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MINIREVIEW

Bacterial self-defence: how *Escherichia coli* evades serum killing

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Abstract

The ability to survive the bactericidal action of serum is advantageous to extra-intestinal pathogenic *Escherichia coli* that gain access to the bloodstream. Evasion of the innate defences present in serum, including complement and antimicrobial peptides, involves multiple factors. Serum resistance mechanisms utilized by *E. coli* include the production of protective extracellular polysaccharide capsules and expression of factors that inhibit or interfere with the complement cascade. Recent studies have also highlighted the importance of structural integrity of the cell envelope in serum survival. These survival strategies are outlined in this review with particular attention to novel findings and recent insights into well-established resistance mechanisms.

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Introduction

The bloodstream vitalizes the organs and tissues of the body. Normally, the entry of microorganisms into the bloodstream is prevented due to the integrity of the vasculature. Despite this, physical barrier microbes can enter the bloodstream and cause infection. The Gram-negative bacillus *Escherichia coli* is one of the leading causes of bloodstream infections. *E. coli* comprises 17–37% of all bacteria isolated from patients with bloodstream infections (Russo & Johnson, 2003). Isolates capable of gaining access to and surviving in the bloodstream are known as extra intestinal pathogenic *E. coli* (ExPEC). The most common extra intestinal site colonized by these bacteria is the urinary tract, which in turn is a common source of origin for bloodstream infections (Russo & Johnson, 2003). The mechanisms underlying the pathogenesis of *E. coli* urinary tract infections and host defence were recently reviewed by Ulett *et al.* (2013), and therefore, will not be discussed herein. The presence of *E. coli* in the bloodstream can result in the induction of a vigorous host inflammatory response leading to sepsis which is associated with high morbidity and mortality (Russo & Johnson, 2003; Marx & Reinhart, 2008).

The virulence factors expressed by ExPEC allow it to survive and colonize sites outside the gastrointestinal tract. For example, colonization of the urinary tract requires the iron acquisition systems and type 1 fimbriae (Snyder *et al.*, 2004). Other factors are required to survive in the blood and overcome the defence mechanisms of the host. Serum resistance is a trait associated with strains that cause bacteraemia (Johnson, 1991). In general, *E. coli* isolated from blood are more serum-resistant than strains that cause urinary tract infections or strains isolated from faecal samples (Johnson, 1991; Jacobson *et al.*, 1992). Septic shock and death are also more associated with serum-resistant rather than serum-susceptible bacteraemia isolates (Johnson, 1991). Multiple virulence factors of *E. coli* have been shown to be involved in serum survival. In this review, we outline the mechanisms utilized by *E. coli* to resist the killing activity of serum.

The bactericidal factors of serum

Serum contains more than thirty proteins of the complement system, a crucial component of the host innate immune response which can also initiate the adaptive response (Sarma & Ward, 2011). Deposition of complement

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factors on the bacterial surface activates the complement cascade and results in the formation of the membrane attack complex (MAC). This complex forms trans-membrane pores in the membranes of susceptible bacteria, thus leading to bacterial death (Sarma & Ward, 2011).

Complement can be activated by three different pathways. Activation of the first pathway, the classical complement pathway, is triggered by recognition of antigen-bound IgG by the C1 complex. The C1 complex is composed of components C1q and two molecules of C1r and C1s. Binding of C1q to antibody-antigen complexes activates C1r and C1s. C1s cleaves complement protein C4, of which fragment C4b covalently binds to the target surface. Surface-associated C4b is bound by complement protein C2 which is subsequently cleaved by C1s. Fragment C2a remains bound to C4b to form the C4b2a complex, the C3 convertase of the classical complement pathway (Fig. 1) (Sarma & Ward, 2011).

The second pathway of complement activation, the mannose-binding lectin pathway is triggered by conserved sugar residues found on microbial cell surfaces. Mannose-binding lectin-pathway-associated proteins act in a similar manner to the C1 complex of the classical complement pathway to form the C3 convertase, C4b2a (Sarma & Ward, 2011).

The third complement pathway does not require a specific pathogen recognition molecule for formation of the C3 convertase to occur. This alternative complement pathway is activated by spontaneous hydrolysis of C3 in plasma which generates C3b. Complement fragment C3b binds to the bacterial surface or is inactivated if no pathogens are present. Factor B binds to bacterial-bound C3b and is cleaved by complement factor D into fragments Ba and Bb. Fragment Bb remains bound to C3b to form the C3 convertase, C3bBb (Fig. 2) (Sarma & Ward, 2011).

Following formation of the C3 convertase, the three complement pathways converge. Complement protein C3 is cleaved by the C3 convertase complexes into fragments C3a and C3b. C3b can bind to C3 convertase complexes to form a C5 convertase (C4b2a3b or $C3b_2Bb$). Cleavage of complement protein C5 by the C5 convertase produces C5b and C5a. Fragment C5b binds to complement proteins C6, C7 and C8 in succession. The resulting C5b-8 complex inserts into target membranes. Polymerization of component C9 on C5b-8 complex produces the MAC (Figs 1 and 2) (Sarma & Ward, 2011).

Activation of the complement system by the mechanisms described can have a profound impact on the development of sepsis. Release of the pro-inflammatory fragments C3a, C4a and C5a during complement activation promotes inflammatory cascades, chemotaxis and histamine release. In particular, C5a has been shown to be a crucial mediator of the inflammatory response affecting multiple processes associated with sepsis (Rittirsch *et al.*, 2008).

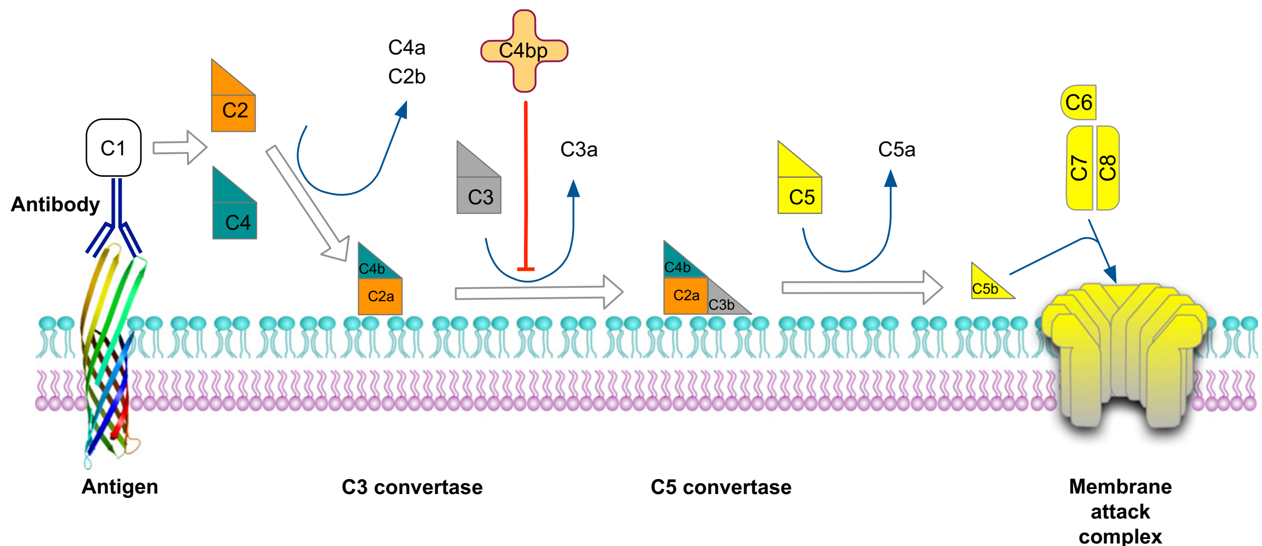

Fig. 1. Classical complement system. The classical complement system is activated by C1 complex recognition of bacterial-bound antibody. The C1s component of the C1 complex cleaves complement proteins C4 and C2. Fragments C4b and C2a form the C3 convertase which remains bound to the bacterial cell surface. The C3 convertase cleaves circulating C3 into C3a and C3b. C3b binds to the C3 convertase complex to from the C5 convertase. The C5 convertase cleaves complement protein C5. Fragment C5b forms a complex with complement proteins C6–C9 to form the membrane attack complex. The complement regulatory protein, C4b-binding protein (C4bp), inhibits formation of the C3 convertase of the classical complement pathway.

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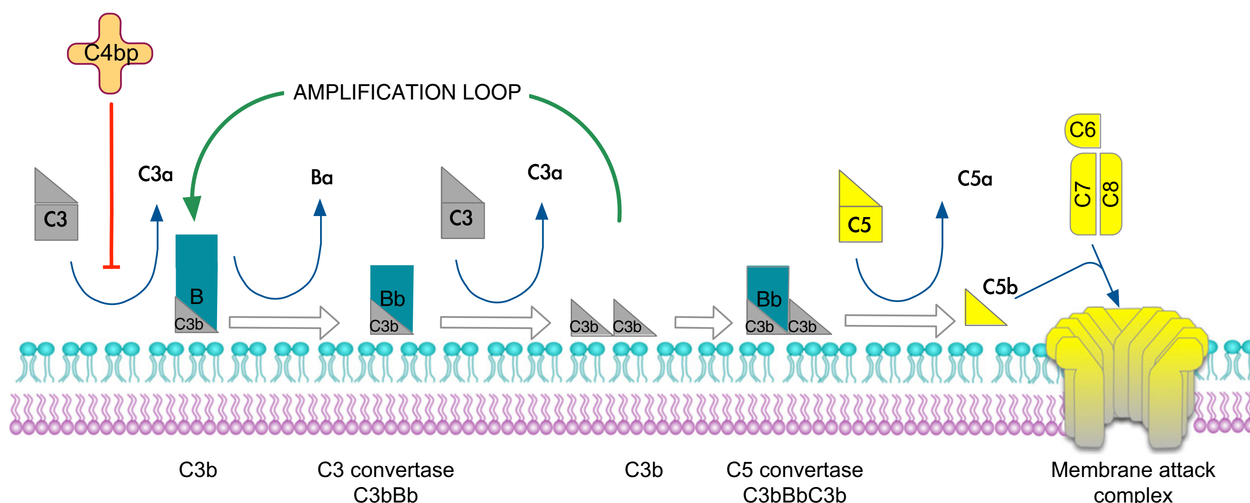

Fig. 2. Alternative complement pathway. The alternative complement pathway is activated by spontaneous hydrolysis of circulating C3b into fragments C3a and C3b. Complement fragment C3b binds to bacterial surfaces and is subsequently bound by factor B. Cleavage of factor B by complement factor D results in fragments Ba and Bb. Fragment Bb remains bound to C3b to form the C3 convertase, C3bBb. C3b can bind to C3 convertase complexes to form a C5 convertase complex ($C3b_2Bb$). Following formation of the C5 convertase, the alternate complement pathway is identical to the classical.

Lysis of susceptible bacteria by complement has been reported to be enhanced in the presence of antimicrobial peptides and proteins (Bugla-Ploskonska *et al.*, 2009). In the blood, these peptides and proteins are mainly contained within the granules of circulating leucocytes and platelets and are released following migration to sites of infection (Hancock & Scott, 2000; Levy, 2000). Systemic levels of antimicrobial peptides are generally low, with the exception of certain protein and peptides induced by the acute phase response (Levy, 2000). Bacterial infection triggers a strong acute phase response, altering the blood concentration of inflammatory mediators and plasma proteins. For example, serum levels of the antimicrobial protein phospholipase A2 are increased significantly during the acute phase. This enzyme hydrolyses bacterial membrane phospholipids and in combination with other neutrophil-derived antimicrobial peptides has been shown to have activity against *E. coli* (Levy *et al.*, 1994; Degousee *et al.*, 2011; Dennis *et al.*, 2011).

The antimicrobial protein lysozyme targets the bacterial cell wall, hydrolysing β-(1-4) glycosidic bonds between N-acetylmuramic acid and N-acetylglucosmine (Callewaert & Michiels, 2010). Mammalian c (conventional)-type lysozyme is found in bodily fluids including serum where it is proposed to enhance lysis of susceptible cells in the presence of an activated complement system (Bugla-Ploskonska *et al.*, 2009; Callewaert & Michiels, 2010). *Escherichia coli* are resistant to lysozyme in the absence of agents that disrupt the protective outer membrane such as complement, lactoferrin and defensins (Callewaert & Michiels, 2010).

In addition to their ability to disrupt bacterial membranes, the antimicrobial peptide cathelicidin LL-37 and bactericidal permeability increasing protein (BPI) protect against sepsis by neutralizing endotoxins (Levy, 2000). LL-37 binds and neutralizes bacterial lipopolysaccharide and lipoteichoic acid inhibiting production of proinflammatory cytokines (Bowdish *et al.*, 2005). Similarly, BPI has a high affinity for lipopolysaccharide and is protective in animal models of sepsis (Elsbach, 1998; Levy, 2000).

Although there is evidence that BPI and lysozyme are active in serum, the activity of many other antimicrobial peptides such as defensins and cathelicidins in physiological conditions has been called into question (Maisetta *et al.*, 2008). High salt, divalent cations and serum proteases have a negative effect on the functionality of these peptides (Maisetta *et al.*, 2008). It is possible that antimicrobial proteins and peptides may act synergistically *in vivo*, increasing their effectiveness (Bowdish *et al.*, 2005).

Action of serum and its components on *E. coli*

Bactericidal serum factors have been shown to work in concert to cause lysis of serum-sensitive cells. In an *ex vivo* study by Donaldson *et al.*, serum lacking lysozyme and beta lysin was unable to cause lysis of a serum-sensitive *E. coli* strain despite the presence of an intact complement system. Platelet-derived beta lysin is a cationic antimicrobial protein which disrupts cytoplasmic membranes (Donaldson *et al.*, 1974). Purified lysozyme and beta lysin had no effect on survival in the absence of complement.

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Electron micrographs showed lysozyme acting on the bacterial cell wall and beta lysin induced damage of the cytoplasmic membrane. Complement-mediated damage was evident on the cytoplasmic membrane (Donaldson *et al.*, 1974).

The sequence of events leading to MAC killing of *E. coli* has been determined in several studies. Taylor and Kroll showed that deposition of complement factors on the outer membrane of *E. coli* was followed by degradation of cytoplasmic membrane phospholipids (Taylor & Kroll, 1984). Later events in the complement cascade were shown to affect respiration and cytoplasmic membrane potential. Specifically, C5b-8 complex formation on *E. coli* was found to inhibit respiration and transiently dissipate cytoplasmic membrane potential (Dankert, 1989). This transient dissipation in potential became irreversible when C9 was added to C5b-8 to form the MAC (Dankert & Esser, 1986; O'Hara *et al.*, 2001). Parallels have been drawn between the action of the active fragment of C9 (C9b) and colicins which both require an energized inner membrane to exert their effects (Dankert & Esser, 1986).

Serum-resistant *E. coli* are able to evade killing mediated by complement and other serum components. In contrast to serum-sensitive strains, reduced deposition of terminal MAC components has been reported, and cytoplasmic membrane is not degraded (Taylor & Kroll, 1984). Study of the metabolic responses of resistant cells to complement attack found that only transient inhibition of respiration occurred, implying that resistant bacteria recover from the inhibitory effects exerted by complement (Dankert, 1989). Protoypic *E. coli* strains reported to be serum-resistant include: CFT073, RS218, 536, CP9 and EC958 (Russo *et al.*, 1993; Grozdanov *et al.*, 2002; Prasadarao *et al.*, 2002; Buckles *et al.*, 2009; Phan *et al.*, 2013). Other isolates such as Nissle 1917, MG1655, HB101 and W3110 are known to be serum-sensitive (Bhakdi *et al.*, 1987; Grozdanov *et al.*, 2002; Miajlovic *et al.*, 2014, Phan *et al.*, 2013).

The role of polysaccharides in serum resistance

Production of a surface-associated polysaccharide layer known as capsule is common among *E. coli* that cause bacteraemia. Expression of capsule provides a steric barrier protecting the outer membrane from host defences including deposition of complement factors (Fig. 3, Buckles *et al.*, 2009). Capsules are firmly associated with the cell surface and in most cases consist of capsular (K) antigen. Polysaccharide layers composed of O antigen lacking a lipid A-core can also act as capsules (Whitfield, 2006).

Over 80 serologically distinct K-antigen polysaccharides have been identified belonging to four distinct groups (Whitfield, 2006). ExPEC strains typically have K antigens belonging to groups 2 and 3 (Whitfield, 2006). The K2 capsule of strain CFT073 and the K1 capsule associated with *E. coli* strains causing neonatal meningitis are major determinants of serum resistance (Leying *et al.*, 1990; Buckles *et al.*, 2009). The K54 antigen has also been implicated in human serum survival (Russo *et al.*, 1993). Not all K antigens confer serum resistance; studies carried out with K27 and K15 found no protective effect (Opal *et al.*, 1982; Schneider *et al.*, 2004). The association of capsule with pathogenesis in the urinary tract and bloodstream makes it an attractive target for novel therapeutics. Small molecule inhibitors of K-antigen synthesis have been developed and tested in *E. coli* expressing K1 and K5 capsule (Goller & Seed, 2010; Noah *et al.*, 2010a, b). One such inhibitor increased binding of C3 to the bacterial cell surface and significantly decreased serum survival (Goller & Seed, 2010).

Several O-antigen serotypes are associated with serum resistance (Johnson, 1991). Mutations which eliminate or reduce the number of O-antigen side chains associated with lipopolysaccharide also lead to increased serum sensitivity (Taylor & Robinson, 1980; Burns & Hull, 1998). This corresponds to the observation that rough strains of *E. coli*, in which lipopolysaccharide lacks O-antigen side chains, are generally more sensitive to serum than O-antigen-rich smooth strains (Johnson, 1991). O antigen may protect against serum killing by activating complement away from target sites on the outer membrane or by blocking antibody-binding sites (Sansano *et al.*, 1985; Johnson, 1991).

The protective role of lipopolysaccharide and O antigen in serum resistance was highlighted by a recent study investigating the serum resistome of *E. coli* ST131, a globally disseminated, multidrug resistant clone (Phan *et al.*, 2013). Utilizing transposon-directed insertion-site sequencing (TRADIS), Phan *et al.*, identified 56 genes required for human serum survival of ST131 strain EC958. Many of these were responsible for lipopolysaccharide production, including lipid A-core biosynthesis and O25-antigen biosynthesis and processing (Phan *et al.*, 2013).

Additional polysaccharides can be expressed by *E. coli* in conjunction with the K and O antigens under particular environmental conditions (Whitfield, 2006). Exopolysaccharide capsules have a distinct sugar composition and are loosely associated with the cell surface (Whitfield, 2006). The exopolysaccharide colanic acid is regulated by the two-component Rcs phosphorelay and has traditionally been associated with biofilm formation and protection from dessication (Clarke, 2010). Recent studies have shown that colanic acid also contributes to serum survival (Li *et al.*, 2005; Phan *et al.*, 2013; Miajlovic *et al.*, 2014). In a transcriptomic study investigating the response of ExPEC to human serum, increased gene expression of colanic acid biosynthesis operon was observed (Miajlovic

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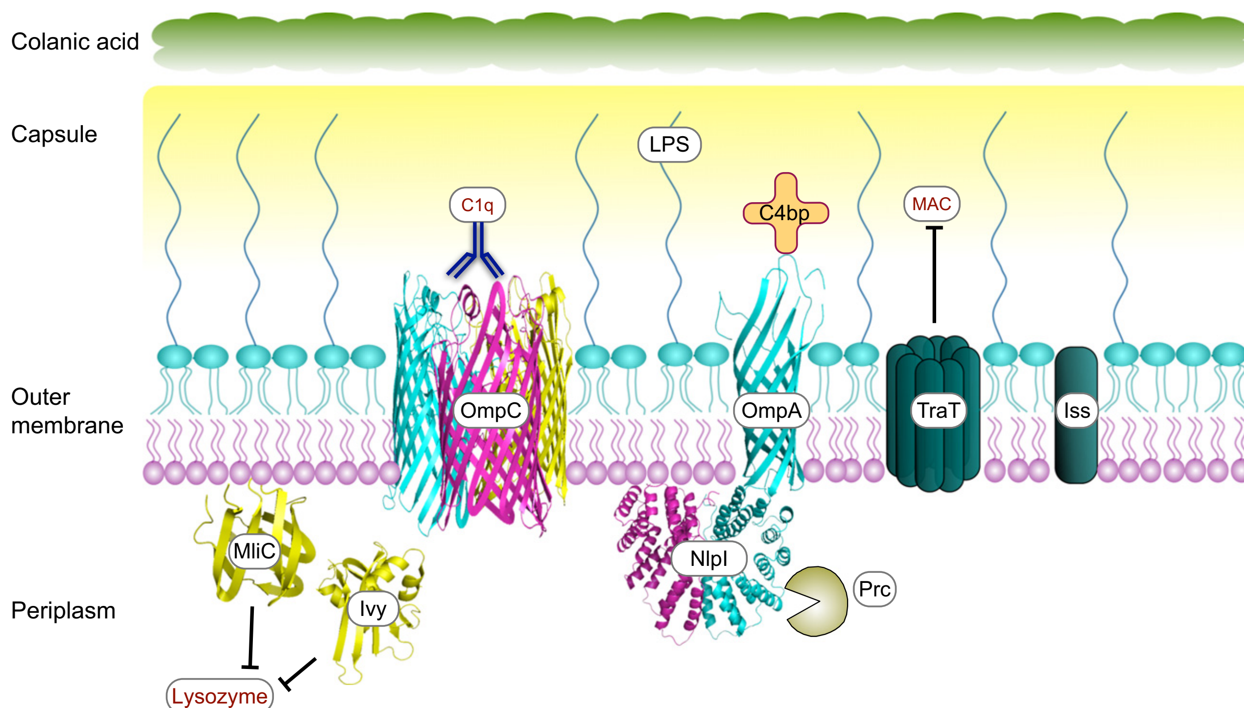

Fig. 3. Overview of *Escherichia coli* factors contributing to serum resistance. Production of K- or O-antigen polysaccharide capsule provides a protective barrier against the deposition of complement factors on the bacterial outer membrane. Colanic acid is an additional exopolysaccharide that can augment this protective effect. OmpA interferes with the classical complement cascade by binding to the complement regulatory protein C4bp which militates against the formation the C3b convertase that is essential for the eventual elaboration of the MAC. OmpA is known to interact with NlpI, and this protein is in turn activated by the Prc protease. OmpC acts as a major immunogen on the surface of *E. coli* promoting deposition of C1q and activation of the classical complement pathway, thus decreased expression of this protein assists in evading complement. TraT and Iss are outer membrane proteins that are predicted to inhibit the later stages of MAC formation. Finally, periplasmic lysozyme inhibitor MliC may inactivate lysozyme that gains access to the periplasmic space.

et al., 2014). Colanic acid was protective against the bactericidal effects of human serum in ExPEC strains CFT073 and RS218 (Miajlovic *et al.*, 2014). Genes involved in colonic acid synthesis were also implicated in fitness of an ExPEC strain in a mouse bacteraemia model (Subashchandrabose *et al.*, 2013). Rcs-regulated colanic acid is required for recovery and survival of *E. coli*-lacking peptidoglycan while *de novo* synthesis of cell wall occurs (Clarke, 2010). Similarly, serum-resistant *E. coli* strains may express colanic acid capsule as a protective measure while the cell recovers from damage induced by bactericidal factors of serum (Fig. 3).

Outer membrane integrity is important for serum resistance

Analysis of the transcriptional response of CFT073 to human bactericidal serum revealed induction of three extracytoplasmic stress response pathways including the Rcs and Cpx two-component systems and the alternate sigma factor, σE (Miajlovic *et al.*, 2014). These were also found to be protective against the bactericidal effects of serum (Miajlovic *et al.*, 2014). The Rcs two-component system promotes serum survival via induced expression of colanic acid (Miajlovic *et al.*, 2014). The Cpx and σE pathways may contribute to serum resistance by affecting outer membrane structure and integrity. Activation of the Cpx and σE pathways occurs following perturbations to the outer membrane, which acts as an indicator of extracytoplasmic stress (Bury-Mone *et al.*, 2009). In a similar manner, serum exposure seems to act as an envelope stress inducing extracytoplasmic stress response pathways (Miajlovic *et al.*, 2014). These pathways regulate the expression of proteins involved in assembly and maintenance of the cell envelope (Rhodius *et al.*, 2006; Vogt & Raivio, 2012). Other *E. coli* factors that are reported to contribute to serum resistance by affecting outer membrane integrity are listed in Table 1.

Interaction of *E. coli* with specific serum complement factors

Outer membrane protein A (OmpA) is a multifaceted protein that has been implicated in serum resistance

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Table 1. Additional *Escherichia coli* factors with a reported role in serum resistance

Bacterial factor	Proposed mechanism of resistance	Reference
Enterobacterial common antigen	Effect on O-antigen biosynthesis	Phan *et al.* (2013)
Tol-Pal system	Contribution to outer membrane integrity	Gaspar *et al.* (2000)
Phosphate transport system	Contribution to outer membrane integrity	Lamarche *et al.* (2005)
Lpp lipoprotein	Contribution to outer membrane integrity	Phan *et al.* (2013)
Bam lipoprotein	Contribution to outer membrane integrity	Phan *et al.* (2013)
Prc periplasmic protease	Proteolytically alters protein profile of the outer membrane	Wang *et al.* (2012)
Membrane-bound inhibitor of c-type lysozyme	Inhibition of serum lysozyme	Vanderkelen *et al.* (2012)
α-Haemolysin	Neutralization of unspecified serum component	Siegfried *et al.* (1992)

(Smith *et al.*, 2007). This protein has four surface-exposed loops of which regions in loops 1, 2 and 4 contribute to the serum resistance phenotype (Maruvada & Kim, 2011; Mittal *et al.*, 2011). Expression of OmpA promotes serum survival of *E. coli* and increased incidence of bacteraemia in neonatal rats (Weiser & Gotschlich, 1991). OmpA was shown to bind to the C4b-binding protein (C4bp), a complement regulatory protein (Fig. 3, Wooster *et al.*, 2006). C4bp is a cofactor for Factor I, a serine protease that degrades C4b and C3b inhibiting formation of the C3 convertase of the classical complement pathway (Prasadarao *et al.*, 2002; Wooster *et al.*, 2006). Recently, *E. coli* lipoprotein NlpI has also been shown to recruit C4bp to the bacterial cell surface inhibiting the classical complement pathway (Tseng *et al.*, 2012). Since this interaction did not appear to be direct, Tseng *et al.*, postulated that NlpI interacts with OmpA and that this interaction may be necessary for OmpA functionality. Alternatively, it was suggested that NlpI may affect outer membrane stability affecting correct surface expression OmpA (Tseng *et al.*, 2012).

Whilst some outer membrane proteins can antagonize complement action, others may be targets for the initiation of complement. OmpC is a major immunogen on the surface of *E. coli* which acts as a target for anti-OmpC IgG (Liu *et al.*, 2012). This promotes deposition of C1q and induction of the antibody-dependent classical complement pathway (Fig. 3). Loss of OmpC in *E. coli* increases resistance to complement-mediated killing (Liu *et al.*, 2012). Therefore, bacterial cells lacking OmpC exhibit increased survival in human serum by reduced activation of the classical complement pathway (Liu *et al.*, 2012). Repression of *ompC* expression may be advantageous in certain environments. Indeed, *ompC* is downregulated in response to bactericidal serum in *E. coli* strain CFT073 (Miajlovic *et al.*, 2014).

Plasmid-encoded serum resistance factors

A number of factors that thwart the action of complement are plasmid-encoded, and this has implications for the transmissibility of these genes leading to bacteria that exhibit enhanced serum resistance. TraT is one such factor, and it is an outer membrane lipoprotein encoded by some ColV plasmids and incompatibility group F plasmids (Johnson, 1991). TraT expression has been associated with increased resistance to serum although the mechanism underlying this property has not been conclusively determined (Johnson, 1991). Studies have suggested that TraT inhibits later stages of MAC activity (Binns *et al.*, 1982) and the formation or structure of the C5b6 complex (Pramoonjago *et al.*, 1992). TraT may also alter C3 deposition on the bacterial surface and affect outer membrane permeability (Aguero *et al.*, 1984; Sukupolvi & O'Connor, 1987).

The gene *iss* is found on ColV/BM plasmids and encodes the increased serum survival (Iss) protein. Expression of Iss increases survival in animal serum by an undetermined mechanism (Johnson, 1991). There is some evidence that Iss interferes with the action of MAC terminal complexes (Binns *et al.*, 1982). Three alleles of *iss* exist of which type 3 is associated with ExPEC strains while type 1 is most often found in APEC and NMEC (Johnson *et al.*, 2008). The phage λ gene *bor* shares 93% sequence identity with *iss* and 79% protein identity and also increases serum resistance of *E. coli* (Barondess & Beckwith, 1995).

Highjacking of a host molecule to avoid complement

A novel means of serum resistance involves the highjacking of a host cell regulatory molecule by *E. coli*. Incorporation

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of host cell protectin (CD59) into the bacterial cell membrane inhibits MAC formation (Rautemaa *et al.*, 1998). Protectin is an inhibitor of the complement system found on human cells, which prevents insertion of C5b-8 into cell membranes, thereby inhibiting host cell destruction (Rautemaa *et al.*, 1998). Rautemaa *et al.*, demonstrated that in the presence of Ca^{2+}, protectin released from human cells could incorporate into the outer membrane of *E. coli* and promote resistance to complement-mediated lysis. Whether specific bacterial factors are required for this process to occur is currently unknown.

Conclusions

Investigation into serum resistance of *E. coli* has identified multiple factors that contribute to this phenotype. Mechanisms promoting resistance include production of protective polysaccharide layers, interference with the complement cascade and expression of factors that contribute to the structural integrity of the cell envelope.

One of the most studied mechanisms of resistance is the production of a protective extracellular polysaccharide capsule. While the involvement of K- and O-antigen capsules in protection from serum killing is well established, new insights have emerged. The elucidation of the entire serum resistome of strain EC958 highlighted the importance of O25 antigen and lipopolysaccharide in serum resistance (Phan *et al.*, 2013). Colanic acid, an exopolysaccharide capsule which was thought to be involved in survival outside of the host, was also shown to contribute to serum survival in several ExPEC strains (Phan *et al.*, 2013; Miajlovic *et al.*, 2014).

Recent work has also furthered knowledge of well-established mechanisms of resistance such as the contribution of NlpI to OmpA-mediated resistance (Tseng *et al.*, 2012). While factors such OmpA are able to directly interfere with the complement cascade, other more general mechanisms of resistance may be of equal importance. Cell envelope structural integrity appears to be crucial for serum survival, and factors which contribute to synthesis and maintenance of outer membrane have a protective effect (Lloubes *et al.*, 2001; Phan *et al.*, 2013; Miajlovic *et al.*, 2014).

The availability of complete genome sequences for many serum-resistant *E. coli* and the application of techniques such as TRADIS and RNAseq should lead to an even greater understanding of the multiplicity of mechanisms deployed by this pathogen to evade complement and defend itself against other host factors.

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