its difference in motility, *Y. pestis* causes a distinctive disease and differs in ecology and epidemiology from the enteropathogenic yersiniae.

1.6.1 *Y. pestis*

*Y. pestis* is a causative agent of plague, a systemic infection in mammals. Bubonic plague is initiated by the bite of an infected flea. As the proventriculus of the flea is blocked by *Y. pestis* aggregates, the flea regurgitates the bacterial mass into the bite site when it attempts to feed (Bacot and Martin, 1914). By means of the flea bite, bacteria are injected intradermally into the mammalian host, where they begin to multiply at the site of infection. Via the lymphatic system and bloodstream, bacteria disseminate to the lymph nodes (Perry and Fetherston, 1997). The bacteria multiply in the lymph nodes and induce an inflammatory response resulting in swollen and painful lymph nodes, termed buboes. As the infection proceeds, bacteria enter the bloodstream, causing septicemia, and infect deeper organs, such as the liver and spleen (Brubaker, 1991; Perry and Fetherston, 1997). When bacteria reach the lungs, the disease takes the form of highly infectious pneumonic plague. Individuals with pneumonic plague transmit the bacteria by spreading infectious droplets. Inhalation of *Y. pestis* aerosols results in development of a pneumonic plague and death due to rapidly progressing sepsis.

*Y. pestis* harbors, in addition to the pYV, common in enteropathogenic yersiniae, two extra plasmids pMT1 (pFra) and pPla (pPCP1) (Hu *et al.*, 1998; Lindler *et al.*, 1998). Ability to survive in the flea gut is provided by pMT1-encoded phospholipase D (*Yersinia* murine toxin) while pPla-encoded plasminogen activator, Pla, is required for
the bacteria to penetrate mammalian tissues and disseminate from the site of the inoculum (Brubaker, 1991; Hinnebusch et al., 2002). Flea-borne transmission requires a chromosomally located hemin storage locus (hms). Hms-encoded outer membrane proteins contribute to biofilm formation and blocking of the flea proventriculus (Hinnebusch et al., 1996).

The three biovars of *Y. pestis* are differentiated on the basis of their ability to ferment glycerol and reduce nitrate: Antiqua, Mediaevalis and Orientalis. Historically, each biovar is associated with one of the three pandemics, the Justinian plague (541-767) has been assigned to the biovar Antiqua, the Black Death (1346-1850) to Mediaevalis (Devignant, 1951; Guiyoule et al., 1994), and Biovar Orientalis is responsible for the most recent plague epidemics (1894-present). This pandemic-biovar relationship has been recently substantiated by ribotyping (Guiyoule et al., 1994) and by IS100 insertion element RFLP (restriction fragment length polymorphism) analyses of *Y. pestis* strains (Achtman et al., 1999). *Y. pestis* strains subjected to comparative sequencing analyses show negligible nucleotide variation, as *Y. pestis* is a recently emerged pathogen (Achtman et al., 1999; Adair et al., 2000). However, IS100-based, multi locus VNTR (variable-number tandem repeat) and single nucleotide polymorphism (SNP) marker analyses provide the high resolution required for estimation of genetic relationship of *Y. pestis* strains (Achtman et al., 1999; Adair et al., 2000; Touchman et al., 2007).

### 1.6.2 Enteropathogenic *Yersinia*

The enteropathogenic *Yersinia* are widespread. *Yersinia* infect an array of animal hosts, including mammals and avian species. *Y. pseudotuberculosis* and *Y. enterocolitica* are usually acquired by ingestion of contaminated food or water (Naktin and Beavis, 1999). They pass through the acidic content of the stomach and reach the small intestine. There, they are shuttled across the intestinal epithelium by the M cells of Peyer’s patches and gain access to subepithelial space (Autenrieth and Firsching, 1996; Marra and Isberg, 1997; Simonet et al., 1990). Subsequently, the bacteria reach the mesenteric lymph nodes and in some cases spread further to the liver and spleen, where they multiply and induce an inflammatory response resulting in gastroenteritis with mesenteric lymphadenitis and terminal ileitis. *Y. enterocolitica* due to expression of the heat-stable toxin (Yst) causes more severe and often bloody diarrhea in children than does *Y. pseudotuberculosis* (Delor and Cornelis, 1992). Although the disease is
mostly self-limiting and restricted to the gastrointestinal tract, bacteria may occasionally induce secondary post-infectious sequelae such as arthritis, erythema nodosum, myocarditis or glomerulonephritis (Baba et al., 1991; Bottone, 1997; Davenport et al., 1987; Krober et al., 1983; Leino et al., 1980; Takeda et al., 1991; Tertti et al., 1984).

1.6.2.1 *Y. pseudotuberculosis*

Both *Y. pseudotuberculosis* and *Y. enterocolitica* are soil- and water-borne enteropathogens causing gastroenteritis. *Y. pestis*, which causes a distinct disease, and is an obligate pathogen found solely in arthropod vectors and mammalian hosts, is paradoxically very closely related to *Y. pseudotuberculosis*. In fact, population genetic studies have suggested that *Y. pestis* evolved from *Y. pseudotuberculosis* 9,000 to 40,000 years ago (Achtman et al., 1999; Achtman, 2004). Furthermore, *Y. pestis* and *Y. pseudotuberculosis* genome sequences show nearly 97% homology (Chain et al., 2004). As the LPS O-ag gene cluster of *Y. pseudotuberculosis* serotype O:1b shows 98.9% identity to the cryptic O-ag gene cluster of *Y. pestis*, evidence is strong, that the plague bacterium evolved from *Y. pseudotuberculosis* serotype O:1b (Skurnik et al., 2000). *Y. pseudotuberculosis* is grouped into 21 serotypes based on differences in LPS O-ag profiles (Skurnik et al., 2000). Based on the distribution of superantigen toxins (YPMa-c), high-pathogenicity island (HPI) and R-HPI (only the right-hand part of the HPI present), *Y. pseudotuberculosis* strains can be further divided into six (genetic) subgroups (Fukushima et al., 2001) (Table 4). Serotypes of subgroups 1, 3, and 4 are predominantly found in the Far East (Russia, China, Korea, and Japan); serotypes of subgroup 2 in the West (Europe, America, Australasia); and strains belonging to subgroups 3 and 5 can be isolated throughout the world.

Many of the insecticidal toxin genes occur in *Y. pseudotuberculosis*, which has, among *Enterobacteriaceae*, a unique ability to infect fleas. Interestingly, in *Y. pestis* many insecticidal toxin genes are pseudogenes (Hinchliffe et al., 2003; Parkhill et al., 2001) because the plague bacterium has to persist in the flea gut and needs the living flea for its life cycle.
1.6.2.2 Y. enterocolitica

Y. enterocolitica has evolved independently from Y. pseudotuberculosis and Y. pestis (Wren, 2003). Based on their biochemical heterogeneity, six Y. enterocolitica biogroups are identifiable (Table 5). Non-pathogenic bacteria lacking the pYV belong to the biogroup 1A, whereas pathogenic pYV-bearing Y. enterocolitica are divided into two lineages: low-virulence bacteria including biogroups 2 to 5 (non-lethal in mice).
and high-virulence biogroup 1B (lethal in mice) (Bottone, 1997). These biogroups differ in their geographic distribution; strains belonging to biogroup 1B are found predominantly in North America, biogroups 2 to 5 strains mainly in Europe and Japan, and strains belonging to biogroup 1A are distributed throughout the world (Bottone, 1997). These biogroups are further subgrouped into serotypes based on variation in the LPS O-ag (Table 5).

**Table 5.** *Y. enterocolitica* biogroups and serotypes (modified from Bottone, 1999).

<table>
<thead>
<tr>
<th>Y. enterocolitica</th>
<th>avirulent</th>
<th>low virulence</th>
<th>high virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>biogroups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1A</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>serotypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O:5</td>
<td>O:9</td>
<td>O:1,2,3</td>
<td>O:3</td>
</tr>
<tr>
<td>O:6,30</td>
<td>O:5,27</td>
<td>O:5,27</td>
<td></td>
</tr>
<tr>
<td>O:7,8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O:18</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>O:46</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
</tbody>
</table>

### 1.6.3 Virulence determinants of pathogenic *Yersinia*

The three pathogenic *Yersinia* species require a 70-kb virulence plasmid (pYV), encoding the proteins of the type III secretion system for full virulence (Viboud and Bliska, 2005). However, pYV, although necessary, alone is not sufficient to cause disease (Heesemann and Laufs, 1983; Heesemann *et al.*, 1984). *Yersinia* pathogenesis additionally involves multiple genes located in the chromosome of the bacterium. Since *Y. enterocolitica* and *Y. pseudotuberculosis* lifestyle differs dramatically from that of *Y. pestis*, several plasmid- and chromosome-located genes required for enteropathogenicity are pseudogenes in the plague bacterium (Tables 6 and 7).
Table 6. Overview of the most important chromosomally-encoded virulence factors of pathogenic *Yersinia*.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Function</th>
<th><em>Y. enterocolitica</em></th>
<th><em>Y. pseudotuberculosis</em></th>
<th><em>Y. pestis</em></th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ail</strong> (attachment and invasion locus)</td>
<td>attachment to and invasion into tissue culture cell</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Bartha <em>et al.</em>, 2007; Bliska and Falkow, 1992; Kolodziejek <em>et al.</em>, 2007; Miller and Falkow, 1988; Yang <em>et al.</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>resistance to complement-mediated killing</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><strong>Inv</strong> (invasin)</td>
<td>binds β1-integrins to promote bacterial translocation across intestinal epithelium</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
<td>Isberg and Leong, 1990; Marra and Isberg, 1997; Pepe and Miller, 1993; Simonet <em>et al.</em>, 1996</td>
</tr>
<tr>
<td></td>
<td>required for colonization of regional lymph nodes</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td><strong>Psa</strong> (pH-6 antigen)</td>
<td>hemagglutination of erythrocytes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Bichowsky-Slomnicki and Ben-Efraim, 1963; Huang and Lindler, 2004; Iriarte <em>et al.</em>, 1993; Iriarte and Cornelis, 1995; Liu <em>et al.</em>, 2006; Marra and Isberg, 1997; Yang <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>or Myf (mucoid <em>Yersinia</em> factor)</td>
<td>adhesion to eukaryotic cells</td>
<td>?</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance to phagocytosis</td>
<td>?</td>
<td>?</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td><strong>Yst</strong> (Yersinia heat-stable toxin)</td>
<td>activates guanylate cyclase to increase cyclic GMP concentration in intestinal cells; induces fluid loss from the cells and diarrhea</td>
<td>Yes</td>
<td>–</td>
<td>–</td>
<td>Delor <em>et al.</em>, 1990; Delor and Cornelis, 1992; Robins-Browne <em>et al.</em>, 1979</td>
</tr>
<tr>
<td><strong>Sod</strong> (superoxide dismutase)</td>
<td>detoxification of reactive oxygen species, resistance to bactericidal activity of phagocytic cells</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
<td>Roggenkamp <em>et al.</em>, 1997; Sebbane <em>et al.</em>, 2006</td>
</tr>
<tr>
<td></td>
<td>required for full virulence</td>
<td>Yes</td>
<td>?</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td><strong>YPM</strong> (Y. pseudotuberculosis-derived mitogen)</td>
<td>superantigenic toxin that stimulates proliferation of polyclonal T lymphocytes</td>
<td>–</td>
<td>Yes</td>
<td>–</td>
<td>Abe <em>et al.</em>, 1993; Miyoshi-Akiyama <em>et al.</em>, 1993</td>
</tr>
<tr>
<td>LPS (lipopolysaccharide)</td>
<td>O-antigen</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>effective colonization of host tissues in early stage of infection</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance to complement-mediated killing</td>
<td>Yes/No</td>
<td>No</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Core</td>
<td>invasion of deeper tissues</td>
<td>Yes</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance to cationic antimicrobial peptides</td>
<td>Yes</td>
<td>?</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>resistance to complement-mediated killing</td>
<td>Yes/No</td>
<td>?</td>
<td>?</td>
<td></td>
</tr>
<tr>
<td>LipidA</td>
<td>resistance to cationic antimicrobial peptides</td>
<td>Yes (21°C &lt; 37°C)</td>
<td>Yes (21°C &lt; 37°C)</td>
<td>Yes (21°C &gt; 37°C)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>deacetylated lipid A at 37°C: reduced biological activity</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>required for the systemic spread</td>
<td>?</td>
<td>?</td>
<td>Yes</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HPI – encoded: (high-pathogenicity island)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>HPI present in highly pathogenic <em>Yersinia</em></td>
<td>biogroup 1B</td>
</tr>
<tr>
<td>Ybt (yersiniabactin)</td>
<td>siderophore that captures iron molecules bound to eukaryotic proteins</td>
</tr>
<tr>
<td><em>hms</em> locus (hemin storage)</td>
<td>biofilm formation in the flea and blocking of flea proventriculus</td>
</tr>
</tbody>
</table>

* a lack of expression, b to be determined, c different serotypes, d for details see Table 4

Sources:
- Anisimov *et al.*, 2005;
- Bengoechea *et al.*, 2003, 2004;

Sources:
<table>
<thead>
<tr>
<th>Plasmid/Factor</th>
<th>Function</th>
<th>Y. ent</th>
<th>Y. pestis</th>
<th>Y. pestis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( pYVV )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textbf{Yops}</td>
<td>\textit{(Yersinia outer proteins)}</td>
<td></td>
<td></td>
<td></td>
<td>Black and Bliska, 1997; Black et al., 1998; Black and Bliska, 2000;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Boland and Cornelis, 1998; Cabellos et al., 1992; Cornetis et al., 1987;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Denecker et al., 2001; Dukuzumuremyi et al., 2000; Erfurth et al., 2004;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Evdokimov et al., 2002; Galyov et al., 1993; Grosdent et al., 2002;</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hamid et al., 1999; Iriarte and Cornelis, 1998; Juris et al., 2000;</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>McDonald et al., 2003; Mills et al., 1997; Monack et al., 1997;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Navarro et al., 2007; Palmer et al., 1998, 1999; Persson et al., 1997;</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Schesser et al., 1998; Shao et al., 2003; Skrypek et al., 2003; Straley</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>and Bowmer, 1986; Trübsch et al., 2005; Viboud and Bliska, 2001; Yao</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>et al., 1999</td>
</tr>
<tr>
<td>\textbf{YopT}</td>
<td>cysteine protease with antiphagocytic activity; inactivates Rho GTPases</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(RhoA, Rac, Cdc42) by removal of lipid modifications and by disruption of</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>actin filaments</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textbf{YopE}</td>
<td>antiphagocytic activity due to inactivating GAP activity towards Rho GTPases</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>disrupts the actin cytoskeleton</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textbf{YopO}</td>
<td>antiphagocytic activity; autophosphorylating serine-threonine kinase</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>or \textbf{YpkA}</td>
<td>\textit{(Yersinia protein kinase ( \Delta ))}</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>thereof inactivating ( G_{\alpha q} ) family of ( G ) proteins,</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>thereby inhibiting ( G_{\alpha q} )-mediated signaling pathways</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>\textbf{YopH}</td>
<td>antiphagocytic protein tyrosine phosphatase; targets p130Cas, focal adhesion</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>kinase (FAK), paxillin, and Fyn-binding protein to disrupt focal adhesions;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>reduces ( \beta 1 ) integrin pathway activated by interaction with</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>invasin; blocks Fe-mediated respiratory burst of phagocytic cells;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>suppresses T- and B-cell activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**YopM**

- a leucine-rich protein of unknown precise cellular function;
- traffics to the nucleus via a vesicle-associated pathway;
- forms a complex with two cytoplasmic kinases RSK1 and PRK2 thereby activating them

**YopP/YopJ**

- an anti-inflammatory cystein protease; induces apoptosis in phagocytic cells, inhibits MAPK signaling pathways via binding to multiple members of the MAPK superfamily, e.g. MKKs and IKKβ; blocks NF-κB activation through inhibition of IκB degradation, inhibits TNF-α and IL-8 production, suppresses CD8 T cell response

<table>
<thead>
<tr>
<th><strong>YadA</strong> (Yersinia adhesin Δ)</th>
<th>adhesion to host cells, epithelial brush border, and mucus</th>
<th>Yes</th>
<th>Yes</th>
<th>– a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>entry into host cells via fibronectin-bound interaction with β1-integrin receptors</td>
<td>No</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>hemagglutination of erythrocytes</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>autoagglutination</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>serum resistance</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>induction of IL-8 production by epithelial cells</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>binding to ECM poteins:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>collagen types I, II, IV</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>fibronectin</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>laminin</td>
<td>Yes</td>
<td>Yes</td>
<td>–</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>references</th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Balligand et al., 1985; Bliska et al., 1993; Brubaker, 1967; China et al., 1993; Eitel and Dersch, 2002; Eitel et al., 2005; El Tahir et al., 2000; Emödy et al., 1989; Flügel et al., 1994; Heesemann and Grütter, 1987; Heise and Dersch, 2006; Kapperud et al., 1987; Mantle et al., 1989; Martinez, 1989; Mota et al., 2005; Paerregaard et al., 1991a, 1991b; Pilz et al., 1992; Roggenkamp et al., 1996; Rosqvist et al., 1988; Ruckdeschel et al., 1996; Schmid et al., 2004; Schulze-Koops et al., 1992, 1993; Skurnik et al., 1984; Skurnik and Wolf-Watz, 1989; Tamm et al., 1993; Tertti et al., 1992; Yang and Isberg, 1993</td>
</tr>
<tr>
<td>Protein/Phenotype</td>
<td>Description</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>LcrV or V-antigen</strong></td>
<td>anti-inflammatory, suppresses TNF-α and IFN-γ production and induces IL-10 release, inhibits neutrophil chemotaxis</td>
</tr>
<tr>
<td><strong>pPla (pPCP1)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Pla</strong> (plasminogen activator)</td>
<td>converts plasminogen into plasmin and inactivates the main plasmin inhibitor (anti-protease α2-antiplasmin)</td>
</tr>
<tr>
<td></td>
<td>mediates adhesion to extracellular matrix</td>
</tr>
<tr>
<td></td>
<td>degrades fibrin clots and extracellular matrix</td>
</tr>
<tr>
<td></td>
<td>cleaves complement factor C3</td>
</tr>
<tr>
<td></td>
<td>reduces chemoattraction of the inflammatory cells</td>
</tr>
<tr>
<td></td>
<td>required for the development of primary pneumonic plague</td>
</tr>
<tr>
<td></td>
<td>enhances bacterial adhesion to epithelial cell lines</td>
</tr>
<tr>
<td><strong>pMT1 (pFra)</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Ymt</strong> (Yersinia murine toxin)</td>
<td>phospholipase involved in the colonization and survival of Y. pestis in the midgut of the flea</td>
</tr>
<tr>
<td><strong>F1 capsule</strong></td>
<td>antiphagocytic, reduces bacterial binding to phagocytic cells</td>
</tr>
</tbody>
</table>

*a lack of expression*
1.6.4 Y. enterocolitica surface factors shaping serum resistance phenotype

1.6.4.1 YadA

YadA, formerly known as Yop1 or P1, forms lollipop-like structures (approx. 30 nm or 23 nm in length in Y. enterocolitica serotypes O:3 and O:8, respectively) on the surface of enteropathogenic Yersinia (Bölin et al., 1982, 1985; Hoiczyk et al., 2000). YadA is the most important virulence factor of Y. enterocolitica in the initial intestinal stage of the infection (Pepe et al., 1995). The role of YadA in Y. pseudotuberculosis infection remains unclear, however, as the yadA mutant is as virulent as the wild type Y. pseudotuberculosis strain (Bölin and Wolf-Watz, 1984). In Y. pestis, YadA is silenced as a result of a single nucleotide deletion in the yadA gene (Rosqvist et al., 1988; Skurnik and Wolf-Watz, 1989). The Y. pestis lifestyle differs dramatically from that of Y. enterocolitica and Y. pseudotuberculosis. The YadA required for adhesion to gut tissue appears to be redundant for the plague bacterium and therefore is represented by the pseudogene.

Expression of the yadA gene is thermodependent and under the control of the virF/lcrF gene which encodes for a transcriptional activator driving YadA expression at 37°C but not below 30°C (Lambert de Rouvroit et al., 1992; Skurnik and Toivanen, 1992).

YadA is a homotrimeric 200- to 240-kDa outer membrane protein with a monomer size of about 44 kDa (Mack et al., 1994; Nummelin et al., 2004; Skurnik et al., 1984). YadA belongs to the family of trimeric autotransporters and consists of an internal passenger domain and a C-terminal translocation unit (Fig. 8) (Cotter et al., 2005; Hoiczyk et al., 2000; Roggenkamp et al., 2003; Skurnik and Wolf-Watz, 1989; Surana et al., 2004). The YadA passenger domain consists of N-terminal head, neck, and coiled-coil stalk domains and is followed by a C-terminal translocation and membrane-anchoring unit (Hoiczyk et al., 2000). A detailed description of the YadA structural units is given below for the Y. enterocolitica serotype O:3. The membrane anchor represents a 12-stranded β-barrel closed by a stalk segment of four heptamers (residues 368-395) (Koretke et al., 2006). The left-handed coiled-coil segment continues into a right-handed coiled-coil (residues 214-367) stalk built of 15-residue repeats (pentadecads) (Hoiczyk et al., 2000). The number of these degenerate repeats is strain-specific. The right-handed part of the stalk of Y. enterocolitica serotype O:3 has been predicted to consist of one exceptional 19-residue unit following the left-handed segment, and nine pentadecads (Koretke et al., 2006). The right-handed part of the
stalk is connected to the neck region (residues 192-213) that comprises a safety pin-like element of each monomer (Nummelin et al., 2004) and makes the transition from the rod-like stalk to the globular head. The head domain, about 5 nm long, is a tight cylindrical trimeric structure formed by left-handed $\beta$-rolls (Nummelin et al., 2004).

The export of autotransporters through the inner membrane, involving the Sec machinery, is initiated by the signal peptide. Once through the inner membrane, the signal sequence is cleaved off, and the translocation domain is inserted into the outer membrane, forming a $\beta$-barrel structure (Cotter et al., 2005). This structure constitutes a pore in the outer membrane through which the YadA passenger domain extrudes. The exact mechanism by which the passenger domain is translocated through the pore remains, however, poorly understood. Once all three YadA subunits have been translocated, the protein folds completely and achieves its native conformation. As YadA has no cysteines (Skurnik and Wolf-Watz, 1989), its trimerization depends strongly on ionic or hydrophobic interaction, or both, between the polypeptide chains.

Figure 8. Schematic representation of YadA domains and their functions (modified from Koretke et al., 2006); TU, translocation unit.
YadA appears on bacterial surface within minutes after a temperature-shift to 37°C (Bölin et al., 1982). Because YadA molecules on neighboring bacteria interact with each other in a zipper-like fashion, involving the head and neck domains, bacteria tend to autoagglutinate (Balligand et al., 1985; Hoiczyk et al., 2000).

YadA is unusual in a number of its functions (Table 7). It binds to mucin (Paerregaard et al., 1991a) to facilitate bacterial colonization of the intestine, and it mediates the binding of bacteria to β1-integrins on the host cells via a fibronectin bridge (Eitel and Dersch, 2002). The latter activity, specific for Y. pseudotuberculosis YadA, which is bearing a unique N-terminal sequence (Heise and Dersch, 2006), may stimulate phagocytic activity of the M cells to transport Yersinia across the intestinal epithelium. Y. enterocolitica YadA binds fibronectin (Schulze-Koops et al., 1993; Tertti et al., 1992) but does not mediate invasion of human epithelial cells (Heesemann and Grüter, 1987; Schmid et al., 2004). In addition to fibronectin, YadA also binds to other extracellular matrix proteins such as collagen types I, II, III, IV, V, and XI (Emödy et al., 1989; Schulze-Koops et al., 1992), and laminin types 1 and 2 (Flügel et al., 1994).

YadA also mediates adherence to cultured human epithelial cells (Bliska et al., 1993; Heesemann and Grüter, 1987), rabbit intestinal tissue (Paerregaard et al., 1991b), guinea pig erythrocytes (Kapperud and Lassen, 1983; Kapperud et al., 1985), human intestinal submucosa (Skurnik et al., 1994), and phagocytic cells such as neutrophils (Roggenkamp et al., 1996) and macrophages (Mota et al., 2005). YadA binding to macrophages has been shown to anchor the bacteria so that the type III secretion needle can reach the host cell membrane, and the effector proteins can be translocated to the cytoplasm of the host cell (Mota et al., 2005).

1.6.4.1.2 YadA-mediated serum resistance

One of the multiple functions of YadA is bactericidal serum resistance. This functional characteristic is shared by several members of the oligomeric coiled-coil adhesin (Oca) family (Table 8). YadA protects bacteria against bactericidal action of serum as transposon insertion yadA mutants prove susceptible to human serum, and, in addition, YadA expressed in E. coli K12 confers serum resistance upon this normally serum-sensitive bacterium (Balligand et al., 1985; Martinez, 1989). Attempts to identify the mechanism of YadA-mediated complement resistance implicated YadA in the
inhibition of complement activation at both C3b and C9 levels (China et al., 1993; Pilz et al., 1992). Bacteria expressing YadA were also shown more rapidly degrade surface-bound C3b to iC3b, and accordingly, display higher resistance to opsonin-mediated phagocytosis (Grosdent et al., 2002; Pilz et al., 1992). It has been proposed that YadA may silence the complement activation and reduce C3b-opsonization by coating the bacterial surface with FH (China et al., 1993; Roggenkamp et al., 1996). No direct binding of FH to YadA, however, has been demonstrated.

<table>
<thead>
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<th>Protein</th>
<th>Organism</th>
<th>Serum resistance mechanism</th>
<th>Reference</th>
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<td>Actinobacillus actinomycetemcomitans</td>
<td>FH binding</td>
<td>Asakawa et al., 2003</td>
</tr>
<tr>
<td>DsrA</td>
<td>Haemophilus ducreyi</td>
<td>C4bp binding, vitronectin binding</td>
<td>Abdullah et al., 2005</td>
</tr>
<tr>
<td>UspA1 and UspA2</td>
<td>M. catarrhalis</td>
<td>C4bp binding, vitronectin binding</td>
<td>Nordström et al., 2004; Attia et al., 2006</td>
</tr>
<tr>
<td>Eib</td>
<td>E. coli</td>
<td>unknown</td>
<td>Sandt and Hill, 2000</td>
</tr>
<tr>
<td>Hsf</td>
<td>Haemophilus influenzae</td>
<td>vitronectin binding</td>
<td>Hallström et al., 2006</td>
</tr>
</tbody>
</table>

A study aimed at identification of YadA segments contributing to serum resistance argued that this function could not be ascribed to a single YadA domain (Roggenkamp et al., 2003). Both the head domain and the neck region appeared to be required for the YadA-mediated complement resistance (Roggenkamp et al., 2003). It has been proposed, however, that regions within the stalk and the translocation domain function as serum-resistance determinants (Ackermann et al., 2008; Roggenkamp et al., 2003).
1.6.4.2 Ail

Ail is a 17-kDa outer membrane protein encoded by the chromosomally located *ail* gene (Miller and Falkow, 1988; Miller *et al.*, 1990). The presence of this gene has been associated exclusively with the pathogenic *Y. enterocolitica* species (Miller *et al.*, 1989). Transcription of the *ail* gene is regulated by the temperature and the growth phase of the bacteria. In log-phase bacteria, the *ail* transcript is detectable at 22°C, 28°C, and 37°C, whereas in the stationary-phase bacteria, Ail is expressed exclusively at 37°C (Pierson and Falkow, 1993). In addition, Ail expression is reduced when bacteria grow under limited oxygen conditions (Pederson and Pierson, 1995).

Ail belongs to a family of proteins predicted to form eight outer membrane-spanning amphipatic β-strands and four short extracellular loops (Fig. 9) (Beer and Miller, 1992; Stoorvogel *et al.*, 1991; Vogt and Schulz, 1999). These loops appear to be masked to some extent by the full-length LPS (Pierson, 1994).

Ail mediates attachment to, and subsequent invasion of *yersinia* into eukaryotic cells (Miller and Falkow, 1988) but the exact mechanism of Ail-mediated invasion is unknown. The role for Ail *in vivo* also remains to be determined, since *ail*-defective bacteria cross the gastrointestinal epithelium in a similar fashion as do the wild type bacteria (Wachtel and Miller, 1995). Nevertheless, Ail is indeed produced by bacteria *in vivo* during the first two days of infection (Wachtel and Miller, 1995).

1.6.4.2.1 Ail-mediated serum resistance

The family of outer membrane proteins structurally related to Ail includes *E. coli* and *Enterobacter cloacae* OmpX, and *Salmonella typhimurium* PagC and Rck (Beer and Miller, 1992; Heffernan *et al.*, 1992; Mecsas *et al.*, 1995; Pulkkinen and Miller, 1991; Stoorvogel *et al.*, 1991). Only Rck, however, shares functional homology with Ail; namely its resistance to complement (Heffernan *et al.*, 1994). Ail has been implicated in resistance to complement-mediated killing based on the following observations: (i) pathogenic *Y. enterocolitica ail*-defective mutant bacteria became sensitive to serum killing and the serum-resistance phenotype of the strain was restored by complementation by a wild-type copy of the *ail* gene, (ii) introducing *ail* into laboratory *E. coli* strains and non-pathogenic strains of *Y. enterocolitica* resulted in
increased resistance to killing by human serum (Bliska and Falkow, 1992; Pierson and Falkow, 1993). The serum resistance-conferring regions of *Y. enterocolitica* serotype O:8 Ail have been mapped to the C-terminal end of loop 2 and the N-terminal end of loop 3 (Miller *et al.*, 2001).

![Figure 9](image)

**Figure 9.** Schematic representation of the *E. coli* OmpX structure (modified from Fernandez *et al.*, 2002); loops 1-4 (L1-4).

### 1.6.4.3 LPS

LPS is the major component of the outer membrane of *Y. enterocolitica*; it has three main structural components: lipid A, the core (inner and outer), and O-ag. Unlike in many other Gram-negative bacteria, in *Y. enterocolitica* serotypes O:3 and O:9, the O-ag and the OC are linked to the inner core (IC), forming a branched structure on the bacterial surface (Fig. 10) (Skurnik *et al.*, 1995; Skurnik and Bengoechea, 2003).

### 1.6.4.3.1 O-antigen

O-ags of *Y. enterocolitica* serotypes O:3 and O:9 are homopolymers of 6-deoxy-L-altrose and *N*-formylperosamine, respectively (Caroff *et al.*, 1984; Hoffman *et al.*, 1980).
Homopolymeric O-ags are fully synthesized at the cytoplasmic face of the inner membrane, on the membrane-bound carrier undecaprenyl phosphate (Und-P). Und-P accepts the first sugar moiety from nucleotide diphosphate (NDP)-activated sugar precursors that function as sugar donors. This reaction and the sequential transfer of sugar moieties to the growing polysaccharide polymer is carried out by specific glycosyltransferases. The full-length O-ag polymer is translocated to the periplasm via ATP-driven transporter system formed of the members of ABC transporter family. Subsequently the polymer is ligated by the O-ag ligase to pre-formed lipid A-core and translocated to the outer membrane (Raetz and Whitfield, 2002).

The *Y. enterocolitica* serotype O:3 O-ag gene cluster is composed of two operons (Fig. 11), both essential for O-ag synthesis and both transcribed from tandem promoters (Al-Hendy *et al.*, 1991a, 1992; Zhang *et al.*, 1993). These operons are likely to encode: (i) enzymes involved in the biosynthesis of the NDP-activated sugar precursor, dTDP-6-deoxy-L-altrose (*wbbS, wbbV* and *wbbW*), (ii) two glycosyltransferases to initiate the O-ag synthesis and to extend the homopolymer (*wbbT and wbbU*), and (iii) ABC transporter system (*wzt* and *wzm*) (Skurnik, 1999; Zhang *et al.*, 1993). The genomic location of the O-ag gene cluster is unknown. The organization and characterization of the O-ag gene cluster of serotype O:9 is presented in Study II.
O-ag expression is regulated by the temperature and the growth phase. Below 30°C, O-ag expression by bacteria in the stationary phase (or near the start of the stationary phase of growth) is maximal. Bacteria in the exponential phase, however, express O-ag constitutively and regardless of the growth temperature (Lahtinen et al., 2003).

1.6.4.3.2 Outer core

OC gene clusters of Y. enterocolitica serotypes O:3 and O:9 are very closely related and are located between the hemH and gsk genes. Interestingly, this locus in Yersinia is usually occupied by heteropolymeric O-ag gene clusters (Skurnik et al., 1995; Skurnik and Bengoechea, 2003). OC of Y. enterocolitica serotype O:3 is, however, thought to be an ancestral O-unit which serves as a receptor for bacteriophage φYeO3-12 and enterocoliticin (Skurnik et al., 1995; Strauch et al., 2003).

Hexasaccharide OC of Y. enterocolitica serotype O:3 forms a branch consisting of two glucose, two N-acetyl-D-galactosamines, galactose, and 2-acetamido-2,6-dideoxy-D-xylo-4-hexulose (Duda, 2007; Skurnik, 1999). The latter sugar links the OC to the IC. OC biosynthesis begins at the cytoplasmic face of the inner membrane on the Und-P. The transfer of sugar residues—from NDP-sugar precursors—to the growing hexasaccharide is carried out by glycosyltransferases. Completed hexasaccharide is flipped to the periplasmic space, and subsequently ligated to the lipidA-inner core structure (Skurnik, 1999).
The *Y. enterocolitica* serotype O:3 OC gene cluster consists of nine genes presumably encoding: (i) enzymes involved in the biosynthesis of the NDP-activated sugar precursors (*wbcP* and *gne*), (ii) six glycosyltransferases (*wbcK, wbcL, wbcM, wbcN, wbcO, and wbcQ*), and (iii) a flippase (*wzx*) (Fig. 12) (Skurnik *et al.*, 1995; Skurnik and Bengoechea, 2003).

**Figure 12.** OC gene cluster of *Y. enterocolitica* serotype O:3, schematic drawing (modified from Skurnik and Bengoechea, 2003). Individual genes are drawn as arrows but not to scale. The color of each arrow indicates gene function; gray, biosynthetic enzyme; white, glycosyltransferase; and black, flippase. Dashed-line arrows represent genes flanking the OC cluster.

OC is required for the full virulence of *Y. enterocolitica* serotype O:3, for colonization of the deeper organs, and for prolonged persistence of the bacteria in the Peyer’s patches (Skurnik *et al.*, 1999). OC provides also resistance against antimicrobial peptides and that suggests its role in resistance to innate host defense mechanisms (Skurnik *et al.*, 1999).

### 1.6.4.3.3 LPS and serum resistance

Although a role for O-ag of *Y. enterocolitica* in the inhibition of AP activation is suggested based on the studies using the guinea pig serum, studies using human serum do not provide evidence for involvement of O-ag and OC in serum resistance (Skurnik *et al.*, 1999; Wachter and Brade, 1989).
2. AIMS OF THE STUDY

This thesis work was undertaken to increase our understanding of molecular details of the interactions between the *Y. enterocolitica* surface factors and serum complement components. The specific aims of this study were:

1. to determine the role of *Y. enterocolitica* surface factors YadA, Ail, and LPS in bacterial resistance against complement-mediated killing.
2. to investigate whether *Y. enterocolitica* utilizes the AP inhibitor FH and the CP inhibitor C4bp, to resist the complement system.
3. to identify and map the regions of the bacterial complement resistance factors interacting with the complement regulators.
3. MATERIALS AND METHODS

The materials and methods of this study are listed below, with detailed descriptions in the original publications, which are here referred to by Roman numerals.

Table 9. Bacterial strains used in this study

<table>
<thead>
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<th>Bacterial strains</th>
<th>Description</th>
<th>Source or reference</th>
<th>Used in</th>
</tr>
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<td><em>Y. enterocolitica</em> O:3</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>6471/76 (YeO3)</td>
<td>Serotype O:3, patient isolate, wild type</td>
<td>Skurnik, 1984</td>
<td>I, III, V</td>
</tr>
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<td>31761</td>
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<td>HUSLAB, Helsinki, Finland</td>
<td>V</td>
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<td>49008</td>
<td>Serotype O:3, blood isolate</td>
<td>HUSLAB, Helsinki, Finland</td>
<td>V</td>
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<td>49491</td>
<td>Serotype O:3, blood isolate</td>
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<td>YeO3-Ail</td>
<td>Δail::Km-GenBlock, Km'</td>
<td>This study</td>
<td>I, III</td>
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<td>YeO3-Ail-R</td>
<td>Spontaneous rough derivative of YeO3-Ail, Km'</td>
<td>This study</td>
<td>I, III</td>
</tr>
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<td>YeO3-Ail-OCR</td>
<td>Spontaneous OC mutant derivative of YeO3-Ail-R, Km'</td>
<td>This study</td>
<td>I, III</td>
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<td>YeO3-trs-11</td>
<td>Δ(wzx-wbcL)::Km-GenBlock, Km', derivative of YeO3</td>
<td>Skurnik et al., 1995</td>
<td>I, III</td>
</tr>
<tr>
<td>YeO3-trs-11R</td>
<td>Spontaneous rough derivative of YeO3-trs11, Km'</td>
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<td>I, III</td>
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<td>YeO3-OC</td>
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<td>This study</td>
<td>I, III</td>
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<td>YeO3-Ail-OC</td>
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</tr>
<tr>
<td>YeO3-R2</td>
<td>Spontaneous rough derivative of YeO3</td>
<td>Al-Hendy et al., 1992</td>
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<td>YeO3-O28</td>
<td>ΔyadA::Km-GenBlock, Km', derivative of YeO3</td>
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<td>I, III, V</td>
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<td>YeO3-O28-R</td>
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<td>YeO3-O28-OCR</td>
<td>Spontaneous OC mutant derivative of YeO3-O28-R1, Km'</td>
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<td>YeO3-O28-OC</td>
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<td>6471/76-c (YeO3-c)</td>
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<td>YeO3-R1</td>
<td>Spontaneous rough derivative of YeO3-c</td>
<td>Al-Hendy et al., 1992</td>
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### YeO3-c-OC

- $\Delta(wzx-wbcQ)$ derivative of YeO3-c
- This study
- I, III, V

### YeO3-c-OCR

- Spontaneous rough derivative of YeO3-c-OC
- This study
- I, III, V

### YeO3-c-trs8

- $\Delta(wzx-wbcL)::\text{Km-GenBlock}, \text{Km}^t$, derivative of YeO3-c
- Skurnik et al., 1995
- I, III

### YeO3-c-trs8R

- Spontaneous rough derivative of YeO3-c-trs8, Km
- Skurnik et al., 1995
- I, III

### YeO3-c-Ail

- $\Delta(ail)::\text{Km-GenBlock}, \text{Km}^t$, derivative of YeO3-c
- This study
- I, III, IV, V

### YeO3-c-Ail-OC

- Spontaneous OC mutant derivative of YeO3-c-Ail, Km
- This study
- I, III, IV, V

### YeO3-c-Ail-R

- Spontaneous rough derivative of YeO3-c-Ail, Km
- This study
- I, III, V

### YeO3-c-Ail-OCR

- Spontaneous OC mutant derivative of YeO3-c-Ail-R, Km
- This study
- I, III, IV, V

### Y. enterocolitica O:8

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<td>90937 (ATCC 23715)</td>
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### Y. enterocolitica O:9

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<td>34884</td>
<td>Serotype O:9, blood isolate</td>
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<td>Ruokola/71</td>
<td>Patient isolate</td>
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<td>Ruokola/71-c</td>
<td>Spontaneous virulence plasmid cured derivative of Ruokola/71</td>
<td>Skurnik, 1984</td>
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<td>YeO9-R1</td>
<td>$\Delta(per)::\text{Km-GenBlock},$ rough (O-ag negative), Km derivative of Ruokola/71</td>
<td>This work</td>
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<tr>
<td>YeO9-OC</td>
<td>$\Delta(wzx-wbcL)::\text{Km-GenBlock},$ OC negative, Km derivative of Ruokola/71</td>
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<td>YeO9-OC$^p$</td>
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<td>This work</td>
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### E. coli

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<td>DH10B</td>
<td>$F^+ mcrA \Delta(mrr-hsd RMS-mcrBC), \phi 80lacZ\Delta M15 \Delta lacX74, deoR, recA1 endA1 araA139\Delta ara, leu)7697 galU, galK \lambda\text{rpsL}\text{nupG} \lambda\text{tonA}$</td>
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<td>JM103</td>
<td>Sequencing host strain</td>
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### MATERIALS AND METHODS

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<td>S17-1</td>
<td>recA derivative of E. coli 294 (F’ thi pro hsdR) carrying a modified derivative of IncPα plasmid pRP4 (Ap’ Tc’ Km’) integrated in the chromosome, Tp’</td>
<td>Simon et al., 1983</td>
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<td>S17-1λpir</td>
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<td>De Lorenzo and Timmis, 1994</td>
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<td>SY327λpir</td>
<td>λ(lac pro) argE (Am) rif nalA recA56 (λpir)</td>
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### Table 10. Plasmids used in this study

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<tr>
<td>pAil-8</td>
<td>A pBR322 clone carrying a 8-kb insert of YeO3-c containing the <em>ail</em> gene, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pAil-8.1</td>
<td><em>Pvu</em>II deletion derivative of pAil-8, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pAil-8.2</td>
<td><em>Eco</em>RV-Nrul deletion derivative of pAil-8.1, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vector, Amp&lt;sup&gt;i&lt;/sup&gt; Tet&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Bolivar <em>et al.</em>, 1977</td>
<td>I</td>
</tr>
<tr>
<td>pJM703.1</td>
<td>Suicide vector, contains R6K origin of replication and RP4 Mob region; must be replicated in <em>(λpir)</em> hosts, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Miller and Mekalanos, 1988</td>
<td>I</td>
</tr>
<tr>
<td>pL2.1</td>
<td>pBR322 with the <em>P</em>&lt;sub&gt;lac&lt;/sub&gt; promoter, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Viitanen <em>et al.</em>, 1990</td>
<td>III, V</td>
</tr>
<tr>
<td>pRV1</td>
<td>Suicide vector, derivative of pJM703.1, Clm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Skurnik <em>et al.</em>, 1995</td>
<td>I</td>
</tr>
<tr>
<td>pRV7</td>
<td>A pBR322 clone carrying a 12.4-kb insert of YeO3-c covering the OC gene cluster, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Skurnik <em>et al.</em>, 1995</td>
<td>I</td>
</tr>
<tr>
<td>pRV19-GB</td>
<td>Suicide vector to inactivate OC gene cluster, Δ(wzx-wbcL)::Km-GenBlock, Clm&lt;sup&gt;i&lt;/sup&gt; Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Skurnik <em>et al.</em>, 1995</td>
<td>II</td>
</tr>
<tr>
<td>pRV37</td>
<td>Intermediate to construct OC deletion suicide vector; pBR322 carries a <em>Hind</em>III fragment of pRV7 from which the OC gene cluster is deleted by PCR, Amp&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pRV38</td>
<td>2-kb <em>Hind</em>III fragment of pRV37 cloned into pRV1 (O:3 OC gene cluster suicide vector), Clm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pRV42</td>
<td>Suicide vector constructed to inactivate the <em>ail</em> gene; Km-GenBlock inserted between <em>ail</em> flanking fragments and cloned into pRV1, Km&lt;sup&gt;i&lt;/sup&gt;, Clm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>I</td>
</tr>
<tr>
<td>pTM100</td>
<td>Mobilizable vector, pACYC184-ori&lt;sub&gt;T&lt;/sub&gt; of RK2, Clm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Michiels and Cornelis, 1991</td>
<td>I, IV, V</td>
</tr>
<tr>
<td>pTM100-ail</td>
<td><em>ail</em> gene cloned in a 1711 bp PCR fragment (nucleotides 1490-3201**) into <em>Eco</em>RV site of pTM100, Clm&lt;sup&gt;i&lt;/sup&gt;</td>
<td>This study</td>
<td>IV, V</td>
</tr>
<tr>
<td>pTM100-ail1</td>
<td>bp 2459-2461 CAC substituted for GCA in pTM100-ail; expresses Ail with His65Ala substitution (loop2)</td>
<td>This study</td>
<td>IV</td>
</tr>
<tr>
<td>pTM100-ail2</td>
<td>bp 2465-2470 GATCTT substituted for GGAGGT in pTM100-ail; expresses very low levels of Ail with Asp67Gly and Leu68Gly substitutions (loop2)</td>
<td>This study</td>
<td>IV</td>
</tr>
<tr>
<td>pTM100-ail3</td>
<td>bp 2465-2470 GATCTT substituted for GCACGA in pTM100-ail; expresses very low levels of Ail with Asp67Ala and Leu68Arg substitutions (loop2)</td>
<td>This study</td>
<td>IV</td>
</tr>
<tr>
<td>pTM100-ail4</td>
<td>bp 2564-2566 TCA substituted for GAT in pTM100-ail; expresses Ail with Ser100 substituted for Asp (loop3)</td>
<td>This study</td>
<td>IV</td>
</tr>
<tr>
<td>pUC-4K</td>
<td>Origin of the Km-GenBlock cassette, Amp&lt;sup&gt;i&lt;/sup&gt;, Km&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Pharmacia</td>
<td>I, V</td>
</tr>
<tr>
<td>pYMS3221x</td>
<td>yadA cloned as 4.4-kb XbaI-PvuI fragment between EcoRV-XbaI sites of pTM100</td>
<td>This study</td>
<td>V</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------</td>
<td>---------------</td>
<td>---</td>
</tr>
<tr>
<td>pYMS3223</td>
<td>Km-GenBlock inserted in the ClaI site of pYMS3221x</td>
<td>This study</td>
<td>V</td>
</tr>
<tr>
<td>pYMS 4450</td>
<td>promoterless yadA cloned into pL2.1; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Skurnik et al., 1994</td>
<td>III, V</td>
</tr>
<tr>
<td>pYMS 4505</td>
<td>yadA gene of YeO3 cloned as a 1735 bp PCR fragment (nucleotides 478-2213*) into BamHI site of pTM100; Clm&lt;sup&gt;+&lt;/sup&gt;</td>
<td>El Tahir et al., 2000</td>
<td>I</td>
</tr>
</tbody>
</table>

| pYMS4505:ΔNECK | bp 1194-1271 deletion of the yadA gene in pYMS 4505; expresses YadA with 26 aa deletion (aa 193-218) | This study | IV |
| pYMS4505:A | bp 1257-1301 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 214-228) | This study | IV |
| pYMS4505:S | bp 1281-1325 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 222-236) | This study | IV |
| pYMS4505:Δ#1 | bp 1311-1355 deletion of the yadA gene in pYMS 4505; expresses YadA with 16 aa deletion (aa 231-246) | This study | IV |
| pYMS4505:B | bp 1347-1391 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 244-258) | This study | IV |
| pYMS4505:Δ#2 | bp 1356-1403 deletion of the yadA gene in pYMS 4505; expresses YadA with 16 aa deletion (aa 247-262) | This study | IV |
| pYMS4505:C | bp 1359-1403 deletion of the yadA gene in pYMS 4505; does not express YadA due to a frame shift mutation introduced during the construction | This study | IV |
| pYMS4505:D | bp 1371-1415 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 252-266) | This study | IV |
| pYMS4505:E | bp 1383-1427 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 256-270) | This study | IV |
| pYMS4505:Δ#3+6 | bp 1398-1448 deletion of the yadA gene in pYMS 4505; expresses YadA with 17 aa deletion (aa 261-277) | This study | IV |
| pYMS4505:F | bp 1392-1436 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 259-273) | This study | IV |
| pYMS4505:Δ#3 | bp 1404-1448 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 263-277) | This study | IV |
| pYMS4505:H | bp 1416-1460 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 267-281) | This study | IV |
| pYMS4505:I | bp 1428-1472 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 271-285) | This study | IV |
| pYMS4505:Δ#4 | bp 1449-1493 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 278-292) | This study | IV |
| pYMS4505:J | bp 1482-1571 deletion of the yadA gene in pYMS 4505; expresses YadA with 30 aa deletion (aa 289-318) | This study | IV |
| pYMS4505:Δ#5 | bp 1494-1538 deletion of the yadA gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 293-307) | This study | IV |
| pYMS4505:Δ#5-6 | bp 1494-1583 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 30 aa deletion (aa 293-322) | This study | IV |
| pYMS4505:K | bp 1506-1595 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 30 aa deletion (aa 297-326) | This study | IV |
| pYMS4505:L | bp 1518-1607 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 30 aa deletion (aa 301-330) | This study | IV |
| pYMS4505:Δ#6 | bp 1539-1583 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 308-322) | This study | IV |
| pYMS4505:Δ#6+3 | bp 1539-1586 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 16 aa deletion (aa 308-323) | This study | IV |
| pYMS4505:M | bp 1572-1616 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 319-333) | This study | IV |
| pYMS4505:Δ#7-9 | bp 1584-1706 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 41 aa deletion (aa 323-363) | This study | IV |
| pYMS4505:N | bp 1617-1661 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 334-348) | This study | IV |
| pYMS4505:O | bp 1662-1706 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 15 aa deletion (aa 349-363) | This study | IV |
| pYMS4505:P | bp 1707-1727 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 7 aa deletion (aa 364-370) | This study | IV |
| pYMS4505:Δ1-3 | bp 1707-1772 deletion of the *yadA* gene in pYMS 4505; does not express surface-located YadA, 22 aa deletion (aa 364-385) | This study | IV |
| pYMS4505:Q | bp 1728-1748 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 7 aa deletion (aa 371-377) | This study | IV |
| pYMS4505:R | bp 1749-1769 deletion of the *yadA* gene in pYMS 4505; expresses YadA with 7 aa deletion (aa 378-384) | This study | IV |
| pYMS 4506 | Δ*yadA*::Km-GenBlock derivative of pYMS4505; the internal 1.3-kb *Nsi*I fragment of *yadA* gene replaced with Km-GenBlock; Clm', Km' | This study | I |
| pYMS 4509 | The Δ*yadA*::Km-GenBlock fragment cloned into *EcoRI* site of pJM703.1; Amp', Km' | This study | I |
| pYMS 4515 | Suicide vector to inactivate the *yadA* gene; the *cat* gene of pTM100 cloned into the *Pst*I site of pYMS4509; Clm', Km' | This study | I |
| pYV8081 | Virulence plasmid of 8081 | Portnoy *et al.*, 1981 | V |

* in all *yadA* mutants, nucleotide positions refer to the numbering of the 2551 bp sequence containing the *yadA*-gene of *Y. enterocolitica* O:3 under Acc. no. X13882.

** in all *ail* mutants, nucleotide positions refer to the numbering of the 3865 bp sequence containing the *ail*-gene of *Y. enterocolitica* O:3 under Acc. no. AJ605740
## Table 11. Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody name or cat. no</th>
<th>Description</th>
<th>Source or reference</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary antibodies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2A9</td>
<td>Mouse monoclonal antibody against YadA of <em>Y. enterocolitica</em> O:3, epitope within aa 193-244</td>
<td>Skurnik <em>et al.</em>, 1994</td>
<td>III, IV</td>
</tr>
<tr>
<td>2G12</td>
<td>Mouse monoclonal antibody against YadA of <em>Y. enterocolitica</em> O:3, epitope within aa 193-214</td>
<td>Skurnik <em>et al.</em>, 1994</td>
<td>III, IV</td>
</tr>
<tr>
<td>3G12</td>
<td>Mouse monoclonal antibody against YadA of <em>Y. enterocolitica</em> O:3, epitope within aa 236-263</td>
<td>Skurnik <em>et al.</em>, 1994</td>
<td>I, III, IV</td>
</tr>
<tr>
<td>Tom A6</td>
<td>Mouse monoclonal antibody against O-ag of <em>Y. enterocolitica</em> O:3</td>
<td>Pekkola-Heino <em>et al.</em>, 1987</td>
<td>I</td>
</tr>
<tr>
<td>2B5</td>
<td>Mouse monoclonal antibody against OC of <em>Y. enterocolitica</em> O:3</td>
<td>Pekkola-Heino <em>et al.</em>, 1987</td>
<td>I</td>
</tr>
<tr>
<td>755</td>
<td>Mouse monoclonal antibody against α-chain of human C3</td>
<td>Vogel <em>et al.</em>, 1997</td>
<td>I</td>
</tr>
<tr>
<td>C2456</td>
<td>Mouse monoclonal antibody against collagen type I</td>
<td>SIGMA</td>
<td>IV</td>
</tr>
<tr>
<td>A0062</td>
<td>Rabbit antiserum against human C3c</td>
<td>DAKO</td>
<td>III</td>
</tr>
<tr>
<td>A0063</td>
<td>Rabbit antiserum against human C3d</td>
<td>DAKO</td>
<td>III</td>
</tr>
<tr>
<td>A312</td>
<td>Goat antiserum against human FH</td>
<td>QUIDEL</td>
<td>III, IV</td>
</tr>
<tr>
<td>PC026</td>
<td>Sheep antiserum against human C4bp</td>
<td>The Binding Site, Birmingham, UK</td>
<td>V</td>
</tr>
<tr>
<td>A0065</td>
<td>Rabbit antiserum against human C4c</td>
<td>DAKO</td>
<td>V</td>
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<tr>
<td><strong>Secondary antibodies</strong></td>
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<td></td>
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<tr>
<td>P0260</td>
<td>Peroxidase-conjugated rabbit anti-mouse immunoglobulins</td>
<td>DAKO</td>
<td>I, IV</td>
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<tr>
<td>P217</td>
<td>Peroxidase-conjugated swine anti-rabbit immunoglobulins</td>
<td>DAKO</td>
<td>III, IV, V</td>
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<td>P449</td>
<td>Peroxidase-conjugated rabbit anti-goat immunoglobulins</td>
<td>DAKO</td>
<td>III, IV</td>
</tr>
<tr>
<td>713-035-147</td>
<td>Peroxidase-conjugated donkey anti-sheep immunoglobulins</td>
<td>Jackson ImmunoResearch Laboratories</td>
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### Table 12. Bacteriophages and bacteriocins used in this study

<table>
<thead>
<tr>
<th>Bacteriophages and bacteriocins</th>
<th>Description</th>
<th>Source or reference</th>
<th>Used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriophages</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>φYeO3-12</td>
<td>O-antigen specific phage of <em>Y. enterocolitica</em> O:3</td>
<td>Al-Hendy <em>et al.</em>, 1991a</td>
<td>I</td>
</tr>
<tr>
<td>φR1-37</td>
<td>Outer core specific phage of <em>Y. enterocolitica</em> O:3</td>
<td>Skurnik <em>et al.</em>, 1995</td>
<td>I, II, III</td>
</tr>
<tr>
<td>φR8-01</td>
<td>Infects O-ag and OC-negative <em>Y. enterocolitica</em> O:3 bacteria</td>
<td>This study</td>
<td>III</td>
</tr>
</tbody>
</table>

### Table 13. Purified proteins used in this study

<table>
<thead>
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<th>Purified protein</th>
<th>Description</th>
<th>Source or reference</th>
<th>Used in</th>
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</thead>
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<tr>
<td>FH</td>
<td>Human</td>
<td>Calbiochem (341274)</td>
<td>III, IV</td>
</tr>
<tr>
<td>FI</td>
<td>Human</td>
<td>Calbiochem (341280)</td>
<td>III, IV, V</td>
</tr>
<tr>
<td>Collagen type I</td>
<td>Human</td>
<td>SIGMA (C7774)</td>
<td>IV</td>
</tr>
<tr>
<td>C3b</td>
<td>Human; generated from C3 by factor B, factor D in the presence of Mg²⁺</td>
<td>This study and Koistinen <em>et al.</em>, 1989</td>
<td>III, IV</td>
</tr>
<tr>
<td>C4b</td>
<td>Human</td>
<td>Calbiochem (204897)</td>
<td>V</td>
</tr>
<tr>
<td>SCR 1-5</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
</tr>
<tr>
<td>SCR 1-6</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
</tr>
<tr>
<td>SCR 1-7</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
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<tr>
<td>SCR 8-11</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
</tr>
<tr>
<td>SCR 11-15</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
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<tr>
<td>SCR 8-20</td>
<td>Human, recombinant FH construct produced in the baculovirus expression system</td>
<td>This study and Kühn and Zipfél, 1995</td>
<td>III, IV</td>
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</table>
### Table 14. Methods used in this study

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<th>Methods</th>
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<tr>
<td>Site-directed mutagenesis</td>
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<tr>
<td>PCR</td>
<td>I, II, IV</td>
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<tr>
<td>Cycloserine enrichment</td>
<td>I</td>
</tr>
<tr>
<td>Cloning</td>
<td>I, IV</td>
</tr>
<tr>
<td>Extraction of genomic or plasmid DNA or both</td>
<td>I, II, IV, V</td>
</tr>
<tr>
<td>Screening of recombinant plasmids by size</td>
<td>IV</td>
</tr>
<tr>
<td>Conjugation</td>
<td>I, II, IV, V</td>
</tr>
<tr>
<td>Preparation of electrocompetent cells and electroporation</td>
<td>I, IV, V</td>
</tr>
<tr>
<td>Construction of suicide vectors to eliminate the <em>ail</em> and <em>yadA</em> genes</td>
<td>I</td>
</tr>
<tr>
<td>Isolation of spontaneous mutants using bacteriophages</td>
<td>I, II</td>
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<tr>
<td>DOC-PAGE and silver staining</td>
<td>I, II</td>
</tr>
<tr>
<td>Collagen affinity blotting</td>
<td>IV</td>
</tr>
<tr>
<td>Tx-114 or β-octylglucoside isolation of outer membrane proteins</td>
<td>III, V</td>
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<tr>
<td>Bacterial outer membrane preparation</td>
<td>I</td>
</tr>
<tr>
<td>Lipopolysaccharide isolation</td>
<td>I, II</td>
</tr>
<tr>
<td>Radiolabeling of proteins using Iodine ((^{125})I)</td>
<td>III, V</td>
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<tr>
<td>Human serum pool preparation</td>
<td>I, II, III, IV, V</td>
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<tr>
<td>Enzyme-linked immunosorbent assay</td>
<td>III</td>
</tr>
<tr>
<td>SDS-PAGE and Immunoblotting</td>
<td>I, III, IV, V</td>
</tr>
<tr>
<td>Cofactor assay for C3b or C4b inactivation</td>
<td>III, IV, V</td>
</tr>
<tr>
<td>Direct binding of radiolabeled proteins to <em>Yersinia</em></td>
<td>III, V</td>
</tr>
<tr>
<td>Serum bactericidal assay</td>
<td>I, II, IV</td>
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<tr>
<td>Serum adsorption assay</td>
<td>V</td>
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<tr>
<td>Binding of purified FH, FH recombinant constructs, or C4bp to bacteria</td>
<td>III, IV, V</td>
</tr>
<tr>
<td>Ligand blotting analysis of FH or C4bp binding</td>
<td>III, V</td>
</tr>
<tr>
<td>Bacteriophage sensitivity assay</td>
<td>III</td>
</tr>
<tr>
<td>Modeling of the coiled-coil structure of the YadA mutants</td>
<td>IV</td>
</tr>
<tr>
<td>Statistical methods</td>
<td>II, III, IV</td>
</tr>
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4. RESULTS AND DISCUSSION

4.1 Combined action of *Y. enterocolitica* outer membrane proteins and LPS in resistance to complement-mediated lysis

The complement system contributes to innate immune responses by opsonizing and killing microorganisms and by augmenting the inflammatory response. Escape from innate defense mechanisms appears as a prominent evasion strategy of *Y. enterocolitica*. By avoiding the activation of host innate defense mechanisms, *Y. enterocolitica*, like many pathogenic bacteria, is able to resist the bactericidal activity of complement. Several bacterial surface factors, studied individually, have been implicated in the complement resistance. These include YadA, Ail, and LPS O-ag and OC (Balligand *et al.* 1985; Bliska and Falkow, 1992; Martinez, 1989; Pierson and Falkow, 1993; Skurnik *et al.*, 1999; Wachter and Brade, 1989). Studies on the role of LPS in serum resistance have, however, produced conflicting results (Skurnik *et al.*, 1999; Wachter and Brade, 1989).

To study the contribution of these four factors, we constructed 23 mutant strains of *Y. enterocolitica* O:3 expressing different combinations of YadA, Ail, O-ag, and OC (I). Survival of bacteria, in the exponential or stationary phase of growth, was tested by incubating the bacteria in 70% normal human serum (NHS) (CP, LP, and AP functional) or EGTA-Mg-treated serum (only AP functional). The reaction was stopped after 0.5 or 2 hours’ incubation at 37°C, the samples were plated, and the viable counts of the bacteria were determined (I). The results obtained correlated with previous observations pointing to involvement of YadA and Ail in resistance to CP/AP-mediated killing. In addition, the importance of the combination of the factors in the outer membrane into providing optimal protection against serum killing, was evident.

In general, bacteria growing exponentially were more complement-resistant than bacteria grown to the stationary phase (Figs 4 and 5 in I). YadA, as the most potent single-serum resistance factor, provided bacteria with long-term protection against complement-mediated lysis (Fig. 2 in I). Ail, although crucial for bacterial defense against serum killing, conferred short-term resistance and delayed the killing of bacteria. The protective effect of Ail could, however, be seen when YadA-lacking
bacteria were deprived of O-ag and OC (Fig. 3 in I). LPS is predicted to cover ~75% of the bacterial surface (Raetz and Whitfield, 2002). It may thus sterically block small-sized Ail in the outer membrane and, in consequence, interfere with the Ail-exerted functions: serum resistance and, as shown earlier, attachment to/invasion of mammalian cells (Pierson, 1994). Since also the binding of bacteriophages to their receptors was blocked when these were located beneath the LPS “coat” [(Kawaoka et al., 1983; Skurnik et al., 1995) and III], it is apparent that LPS can mask the bacterial surface (Fig. 13).

Although remains unknown, how bacteria modulate LPS expression in vivo, in vitro O-ag and OC expression is downregulated at 37°C (Al-Hendy et al., 1991a, b; Muszynski, 2004). Thus, one can assume that bacteria, when needed, are able to downregulate the expression of LPS in vivo to uncover factors crucial for certain steps of an infection. This hypothesis appears to be quite applicable in the case of Y. enterocolitica O:3 LPS, which provided no protection against complement-mediated killing (Fig. 3 in I). Moreover, bacteria lacking either O-ag or OC displayed enhanced resistance to CP/LP or AP killing, respectively. Interestingly, this dependence was apparent only when the three factors YadA, Ail, and either O-ag or OC were expressed (Fig. 2 in I). Thus, it appears that serum resistance factors, to be fully functional, must be presented in the membrane in the correct context.

**Figure 13.** Schematic model of Y. enterocolitica surface illustrating serum resistance factors.
In contrast to serotype O:3, LPS appears to participate in serum resistance in serotype O:9. The importance of long O-ag chains in resistance against complement lysis has been demonstrated also for other pathogens (Burns and Hull, 1998; Grossman et al., 1987; Joiner et al., 1986). In Y. enterocolitica O:9, however, LPS plays an accessory role in serum resistance by strengthening the YadA-mediated protection; OC appears to contribute to protection in the early exposure to serum, whereas O-ag contributes to prolonged bacterial survival (Fig. 4 in II).

In general, within a population of the wild type bacteria of both serotypes there existed serum-sensitive and serum-resistant fractions. One could speculate that the surviving bacteria are hidden within aggregates typically formed by the YadA-expressing bacteria. In addition, heterogeneity of the bacterial population may result from differential expression of bacterial-surface factors. It is known that LPS molecules isolated from any smooth Y. enterocolitica strain show an extensive variation in the length of their O-ag chains. Moreover, some of these LPS molecules do not contain attached O-ag chains (Skurnik, 2004). Thus, one could envision bacterial cells equipped with fewer or shorter O-ag chains or both as being more resistant to complement due to the greater exposure of YadA and Ail.

4.2 Y. enterocolitica and acquisition of complement regulators

Complement activation, being potentially deleterious for the host tissues, is tightly regulated. Fluid-phase complement regulators are, however, often sequestered by pathogens to prevent complement activation on their surfaces (Berggård et al., 1997; Hellwage et al., 2001; Horstmann et al., 1988; Johnsson et al., 1996; Meri et al., 2002; Nordström et al., 2004; Prasadarao et al., 2002; Ram et al., 1998b, 2001). We thus aimed to verify whether binding of complement regulators such as FH and C4bp would also be a mechanism of the complement resistance of Y. enterocolitica.

4.2.1 Acquisition of FH

First, we wanted to examine the mechanism by which Y. enterocolitica evades AP-mediated killing. Although Y. enterocolitica binds FH from serum (China et al., 1993; Roggenkamp et al., 1996), no direct binding of purified FH to bacteria has been demonstrated (Roggenkamp et al., 2003). We thus aimed to re-examine whether Y. enterocolitica acquires FH to prevent AP activation on its surface.
Bacteria, incubated with human serum in the test tube or in microtiter wells were then washed, and deposited serum FH was analyzed by immunoblotting or ELISA (Fig. 1 in III). In both techniques, goat antiserum that recognizes human FH served for the detection of bound FH. As both methods confirmed the earlier observation that \textit{Y. enterocolitica} acquires serum FH (Fig. 1 in III), we wanted to examine whether \textit{Y. enterocolitica} involves other serum proteins to mediate the interaction with FH. Bacteria were thus incubated with purified FH, washed, and subjected to SDS-PAGE and immunoblotting. The analysis, using goat antiserum against human FH, revealed that \textit{Y. enterocolitica} O:3 bound purified FH directly (Fig. 2 in III). Since earlier attempts to demonstrate a direct binding of purified/\textsuperscript{125}I-labeled to \textit{Y. enterocolitica} had failed (Roggenkamp \textit{et al.}, 2003), we also re-assayed the binding experiment. We did not succeed, however, in showing the \textsuperscript{125}I-FH binding to \textit{Y. enterocolitica}, either (III). We thus examined the effect of buffers used in the binding assay and found that gelatin used to block non-specific binding was likely to inhibit the \textsuperscript{125}I-FH-\textit{Y. enterocolitica} interaction (III). Gelatin is a denatured form of collagen and greatly inhibits YadA-mediated collagen binding (Emödy \textit{et al.}, 1989). One could thus suggest that FH-\textit{Y. enterocolitica} interaction, like that of collagen-\textit{Y. enterocolitica}, involves YadA and thus can be inhibited by gelatin.

The specificity of FH binding to \textit{Y. enterocolitica} was further determined. Bacteria were incubated with purified FH in the presence of bovine serum albumin (BSA). The binding of FH was affected only by the highest BSA concentration tested, suggesting that the binding of FH to \textit{Y. enterocolitica} is specific (III).

We next aimed to study whether FH bound to the \textit{Y. enterocolitica} surface remained in a functional form, by testing the functional activity of \textit{Y. enterocolitica}-bound FH in a cofactor assay (Fig. 2 in III). Bacteria pre-incubated with purified FH were incubated further with FI and C3b. The degree of C3b cleavage was assayed by subjecting the supernatants to SDS-PAGE/immunoblotting with a mixture of anti-C3c and anti-C3d antibodies (Fig. 2 in III). \textit{Y. enterocolitica}-bound FH acted as a cofactor for FI-mediated cleavage of C3b, as the \(\alpha^\prime\)-chain of C3b became cleaved into 67 kDa, 43 kDa, and 41 kDa fragments. Thus, FH deposited on the \textit{Y. enterocolitica} surface retained its regulatory function.
4.2.2 Acquisition of C4bp

As *Y. enterocolitica* resists the CP/LP-mediated killing (Fig. 2 in I), we wanted to examine whether one mechanism underlying this resistance is acquisition of C4bp. A serum adsorption assay was used to test whether *Y. enterocolitica* binds serum C4bp. Bacteria were incubated in 5% heat-inactivated serum at 37°C; bacteria-bound serum proteins were then eluted and subjected to SDS-PAGE/immunoblotting with sheep antiserum against human C4bp (Fig. 3 in V). Because the immunoblotting results revealed the presence of C4bp in the eluted fractions, we next studied whether *Y. enterocolitica* binds C4bp independent of other serum proteins. 125I-labeled C4bp was incubated with *Y. enterocolitica* serotypes O:3, O:8, and O:9. The bacteria that bound radiolabeled C4bp were centrifuged through 20% sucrose, and the binding was quantified with a gamma counter (Fig. 1 in V). All three *Y. enterocolitica* serotypes directly bound C4bp. The specificity of C4bp binding underwent further study in a direct binding assay. Bacteria were incubated with 125I-C4bp in the presence of unlabeled C4bp or BSA. The binding of 125I-C4bp was inhibited in a concentration-dependent manner by unlabeled C4bp, whereas BSA did not affect 125I-C4bp binding. In conclusion, we found that the binding of C4bp to *Y. enterocolitica* to be specific (Fig. 7 in V).

To examine the functional activity of *Y. enterocolitica*-acquired C4bp, a cofactor assay was performed. Bacteria pre-incubated with C4bp were subsequently washed and incubated with C4b and FI. The C4bp-cofactor activity was verified by immunodetection of C4b cleavage products in the collected supernatant by use of immunoblotting with rabbit anti-human C4c antiserum (Fig. 2 in V). The results demonstrated that C4bp bound to *Y. enterocolitica* maintained its cofactor activity and participated in C4b inactivation. In conclusion, we found C4bp to bind to *Y. enterocolitica* in a way that does not interfere with its regulatory function.
4.3 Identification of FH and C4bp receptors on *Y. enterocolitica*

Given the *Y. enterocolitica* - FH and *Y. enterocolitica* - C4bp interaction, we wanted to identify the potential receptors for these complement regulators on the surface of *Y. enterocolitica*. To this end, we used a set of mutant strains of *Y. enterocolitica* O:3 expressing different combinations of YadA, Ail, O-ag, and OC, which had served to identify those bacterial factors that confer serum resistance (I). The ability of the mutant strains to bind serum FH or C4bp was tested by ELISA/immunoblotting or by direct binding assay, respectively (Fig. 1 in III and Fig. 4 in V). In general, FH/C4bp binding was dependent on the presence of those factors that confer complement resistance, YadA and Ail. Importantly, the FH- and C4bp-binding pattern correlated well with the serum sensitivities of the strains; the strains of intermediate and resistant phenotype bound the regulators, whereas the sensitive ones failed to (I, III, V, Fig. 14).

In general, serum-sensitive strains bound negligible amounts of FH and C4bp (Fig. 14). Strains belonging to this group did not express YadA, the most potent single serum resistance factor. It thus became evident that YadA expression is crucial for C4bp/FH binding (Fig. 14). Interestingly, although several serum-sensitive strains expressed Ail—shown earlier to confer serum resistance—Ail’s ability to bind C4bp/FH was inhibited in the presence of O-ag or OC (YeO3-O28, YeO3-c, YeO3-O28-OC, YeO3-c-OC, and YeO3-O28-R, Fig. 14). The distal parts of LPS lacked the ability to bind either of the regulators, and in consequence did not protect the bacteria against serum killing (YeO3-c-Ail, YeO3-c-Ail-OC, and YeO3-c-Ail-R).

All the strains expressing YadA were able to bind both FH and C4bp (Fig. 14). These strains displayed intermediate (YeO3, YeO3-Ail, YeO3-Ail-OC, YeO3-Ail-OCR, YeO3-Ail-R, and YeO3-OCR) or resistant phenotypes (YeO3-OC, YeO3-R2, YeO3-trs11, and YeO3-trs11R). The only two strains that lacked YadA but were able to bind FH and C4bp expressed Ail in the absence of both O-ag and OC (YeO3-c-OCR and YeO3-c-trs8R, Fig. 14). Thus, Ail must be very well exposed in the outer membrane to be able to bind complement regulators.
Interestingly, the ability of YadA-expressing strains to bind FH was greatly enhanced in the absence of O-ag (YeO3-R2, YeO3-trs11R, YeO3-OCR, YeO3-Ail-OCR, and YeO3-AilR, Fig. 14). This suggests that the lack of O-ag might favor FH binding to YadA by exposing its regions crucial for the binding. Lack of OC in the O-ag- YadA-expressing strain did not greatly affect bacterial ability to bind FH (YeO3-OC). YeO3-trs11, however, shown to express less O-ag than did YeO3-OC, bound more FH than did the wild type (I and Fig. 1 in III).
4.3.1 Characterization of YadA- Ail- mediated interactions with FH and C4bp

4.3.1.1 YadA and FH

These findings warranted further studies on the YadA- and Ail-mediated interactions with FH and C4bp (III-V). YadA was initially identified as an FH receptor based on an affinity-blotting experiment using whole serum as the FH source (China et al., 1993). To show a direct interaction between FH and YadA, we re-assayed the affinity-blotting experiment. YadA was extracted from *E. coli* with Triton X-114, and the binding of purified FH to the extracted protein was tested in affinity blotting with goat antiserum against anti-human FH. We observed the binding of purified FH to YadA, thereby confirming that the interaction between YadA and FH is direct (Fig. 3 in III).

To assess the nature of the YadA-FH interaction, we incubated YadA-expressing bacteria with purified FH in the presence of salt (50-650 mM) or heparin (0-1000 μg/ml). As salt did not greatly affect the binding of FH to YadA, the YadA-FH interaction is likely to be hydrophobic in nature (Fig. 5 in III). Heparin had some effect on FH-binding to YadA only at the highest concentrations used (Fig. 5 in III). This suggests that the YadA-FH interaction does not depend primarily on SCR 7, SCR 13, or SCR 20, which have been shown to be heparin-specific (Blackmore et al., 1996; Blackmore et al., 1998b; Pangburn et al., 1991).

To characterize regions of FH involved in the YadA-FH interaction in more detail, we incubated YadA-expressing bacteria with truncated recombinant constructs together representing the entire FH. Binding of FH was detected by immunoblotting with goat antiserum against human FH. All FH fragments bound to YadA-expressing bacteria, suggesting that the interaction with YadA engages SCRs of the entire polypeptide chain of FH (Fig. 15 and Fig. 4A in III). This finding confirmed the heparin inhibition results. Since the SCR 1-5 construct contains the domain responsible for the regulatory functions of FH, *i.e.*, its cofactor and decay-accelerating activities (Gordon et al., 1995; Kühn et al., 1995; Kühn and Zipfel, 1996), we next examined whether FH bound to YadA remained functional. Despite the fact that YadA-FH interaction involves SCRs 1-5, YadA-bound FH retained its regulatory function. Many pathogens like *B. burgdorferi*, *N. gonorrhoeae*, and *C. albicans* target C-terminal SCRs of FH (Hellwage et al., 2001; Meri et al., 2002; Ram et al., 1998b), *S. pneumoniae* targets its
middle portion (Jarva et al., 2002), and *S. pyogenes* and *B. burgdorferi* target its N-terminal region (Kotarsky et al., 1998; Kraiczy et al., 2004; Pandiripally et al., 2003). Binding to the SCRs of the entire FH, however, has not been reported with any other microbe and thus appears to be unique for YadA.

Since we showed that *Y. enterocolitica*-bound FH maintains its function, we wanted to study whether YadA-expressing bacteria use FH to promote FI-mediated cleavage of C3b (Fig. 2 in III). The cofactor assay results demonstrated that YadA-bound FH was fully functional. Our C3b deposition experiments, however, did not support this observation (Fig. 6 in I). The deposition findings not only failed to correlate with resistance phenotypes of the strains, but also pointed to O-ag as a factor preventing the deposition of C3b (Fig. 6 in I). Interestingly, no difference between the wild type and the YadA-negative strains in 125I-C3 deposition from 10% EGTA-Mg serum was observed by Pilz and co-workers either (Pilz et al., 1992). In contrast to this observation, Tertti and co-workers reported that pYV+ bacteria displayed less serum C3b deposited that did pYV- bacteria (Tertti et al., 1987). This difference between strains only appeared, however, with NHS concentration no higher than 5% (Tertti et al., 1987). China and co-workers showed, however, reduced C3b deposition, from 10% EGTA-Mg serum, on bacteria expressing YadA (China et al., 1993). As our data showed no reduction in C3b deposition by YadA-positive strains, one could speculate that the concentration of serum used in the assay was too high. We cannot at present explain these discrepancies.

### 4.3.1.1.1 Mapping of YadA regions involved in FH-binding

As we found that O-ag blocks to some extent the binding of FH to YadA, we wanted to examine whether O-ag blocks the binding of mAb to YadA in an analogous fashion (III). We used the mAbs recognizing epitopes within the neck and the very N-terminal

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**Figure 15.** Schematic representation of FH domains interacting with YadA of *Y. enterocolitica* serotype O:3; lanes indicate recombinant fragments used.
RESULTS AND DISCUSSION

region of the YadA stalk (2A9, 3G12, and 2G12). The access of these mAbs to their epitopes was not blocked, however, by the presence of O-ag. This suggested that YadA regions interacting with FH are located closer to the bacterial outer membrane than are the mAbs epitopes. The indication that regions of the YadA stalk may be involved in FH binding and the fact that the YadA stalk, but not the head domain, mediates serum resistance (Roggenkamp et al., 2003) warranted further studies on characterization of YadA regions involved in both serum resistance and FH binding (IV). To this end, we constructed 28 mutants (Fig. 16) having short internal deletions within the neck and stalk of YadA, based on the proposed coiled-coil structure of the trimeric stalk (Koretke et al., 2006). Plasmids carrying the mutated yadA gene were mobilized to the plasmid-cured Y. enterocolitica O:3 host, YeO3-c. Expression of the trimerized and surface-exposed YadA was detectable in all the mutants, except for the one carrying deletion Δ364-385 (Fig. 1 in IV). This finding was consistent with the earlier finding showing that this region, overlapping the junction between the right-handed and left-handed parts of the stalk, is crucial for YadA translocation across the outer membrane (Roggenkamp et al., 2003). Since all our stalk mutants bound collagen type I, the deletions introduced did not affect the head and neck domains involved in collagen binding (Fig. 1 in IV). Modeling analysis suggests that most deletions do not disturb the structure of the stalk, whereas in certain mutant proteins the local periodicity and thus degree of supercoiling are affected (IV).

The mutants were shown to be useful for the localization of epitopes for the three mAbs specific for YadA: 3G12-15, 2A9, and 2G12. The epitopes were mapped to aa 236-263, 193-244, and 193-214 (Fig. 1 in IV) and appeared to be conformational because mapping attempts using overlapping 16-mer YadA peptides had failed (El Tahir et al., 2000).

The constructed mutants were tested for their ability to bind serum FH (ELISA and immunoblotting using goat antiserum against human FH for detection), and their serum sensitivities were determined in the serum bactericidal assay. One of the constructed YadA mutants that did not express YadA due to an out-of-frame deletion within the stalk-coding region, served as a control in all the experiments.
In general, serum-resistant YadA deletion mutants bound FH (Fig. 3 in IV). However, none of the constructed mutants, even those expressing YadA with broken periodicity, was completely devoid of FH binding activity. This suggests that there exists no single site on the YadA stalk that binds FH. The decrease in FH binding was associated with a decrease in serum resistance for only some mutants (Δ261-277 and Δ301-330). One of these deletions affected the conformation of YadA (Δ261-277), and this may underlie the decrease in both FH binding and serum resistance. The other deletion (Δ301-330), however, was in a pentadecad register, and in addition, was partially covered in those mutants whose ability to bind FH remained unaffected. This observation suggests that a change of distance between the putative FH-binding subsites on YadA, rather than deletion of the actual YadA site binding FH, results in a reduction in FH binding.

Most serum-sensitive mutants did not lose their FH-binding ability (Δ293-322, Δ308-323, Δ323-363, Δ371-377). Moreover, the FH-binding mode of the mutants was similar to that of the wild type YadA and involved SCRs of the entire polypeptide...
chain of FH (Fig. 6 in IV). Although FH bound to YadA mutant proteins displayed cofactor activity for FI-mediated cleavage of C3b (Fig. 5 in IV), one cannot ignore the possibility that its ability to interfere with the formation of the alternative pathway convertases or to accelerate their decay or both remained unaffected. In addition, the excessive binding of FH by the mutants may result in FH depletion from serum and thus trigger uncontrolled activation of the complement.

To conclude, FH does not target one particular segment of the YadA stalk. Instead, FH binding to YadA appears to depend on the recognition of several complementing and discontinuous structural YadA motifs. Moreover, the spacing of these motifs seems crucial for the FH binding. The conformational and discontinuous nature of FH binding has been reported for other proteins predicted to form coiled-coils, streptococcal M protein, OspE and BBA68 of *B. burgdorferi*, and FhbA of *B. hermsii* (Fischetti et al., 1995; Hovis et al., 2006; McDowell et al., 2004, 2005; Metts et al., 2003). The structural motifs of these proteins were crucial for forming the binding site for FH. Distortion of one of the three widely spaced coiled-coils of OspE resulted in attenuated or completely abolished FH binding (McDowell et al., 2004; Metts et al., 2003), whereas destabilization of the BBA68 coiled-coils resulted in reduced FH-binding capacity (McDowell et al., 2005). Since none of the YadA deletions completely abolished FH-binding ability, one could suggest that when one of the YadA sites involved in the interaction is deleted, structural motifs elsewhere on the right-handed part of the YadA stalk, having clear internal symmetry, may find complementary regions on FH.

**4.3.1.2 YadA and C4bp**

Several proteins belonging to the family of trimeric autotransporters, such as *H. ducreyi* DsrA, and *M. catarrhalis* UspA1 and UspA2, were shown to bind C4bp (Abdullah et al., 2005; Nordström et al., 2004). Our receptor-mapping analysis pointed to YadA as one of the C4bp receptors on *Y. enterocolitica*. We thus we wanted to characterize YadA-C4bp interaction in more detail (V).

To show that the interaction between YadA and C4bp is direct, we tested the binding of serum C4bp to TritonX-114-extracted YadA in affinity blotting. That we found
binding of C4bp to YadA trimer suggests a direct interaction between these proteins (Fig. 6 in V).

To study whether the interaction between YadA and C4bp is ionic strength-dependent, we examined the effect of salt on the YadA-C4bp interaction (Fig. 7 in V). Bacteria expressing YadA were incubated with $^{125}$I-C4bp in the presence of salt (50-650 mM). Because salt inhibits YadA-C4bp interaction at a concentration required to inhibit the binding of C4bp to C4b (Blom et al., 2000), it is suggested that the positively charged cluster of amino acids between CCP1 and CCP3, involved in C4b binding, would also interact with YadA (Accardo et al., 1996; Härdig et al., 1997; Ogata et al., 1993). Knowing that heparin competes with C4b for C4bp binding, by binding to CCP1-3 (Blom et al., 1999; Hessing et al., 1990; Villoutreix et al., 1998), we examined whether it could inhibit C4bp-binding to YadA in an analogous fashion. Bacteria were incubated with $^{125}$I-C4bp in the presence of heparin (0-1000 μg/ml), and its influence on the binding was determined. Heparin weakly inhibited C4bp binding to YadA, and a relatively high heparin concentration was required for 50% inhibition of the C4bp binding to YadA (Fig. 7 in V). Although C4b/heparin and YadA do not appear to bind to exactly the same site on C4bp, one cannot exclude the possibility that these binding sites overlap.

We examined the functional activity of the YadA-bound C4bp by a cofactor assay. It clearly showed that C4bp bound to YadA retained its regulatory function (Fig. 2 in V).

**4.3.1.3 Ail and FH**

Knowing that Ail contributed to FH binding when not blocked by LPS, we wanted to study whether Ail-bound FH maintains its regulatory function. The cofactor assay showed that Ail-bound FH was fully functional and displayed cofactor activity for FI-mediated cleavage of C3b (Fig. 2 in III).

Next, we aimed to examine the nature of the interaction between Ail and FH. For that, we incubated the single Ail-positive bacteria with purified FH in the presence of salt (50-650 mM) or heparin (0-1000 μg/ml). That both salt and heparin reduced the FH binding to Ail (Fig. 5 in III) suggests that the interaction between the proteins is ionic
in nature and involves FH region(s) targeted by heparin, i.e. SCR 7, SCR 13, and SCR 20 (Blackmore et al., 1996, 1998b; Pangburn et al., 1991).

To map in more detail the FH regions involved in interaction with Ail, Ail-expressing bacteria were incubated with the recombinant FH fragments that cover the whole FH molecule. The results suggest that the SCR6 and SCR7 may constitute the binding site for Ail (Fig. 17 and Fig. 4A in III).

4.3.1.3.1 Mapping Ail regions involved in FH binding

To identify the Ail region involved in binding of FH, we substituted amino acids shown to be crucial for serotype O:8 Ail-mediated serum resistance (Miller et al., 2001). We thus substituted single amino acids in loop 2 or loop 3, His65Ala and Ser100Asp, respectively (IV, Fig. 18). Two mutants having double amino acid substitutions in loop 2, Asp67Gly Leu68Gly and Asp67Ala Leu68Arg, expressed minimal amounts of Ail and were not studied further (IV, Fig. 18).

Two single substitution mutants were tested for their ability to resist the complement-mediated killing in the bactericidal assay. As the mutant bacteria showed decreased resistance both to NHS and to EGTA-Mg serum, we wanted to examine whether their
ability to bind serum FH was affected by the mutations introduced. The mutants displayed similar levels of bound FH as the wild type (Fig. 5 in IV). This suggested that the Ail-mediated mechanism of serum resistance does not depend primarily on FH binding. The biological significance of FH binding by Ail, a factor promoting *Yersinia* entry into tissue culture cells *in vitro* (Miller and Falkow, 1988), could thus be related to the attachment of bacteria to the glycosaminoglycans and sialic acids on host cells via FH bridging.

### 4.3.1.4 Ail and C4bp

Knowing that Ail binds C4bp in the absence of LPS, we wanted to study whether the interaction between Ail and C4bp is direct. Binding of serum C4bp to β-octylglucoside- and TritonX-114-extracted Ail was tested in a ligand blotting assay. No C4bp binding to Ail could be detected, however, suggesting that its conformational structure is required for the binding to take place (V).

To characterize the Ail-C4bp binding in more detail we tested whether the binding is dependent on charge (Fig. 7 in V). Bacteria expressing Ail were incubated with $^{125}$I-C4bp in the presence of heparin (0-1000 μg/ml) or salt (50-650 mM). Heparin efficiently and dose-dependently inhibited the binding of C4bp to Ail, suggesting that CCP1-3 domains of the C4bp α-chain are involved. That the Ail-C4bp interaction was less salt-sensitive than were YadA-C4bp or C4b-C4bp interactions (Blom *et al.*, 2000) indicates that the interaction is hydrophobic in nature. Thus, Ail binding sites on C4bp, although apparently not equivalent, may still overlap with those involved in C4b- or YadA- C4bp interactions.

To examine whether Ail-bound C4bp remained functionally active, the cofactor assay was performed. The results clearly showed that C4bp bound to Ail-expressing bacteria retained its regulatory function (Fig. 2 in V).
5. CONCLUSIONS AND FUTURE PROSPECTS

In complement activation, FH and C4bp are the key regulators that prevent the complement cascade from attacking host tissues. Some bacteria may bind and deposit these regulators on their own surfaces and thus provide themselves with an efficient means to avoid complement activation. In consequence, bacteria resist complement-mediated lysis and opsonin-dependent phagocytosis.

This study has demonstrated that *Y. enterocolitica*, similar to many other pathogens, recruits both FH and C4bp to its surface to ensure protection against the AP- and CP/LP-mediated killing. YadA and Ail, the most crucial serum resistance factors of *Y. enterocolitica*, mediate the binding of FH and C4bp. FH - YadA interaction involves multiple higher structural motifs on the YadA stalk and the SCRs of the entire polypeptide chain of FH. The Ail binding site on FH has been located to SCRs 6 and 7. The binding site for FH on Ail, however, remains undetermined. Both YadA- and Ail-bound regulators display full cofactor activity for FI-mediated cleavage of C3b/C4b. FH/C4bp-binding characteristics do, however, differ between YadA and Ail. In addition, Ail captures the regulators only in the absence of blocking O-ag and OC, whereas YadA binds FH/C4bp independent of the presence of other surface factors. Independent of mode of binding, however, YadA and Ail provide *Y. enterocolitica* a means to avoid complement-mediated lysis, enhancing chances for the bacteria to survive in the host during various phases of infection.

To investigate whether complement resistance contributes to pathogenesis of yersiniosis *in vivo* would be of major importance. As YadA-mediated collagen binding is essential for full virulence of *Y. enterocolitica* (Tamm et al., 1993), mice should be challenged with serum-sensitive but collagen-binding *Y. enterocolitica* bacteria, expressing YadA with the head and neck domains intact. In fact, Ackermann and co-workers found that serum-sensitive *Y. enterocolitica* bacteria that express YadA comprising the translocation unit of Oca family members showed attenuation in mice (Ackermann *et al.*, 2008). It thus appears that YadA-mediated serum resistance contributes to bacterial virulence *in vivo*. It would be interesting, however, to examine whether the correlation between serum sensitivity and virulence in the mouse model could be observed also with the serum-sensitive YadA mutants generated in this study, ones that carry the deletions within the stalk. It would also be of interest to show that
the binding of FH/C4bp by *Y. enterocolitica* takes place *in vivo*. FH/C4bp-deficient mice could serve to study how the lack of the regulators affects the pathogenesis of yersiniosis.
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