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Lipopolysaccharides and outer membrane proteins as main structures involved in complement evasion strategies of non-typhoidal *Salmonella* strains

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ABSTRACT

Non-typhoidal *Salmonella* (NTS) infections pose a serious public health problem. In addition to the typical course of salmonellosis, an infection with *Salmonella* bacteria can often lead to parenteral infections and sepsis, which are particularly dangerous for children, the elderly and immunocompromised. Bacterial resistance to serum is a key virulence factor for the development of systemic infections. *Salmonella*, as an enterobacterial pathogen, has developed several mechanisms to escape and block the antibacterial effects of the complement system. In this review, we discuss the relevance of outer membrane polysaccharides to the complement evasion mechanisms of NTS strains. These include the influence of the overall length and density of the lipopolysaccharide molecules, modifications of the O-antigen lipopolysaccharide composition and the role of capsular polysaccharides in opsonization and protection of the outer membrane from the lytic action of complement. Additionally, we discuss specific outer membrane protein complement evasion mechanisms, such as recruitment of complement regulatory proteins, blocking assembly of late complement components to form the membrane attack complex and the proteolytic cleavage of complement proteins.

1. Introduction

When attacking the host, microorganisms encounter many defensive mechanisms from the immune system, whose aim is to prevent colonization, proliferation and spread of bacteria in the human body. The complement system, which is a central coordinator of innate immunity, quickly identifies and "marks" bacteria, consequently leading to direct lysis and phagocytic clearance. The interaction between bacteria and components of the immune system creates a selective pressure for bacteria to develop mechanisms to escape the immune response. All pathogens have developed numerous mechanisms of defence during evolution. In this review, we summarize the most successful complement evasion strategies of non-typhoidal *Salmonella* strains, highlighting the contribution of the lipopolysaccharide (LPS) and outer membrane proteins (OMPs) in serum resistance.

2. Overview of the complement system

The complement system (C) consists of a tightly regulated network of proteins and is one of the body's main defence systems. It is a link connecting the non-specific and specific immune response. The basic functions of the complement system are: labelling targets for clearance, inducing inflammatory reactions, attraction of phagocytes by chemotaxis, removal of immune complexes and tissue debris, cell activation and direct killing of microorganisms (Aksamit et al., 1981; Ehrengruber et al., 1994; Klos et al., 2009; Merle et al., 2015). However, excessive activation of the complement system may lead to the development of an uncontrolled inflammatory process and irreversible tissue and organ damage. Complement activation consists of a series of enzymatic and non-enzymatic cascade reactions. Due to the type of factor triggering the complement activation cascade, the initial stages of this cascade follow three different pathways: classical, lectin and alternative (Fig. 1).

The classical pathway (CP) is activated by immune complexes formed after recognition of foreign antigens by antibodies (IgG or IgM

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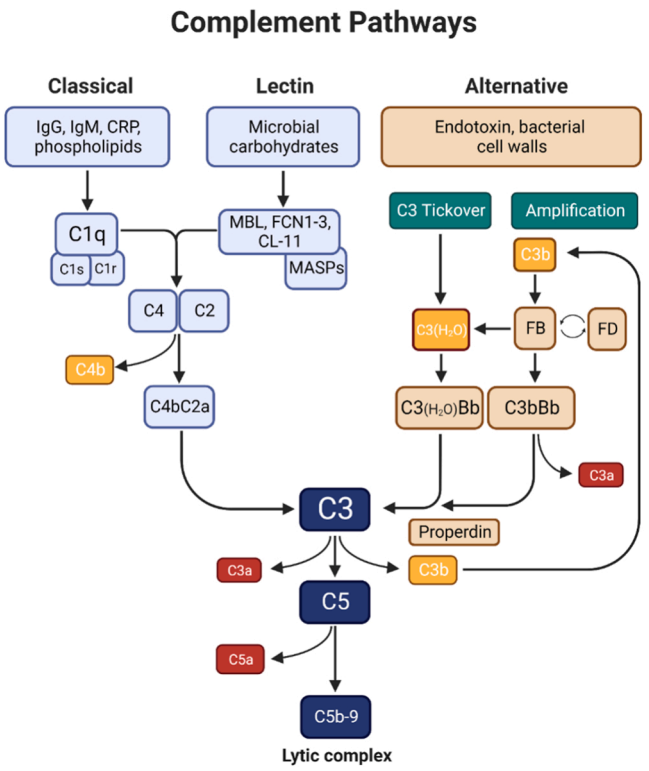

Fig. 1. Complement pathway and its effectors. Three main pathways are: Classical pathway (CP), Lectin Pathway (LP), and Alternative Pathway (AP). CP and LP activation is dependent on suitable pathogen or injury related molecular moieties that bind complement pattern recognition molecules such as C1q, Mannan binding lectin (MBL) or Ficolins (FCN). Recognition of pathogen/ damageassociated molecular patterns leads to complement cascade activation. AP can be activated via three different routes: 1) sporadic hydrolysis of C3 to C3 (H2O) results in short-lived soluble AP C3 convertase, which deposits C3b onto all surfaces at low rate. If the surface is without complement regulation the AP can amplify on the surface, 2) CP and LP initiated complement activation results in C3 cleavage which triggers both AP and terminal pathway, 3) properdin may promote AP activation on surfaces rich in certain types of LPS. There are three types of effectors in the complement cascade: 1) opsonins such as C1q, C4b and C3b/iC3b facilitate phagocytosis of the target, 2) anaphylatoxins C3a and C5a are proinflammatory chemoattractants and activators for white blood cells, 3) lytic C5b-9 complex may permeabilize target cells leading to calcium influxmediated metabolic "storm" or lysis.

immune complexes) or by direct binding of C1q to target structures. The lectin pathway (LP) is activated after binding of sugar residues (mannose, fucose, N- acetylglucosamine) presented in microbial surface polysaccharides by mannose binding lectin (MBL) or by acetylated structures via binding of ficolins 1–3. The alternative pathway (AP), unlike the other two pathways, is in a continuous state of low-grade auto-activation (tick-over) through soluble phase hydrolysis of C3 that leads to generation of C3 convertase enzymes (the initial C3 convertase $C3(H_2O)Bb$ and the "amplification" C3 convertase C3bBb) that subsequently deposit large amounts of C3b on any surface that lacks complement regulatory capacity (Merle et al., 2015). All activation pathways converge at the point, where C3 convertase (C4bC2b for the CP and LP or C3bBb for the AP) breaks down the C3 into biologically active products (C3a, C3b) (Law et al., 1979; Law and Levine, 1977). C3b binds the C3 convertase to form C5 convertase, which cleaves C5 to C5a and C5b. In the next step, C5b recruits C6, C7, C8 and multiple C9 molecules to form the membrane attack complex (C5b-9). The transmembrane channel formed by C5b-9 disrupts the cell membrane integrity and the membrane charge, possibly leading to cell lysis and death of the target microbe (Fig. 1).

3. *Salmonella* bacteria: classification and virulence

Salmonella are Gram-negative bacilli belonging to the family of *Enterobacteriaceae. Salmonella* have a very wide range of hosts and are facultative intracellular pathogens of worldwide epidemiological significance (Silva et al., 2014). In recent years, the nomenclature of the genus *Salmonella* has been the subject of much discussion and controversy. Today, most of the reference centers in the world, including the US Centers for Disease Control and Prevention (CDC), adopt the bacterial nomenclature system recommended by the World Health Organization (WHO). This system classifies *Salmonella* bacteria, based on differences in 16 S rRNA sequence analysis, into two species: *Salmonella enterica* (*S. enterica*) and *Salmonella bongori (S. bongori*). Based on biochemical properties and genomic relatedness, the species of *S. enterica* is further divided into six subspecies. Additionally, apart from classification based on phylogenetic similarity, *Salmonella* bacteria are divided into serovariants (serovars) based on the analysis of main antigen determinants (somatic antigen O and flagellar antigen H). According to the Kauffmann-White-Le Minor scheme developed in 2010 by the WHO, 2659 *Salmonella* serovars are distinguished (Issenhuth-Jeanjean et al., 2014). However, the number is most likely much higher as new serovars are still being discovered.

Salmonella bacteria are among the most prevalent food-borne pathogens. The main transmission route for *Salmonella* is the fecal-oral route and the infection can occur after consumption of contaminated water or food. The infections occur because due to improper preparation of food, or if basic hygiene rules are not followed. *Salmonella* infection may also develop through direct contact with feces of infected animals (Jajere, 2019). The infection with *Salmonella* bacteria may lead to various clinical symptoms, including typhoid/paratyphoid fever or salmonellosis. Based on the clinical symptoms, *Salmonella* strains can be classified as typhoidal *Salmonella* strains (TSS) and non-typhoidal *Salmonella* strains (NTS).

The etiological factors causing typhoid fever include strains of *Salmonella* Typhi (*S.* Typhi). In contrast, the paratyphoid fever is caused by *Salmonella* Paratyphi (*S.* Paratyphi) bacteria from groups A, B and C. *S.* Typhi and *S.* Paratyphi cause infections only in humans. The infection is caused by ingestion of food or water contaminated with waste from infected people. The symptoms and course of the disease are similar for both diseases. The incubation period is 7 days or longer, with prodromal symptoms such as headache and/or abdominal pain and diarrhea. During the disease, a specific, slowly increasing fever occurs, characterized by a temperature of 37.5–38.2 °C in the first week and a temperature of up to 41.5 °C in the second week (Parry et al., 2002; Patel et al., 2010; Thielman and Guerrant, 2004). Apart from the fever, the patients may experience liver and spleen enlargement and muscle pains. In some cases, there is also an additional erythematous maculopapular rash located on the chest and epigastric skin (Parry et al., 2002). In the majority of cases, the patient`s death occurs due to intestinal perforation and peritonitis or severe encephalopathy (de Jong et al., 2012).

Non-typhoidal *Salmonella* strains are the etiological agent of salmonellosis, which may occur in different clinical forms. The most common is the gastrointestinal form of infection. Compared to infections caused by TSS, the incubation period of disease caused by NTS is shorter. The infection presents with several symptoms: sudden diarrhea, vomiting, nausea, abdominal cramps, muscle pains, and low-grade fever not exceeding 39 °C. The symptoms are usually self-limiting, and the disease disappears within a few days of infection. However, it should be noted that immunocompromised and elderly patients or children under 5 years of age are more susceptible to NTS infections and the symptoms may be more severe (Majowicz et al., 2010). Although in most cases NTS causes gastrointestinal inflammation in humans, NTS infections may also take the form of bacteremia or organ form. The main symptom manifesting the presence of bacteria in blood is high fever. In extreme cases, bacteremia can lead to septic shock with high mortality rate. Bacteremia develops in about 5 % of patients with NTS infection. In some cases,

complications may occur, where the bacteria penetrating the organs can cause cholangitis, pneumonia, endocarditis or meningitis (Arii et al., 2002; Shimoni et al., 1999).

Salmonella-induced infections pose a serious public health problem. Infections with non-typhoid *Salmonella* strains are among the most prevalent infections in the world. There are approximately 94 million salmonellosis cases reported annually, of which about 155,000 are fatal (Majowicz et al., 2010). However, the mortality due to salmonellosis is mainly limited to developing countries. These figures are likely to be underestimations, as the short course of the salmonellosis and lack of access to healthcare may lead to under-reporting in developing countries.

Besides *Campylobacter*, *Salmonella* bacteria are the main cause of food poisoning in the European Union (EU). In 2019, EU countries reported 87,923 confirmed cases of infections caused by *Salmonella* bacteria. The five strains most frequently isolated from humans in the EU are: *S.* Enteritidis, *S.* Typhimurium, monophasic *S.* Typhimurium 1.4 [5].12:i:-, *S.* Infantis and *S.* Newport (Authority and European Centre for Disease Prevention and Control, 2021).

The course of infection with *Salmonella* depends largely on the interaction of the strain with the host. TSS and NTS have an overlapping set of virulence factors that enable the bacteria to invade and survive in the host organism. About 90 % of the genome of *S*. Typhi and *S*. Typhimurium is identical. Genes encoding the most important virulence factors are localized on *Salmonella* pathogenicity islands (SPI) and on the virulence plasmid pSLT (Parkhill et al., 2001). Not all serotypes are characterized by the presence of all known 23 SPI. However, SPI-1 - SPI-5 are common to all *S. enterica* serovars, and their contribution has been demonstrated in the pathogenesis of infections caused by *S*. Typhimurium strains.

After ingestion, *Salmonella* bacteria colonize the intestine and invade the intestinal epithelium. However, before bacteria can reach their target host cells in the small intestine, they must survive in the low pH of the stomach, which *Salmonella* accomplishes by maintaining a high intracellular pH (Fàbrega and Vila, 2013). After passing through the mucous layer present in the intestine, *Salmonella* adhere to the host tissues, which is believed to be one of the key events in the pathogenesis. *Salmonella* has been shown to be capable of colonizing a healthy host, but the probability of colonization increases during periods of immunosuppression or physiological stress (Lambert, 2009). The adherence of bacteria to the host cells is mediated, among others, by fimbriae and adhesins. Although *Salmonella* bacteria appear to preferentially adhere to and penetrate the M cells of the epithelial layer, direct invasion of enterocytes and direct uptake of bacteria by dendritic cells from the mucosa is also possible (Fàbrega and Vila, 2013). Additionally, the adhesion process is enhanced by the mobility and chemotactic attraction of the bacterial cell. It has been shown that strains lacking functional fimbriae have a reduced ability to adhere to the epithelial cells in the early stage of infection (Stecher et al., 2004). After recognizing and attaching to M cells, *Salmonella* bacteria begin the invasion process, which involves activation of the host cell effector proteins and remodelling of their cytoskeleton. The invasion process involves mainly bacterial proteins encoded by SPI-1 and proteins encoding the type III secretion system (T3SS). Initiating the remodeling of the cytoskeleton and inducing a pro-inflammatory response in the host is attributed to the effector proteins SopE and SigD, which act as activators of GTPases. SopE is responsible for the activation of the host proteins Cdc42 and Rac-1, while SigD activates the RhoG GTPase. Activation of the signaling cascade leads to rearrangement of actin cytoskeleton, and expression of pro-inflammatory cytokines (IL-8, TNF- α) through NF-kB mediated reprogramming of gene expression. The net effect of *Salmonella*-induced changes is an inflammation of the mucous membranes (Dos Santos et al., 2019; Hardy, 2004). Rearrangement of the cytoskeleton allows endocytic absorption of the bacteria by the host cells. The SipA protein increases the accumulation and stabilizes actin filaments, inhibiting their depolymerization in the early stages of infection (Haneda et al., 2012). Interestingly, the *Salmonella* induced hyperresponsiveness of inflammatory pathways may reduce the survival capacity of *Salmonella* in the host organism. Therefore, *Salmonella* bacteria regulate the level of the inflammatory response by secreting antagonistic anti-inflammatory effectors. SptP inhibits activation of the Cdc42 and Rac-1 proteins, thereby restricting remodelling of the cytoskeleton and the response induced by the SopE and SigD proteins (Fu and Galán, 1999). In turn, the SpvC protein reduces the expression of pro-inflammatory cytokines (Haneda et al., 2012). The process of invasion and rearrangement of the cytoskeleton leads to the formation of salmonella-containing vacuoles (SCV) (Steele-Mortimer, 2008). In SCV, bacterial cells replicate while recruiting phagocytes from the submucosa into the intestinal lumen. The SigD and SsaB effector proteins protect SCVs from degradation by fusion with lysosomes. After passing through the epithelium, mature SCVs are absorbed by phagocytes and the bacteria migrate through the bloodstream to the liver and spleen (Shotland et al., 2003).

4. Lipopolysaccharide: general structure and chemistry

About 70 % of the outer membrane surface of Gram-negative bacteria is composed of lipopolysaccharides. The LPS layer protects the bacterial cell against the host defence mechanisms or antibiotics and is one of the most important virulence factors in Gram-negative bacteria (Lodowska et al., 2007).

The LPS molecule consists of three structural domains: lipid A, core oligosaccharide and O-specific chain (O-antigen) built of repeating polysaccharide units (RU) (Fig. 2). Lipid A, covalently bound to the core oligosaccharide, is responsible for anchoring the LPS into the outer membrane. Lipid A is considered to be the most conservative part of the molecule. Nevertheless, after synthesis the lipid A region may undergo significant modifications, which create the heterogeneity of its structure in various bacterial species (Gunn et al., 1998; Guo et al., 1998; Needham and Trent, 2013). Lipid A is composed of a saccharide component, fatty acids and hydrophilic substitutes (e.g. phosphate groups, ethanolamine). The most conservative part of lipid A is the sugar part, most often composed of two glucosamine molecules connected by a β(1−6) glycoside bond, substituted with non-branched saturated fatty acids (2- and 3-hydroxy acids), which determine the amphiphilic character of the molecule (Lodowska et al., 2007). The oligosaccharide core is linked to lipid A by a ketosidic bond. Due to its unique structure, lipid A is responsible for the biological activity of LPS and confers the LPS molecule toxicity. There are two distinct regions within the core: the outer region - consisting mainly of hexoses, and the inner region - consisting of heptoses and 3-deoxy D-manno-octulosic acid (Kdo).

The O-specific chain is the part of the LPS most exposed to the external environment. It is composed of from a dozen to several dozen repeating units, each containing one to eight sugar residues. These polymers show high variability between bacterial strains and serovars. This is due to the chemical character of individual carbohydrate molecules building up the O-antigen subunits. They differ in chemical composition, sequence of sugar arrangements, types of bonds, side chains or content of non-sugar substitutes (Kaszowska, 2004; Lodowska et al., 2007; Samuel and Reeves, 2003). The above modifications result in a huge variety of O-chains. So far for *Salmonella* enterica 54 different O-antigens have been described (Samuel and Reeves, 2003). The O-specific chain, as the structure most exposed on the surface of the outer membrane, is one of the basic components, determining the survival of bacteria in the host tissues. From the clinical point of view, O-antigen is the crucial factor for differentiating the bacteria within the species and for determining particular serovars.

One of the main protective mechanisms against the lytic action of human serum is the ability to modulate the synthesis and structure of LPS. O-antigens differ not only in their chemical composition but also in the number of repeating subunits in the polysaccharide chain. The analysis of length distribution by polyacrylamide gel electrophoresis (SDS-PAGE) (Goldman et al., 1984; Jann et al., 1975; Switzer et al.,

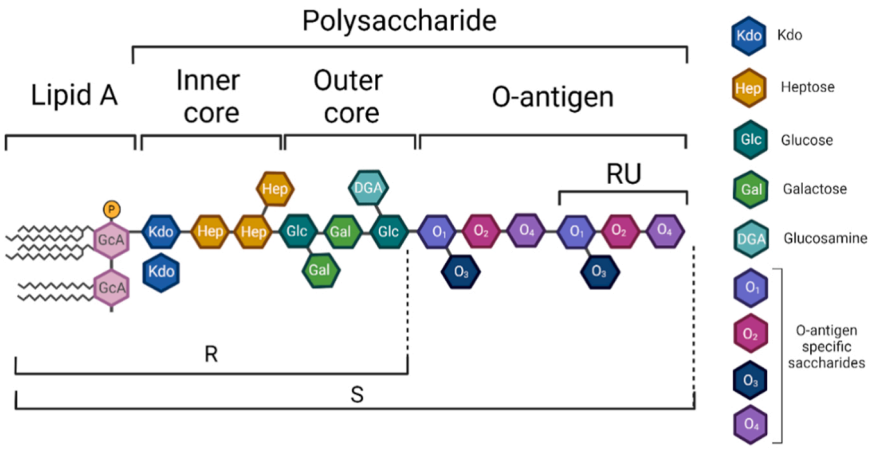

Fig. 2. LPS structure. LPS is anchored in the outer membrane via Lipid A, which may be modified further with inner and outer core oligosaccharides. So called rough (R) phenotypes lack O-antigen and can differ in the core oligosaccharide compositions; smooth (S) phenotype always contains Oantigen. O-antigen is highly variable in its length, repeating unit (RU) composition and relative abundance on the bacterial surface. Both R and S phenotypes convey immunoevasion.

1979) shows that LPS molecules isolated from the same strain of bacteria are not uniform but form a population of molecules of highly differentiated molecular weight, related to different numbers of subunits of polysaccharides (Fig. 3, A). Such a length distribution is caused by the presence of genes responsible for the O-antigen length control (Bastin et al., 1993; Batchelor et al., 1991; Stevenson et al., 1995). *S.* Typhimurium and *S.* Enteritidis LPS molecules are characterized by three clearly distinct fractions, differing in the number of RU. The low molecular weight O-antigen LPS (LMW-OAg LPS) fraction has less than 15 repeating subunits, the long O-antigen LPS (L-OAg LPS) has 16–35 RU, while the very long O-antigen LPS (VL-OAg LPS) has over 100 RU (Fig. 3). It has been demonstrated that the L-OAg LPS synthesis is controlled by the *wzzST* gene, whereas the *wzzfepE* gene is responsible for VL-OAg LPS synthesis (Murray et al., 2003). Homologs of *wzzfepE* protein were identified in *E. coli* O157:H7, *E. coli* K-12, *S. flexneri* and *S.* Typhi (Murray et al., 2003; Ozenberger et al., 1987; Parkhill et al., 2001; Schmitt and Payne, 1988). This indicates that LPS molecules present in three clearly different length fractions are common in

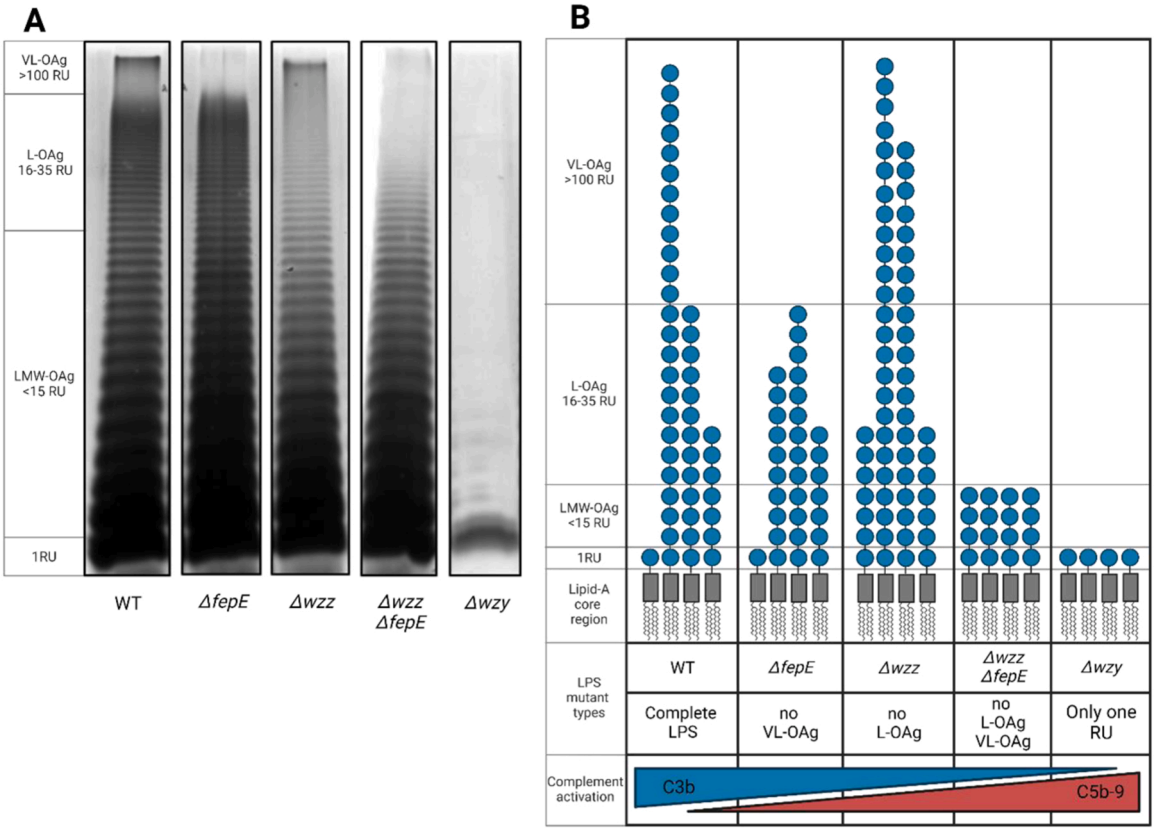

Fig. 3. LPS O-antigen length types in NTS strains and their relation to complement activation. Some NTS strains produce LPS composed of three fractions: VL-OAg, L-OAg and LMW-OAg, varying in the number of repeating units (RU). A) SDS-polyacrylamide gel electrophoresis of LPS isolated from Salmonella O-antigen mutants; B) Schematic overview of the LPS O-antigen length types in NTS strains. The length and density of O-antigen modulate complement deposition; longer O-antigens promote C3 activation but block C5b-9 insertion, shorter O-antigens allow C5b-9 insertion and lytic effect of complement (Liang-Takasaki et al., 1983; Joiner et al., 1986; Crawford et al., 2012; Murray et al., 2006; Grossman et al., 1987).

Enterobacteriaceae, but often, probably due to detection difficulties, VL-OAg LPS are not recognized in isolated LPS preparations. It is assumed that in Gram-negative bacteria smooth (S) strains synthesize LPS consisting of three separate regions: lipid A, core and O-antigen. Unlike smooth strains, the LPS of semi-rough (SR) strains is composed of lipid A, core and one RU in the O-specific chain. In contrast, rough (R) strains synthesize the so-called lipooligosaccharide (LOS) built only of lipid A and the oligosaccharide core (Fig. 2).

5. Contribution of lipopolysaccharide to the protection against the complement system

Complement activation produces covalently linked phagocytosis facilitating opsonins (C3b, C4b) and small soluble proinflammatory peptides called anaphylatoxins (C3a and C5a). Activation of the terminal pathway can result in the formation of lytic C5b-9 complex that decreases the viability or directly lyses affected cells. The formation of lytic C5b-9 (membrane attack complex, MAC), is a major factor influencing serum sensitivity as it can directly damage bacterial cells. In the case of NTS serum resistance, LPS is a major, although complex determinant. Generalizations on the LPS contribution are not easy to establish, even though purified LPS sample interactions with complement can be studied in vitro with relative ease. As a molecule LPS is extremely variable in its chemical structure, length, density and size distribution. These characteristics are strain, isolate and environment-dependent, and the overall contribution to serum resistance is also influenced by other evasion mechanisms such as outer membrane (OM) proteases and complement factor interacting proteins (Fig. 4, A–B, Fig. 5, A–C).

The first studies on carbohydrates and LPS in relation to complement activation date back to the studies of Pillemer in the 50's who established AP-based complement activation by LPS (Pillemer et al., 1954). Furthermore, it has been firmly established that some LPS structures alone may activate AP in solution and on cell surfaces (Gardiner et al., 1991; Vukajlovich et al., 1987). On an organism level, *Salmonella* serum survival may be dependent on alternative, classical or lectin pathway, or on a combination of them (Bugla-Płoskońska et al., 2010; Joiner et al., 1982a; Joiner et al., 1982b). The early studies into the relationship between LPS composition and complement system activation were focused on *S.* Minnesota (*S. minnesota*). This bacterium was an interesting research model due to the availability of different chemotypes, characterized by different compositions of the LPS core region. Key finding was that in the context of *S.* Minnesota, S strains and the core R-chemotypes may differentially trigger complement activation. Bjornson and Bjornson (1977) showed that in comparison to rough strains (Rb, Re chemotypes), the smooth *S.* Minnesota strain readily activated complement through the AP and could trigger bystander lysis of human erythrocytes suggestive of terminal pathway activation. The rough chemotype Rb could activate AP but to lesser extent. Others showed that the Re chemotype preferentially activated CP through direct C1q fixation or natural antibodies, and the O-antigen containing preparations only activated AP, even though they contained the same core structure as Re (Loos et al., 1974; Morrison and Kline, 1977; Vukajlovich et al., 1987). Interestingly, the apparent difference between the R and S chemotype-induced complement activation does not necessarily correlate with serum resistance. Joiner et al. (1982a) and Joiner et al. (1982b) showed that both serum-resistant smooth and serum-sensitive rough *S.* Minnesota strains activated serum complement, leading to opsonisation of the cells and activation of the terminal pathway. However, only the R strain was permeable after serum exposure, suggesting that the O-antigens of S strain may interfere with C5b-9 insertion. Later studies have established that the observed interference was dependent on the density of long O-antigen LPS chains on the outer membrane and on the hydrophilic properties of the O-antigen [51,52]. An appropriate density of long O-specific chains on the bacterial cell surface is necessary for the protection against the complement system by spatial obstruction of C5b-9 insertion into the OM (Grossman et al., 1987) (Fig. 4, A). However, this is not necessarily universal. Some NTS strains with an O-antigen of varying lengths, are not protected by the serum complement cytotoxicity (Bugla-Płoskońska et al., 2010; Dudek et al., 2016). Furthermore, C5b-9 is assembled on the membrane in sequence, where the intermediate C5b-7 complex is the first one to enter the membrane, C8 is the first to penetrate the whole membrane, allowing C9 molecules to bind and polymerize into a membrane spanning ring lesion (Bhakdi and Tranum-Jensen, 1986; Hadders et al., 2012).

The composition of the O-antigen is also an important factor to consider in the complement activation properties of LPS. Grossman and Leive demonstrated that for strains with O-antigens ranging 3–40 RU, the polysaccharide side-chain chemistry, rather than O-antigen length, determined C3 activation (Grossman and Leive, 1984). Furthermore, Grossman et al. (1990) demonstrated that the differences between *S.* Typhimurium and *S.* Enteritidis C3 opsonization most likely correspond to the differences in orientation of -OH groups in the O-antigen repeating unit positions C2 and C4 of abequose and tyvelose residues (Fig. 4, B). It is worth mentioning that also the saccharide composition of the LPS O-antigen can directly affect complement activation. Sialic acid as a component of the O-antigen of *Salmonella* O48 strains impacts C3b binding leading to reduced C3b deposition (Futoma-Kołoch et al., 2015). Mannose as a component of the O-antigen of *S.* Montevideo can be detected by MBL and lead to LP activation (Grossman et al., 1987). However, in *S.* Typhimurium the presence of O-antigen in the LPS

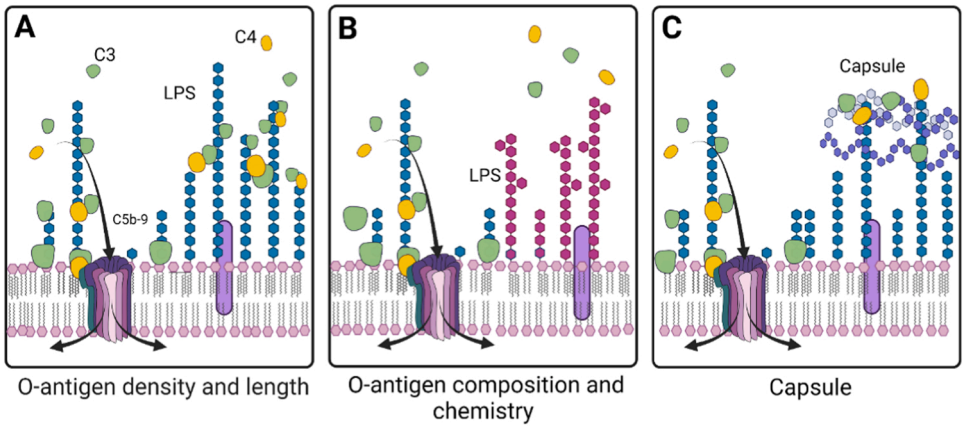

Fig. 4. Polysaccharide-related complement modulation in NTS strains. A) Overall length and density of O-antigen modulates complement deposition, longer O-antigens promote C3 activation but block C5b-9 insertion, shorter LPS allow C5b-9 insertion and lytic effect of complement. B) Various modifications to O-antigen composition can reduce C3 opsonization. C) Capsule may support C3 opsonization but it also shields the outer membrane from lytic complement.

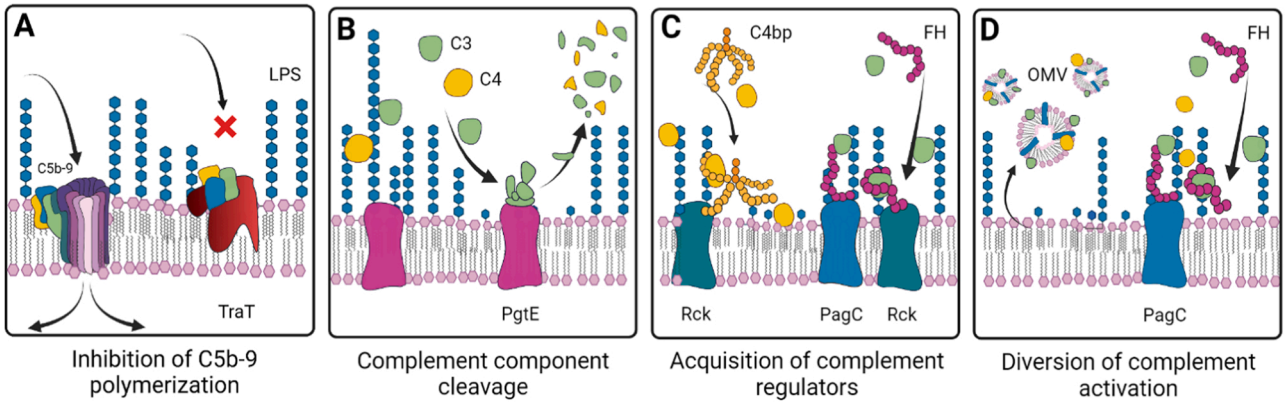

Fig. 5. Specific outer membrane protein complement evasion mechanisms of NTS strains. A) TraT is a membrane-associated protein that has been suggested to block C5b-9 formation on the outer membrane at the level of C5b-6 and C5b-8. B) PgtE is a transmembrane protease that can cleave C4b, C3b, C5, Factor B and Factor H. C) Rck is a transmembrane protein, which can bind the alternative pathway regulator FH and the classical and lectin pathway regulator C4bp. D) PagC is a transmembrane protein, which can bind FH (panel C), additionally OMV enriched in PagC can divert and inactivate complement away from the cell surface.

molecule reduced MBL binding properties and complement activation (Devyatyarova-Johnson et al., 2000). Therefore, evidence of serum complement consumption alone or in conjunction with bacterial opsonization with C3 is not a direct measure of complement-mediated cytotoxicity. Viability after serum exposure remains the clearest endpoint with regard to serum resistance. Furthermore, it is very difficult to generalize serum resistance based on single factors such as O-antigen density, RU distribution or biochemical composition of the O-antigen itself.

5.1. *Serum resistance in the context of O-antigen length*

The variable length and composition of the O-antigen and its contribution to complement activation or evasion has been the focus of decades of research. In NTS strains the O-antigen length can be categorized as LMW-OAg, L-OAg or recently better characterized VL-OAg (Murray et al., 2006, 2005, 2003) (Fig. 3). The length of the O-antigen has been observed to play multiple roles in colonization, environmental survival, cellular invasion, intracellular replication and in serum resistance (Bravo et al., 2008; Crawford et al., 2012; Murray et al., 2005; Nevola et al., 1985). The ability to colonize the mouse intestine is directly related to the length of the LPS molecule, decreasing along with the shortening of the LPS length (Nevola et al., 1985). According to Crawford et al., very long O-specific chains affect the survival of *S.* Typhimurium in bile (Crawford et al., 2012). Liang-Takasaki et al. were the first to demonstrate that *Salmonella* strains with longer O-antigen are more effectively phagocytosed and activate more serum C3 (Liang-Takasaki et al., 1983). Similarly, Joiner et al. demonstrated that C3 preferentially opsonises *S.* Montevideo LPS molecules with over 60 RU, compared to LPS with only 20–21 RU (Joiner et al., 1986). A similar result was obtained for *S.* Typhimurium with 15–38 RU. It consumed more C3 than a strain with 5–15 RU in the O-antigen (Murray et al., 2006). Grossman et al. (1987) demonstrated that serum resistant NTS strain *S.* Montevideo SL5222 had lipopolysaccharides that contained more than 14 O-antigen RU. Murray et al. (2006) showed that for *S.* Typhimurium the minimum complement protective O-antigen length was 5–15 RU. Furthermore, soluble high molecular weight O-antigens can rescue serum-sensitive bacteria and block serum hemolytic activity (Tomás et al., 1988).

Most of the studies describing complement evasion of NTS strains depending on the O-antigen LPS length have been done with mutants that lack specific genes responsible for LMW-OAg, L-OAg and VL-OAg biosynthesis (Fig. 3). Murray et al. confirmed the importance of O-antigen length in *S.* Typhimurium in the pathogenesis process. The contribution of LPS of different lengths to serum resistance was examined by comparing the serum sensitivity of different LPS length mutants to the wild *S.* Typhimurium strain. It was observed that while the wild type strain was very resistant to the complement system, the strain lacking the *wzzST* gene (the strain that did not produce L-OAg LPS) or *wzzfepE* (the strain that did not produce VL-OAg LPS) did not show significant difference in the susceptibility to the complement system. On the other hand, the mutant with two *wzz* genes impaired (the strain did not produce neither L-OAg LPS nor VL-OAg LPS) was highly sensitive to complement-mediated killing (Murray et al., 2003). Hölzer et al. studied the effect of LPS length on the invasion and intracellular replication of *S.* Typhimurium showing that the length of O-antigen is crucial for the functioning of the type III secretion system (T3SS) and the bacterial invasion process. Strains unable to synthesize VL-OAg LPS and L-OAg LPS or VL-OAg LPS showed an increased translocation of the effector protein SPI1-T3SS. However, changes in the length of O-specific LPS did not affect SPI2-T3SS (Hölzer et al., 2009). In contrast to previous observations of Murray et al, (2006) and Hölzer et al. (2009) could not confirm the effect of LPS length on bacterial absorption by macrophages. Murray et al. (2003) also indicated the significance of the length of O-antigen during infection in the mouse model. *S.* Typhimurium C5 *wzzfepE* showed a weaker infective capacity than the wild type strain. Later, Murray et al. (2005) demonstrated that LPS isolated from *S.* Typhimurium C5 and *wzzST* strains after culture in heat-inactivated serum exhibited an increase in the fraction of very long O-specific chains. It was demonstrated that this modified phenotype was directly related to the increased serum resistance. The length of O-antigen in *S.* Typhimurium also plays a major role in the process of bacterial cells absorption by macrophages. Murray et al. (2006) demonstrated that the tendency for bacterial absorption by macrophages increases with the shortening of the O-antigen.

In addition to LPS, the bacterial capsules in *Salmonella* are the second polysaccharide virulence factor. The capsules allow bacteria to avoid recognition by the host's immune system. Hence, they facilitate the spread throughout the host. One of the best-characterized polysaccharide capsules is Vi antigen. This antigen is most often described for *S.* Typhi, but it is also identified for *S.* Dublin and *S.* Paratyphi C (Ryan et al., 2017). Antigen Vi is a linear polymer of N-acetyl-galactosaminuronic acid molecules (Raffatellu et al., 2006). The Vi antigen possesses anti-inflammatory properties, which is the reason for the longer incubation time of typhoid fever in comparison to the infection caused by the NTS strains. The connection between the presence of capsular Vi antigen in bacteria and their resistance to bactericidal activity of human serum has been demonstrated. Looney and Steigbigel have shown that the encapsulated strain *S.* Typhi Quailes is more resistant to serum activity comparing to the non-capsulated *S.* Typhi

0901 (Looney and Steigbigel, 1986). Hashimoto et al. (1993) have demonstrated that the *S.* Typhi strain lacking the Vi antigen is characterized by 100 % mortality after 15 min of incubation in 90 % serum, whereas the encapsulated strain has a 50 % mortality rate. However, Bravo et al. (2008) investigating two *S.* Typhi strains differing on the presence of the capsule could not see an effect of the Vi antigen on the survival rate of the tested strains in human serum. The recognition and binding to free hydroxyl groups of LPS by the C3 protein is the first step of activation of the alternative complement pathway. Looney and Steigbigel have shown that encapsulated *S.* Typhi strains are not as effectively opsonized as non-capsulated strains (Looney and Steigbigel, 1986). In later studies, also Wilson et al. (2011) have demonstrated that the expression of Vi antigen reduces C3b opsonization to the bacterial cell surface. In recent years, more and more attention has been paid to group IV polysaccharide capsules, which have a high degree of similarity in structure with the O-antigen of the LPS molecule (Caboni et al., 2015; Freudenberger Catanzaro and Inzana, 2020; Marshall and Gunn, 2015; Patrick et al., 2009). It has been shown that in *Salmonella* the O-antigen capsules are important in the processes of adhesion and in the persistence of bacteria in the environment (Barak et al., 2007; Crawford et al., 2008). In *S.* Enteritidis and *S.* Typhimurium the O-antigen capsule biosynthesis is encoded by the *yshA-yihU* (Gibson et al., 2006; Zakikhany et al., 2010). Marshall and Gunn (2015) have shown that in *S.* Typhimurium the O-antigen capsule plays an important role in facilitating pathogenesis by modulating phase variation of flagella and protection of LPS from recognition by specific antibodies. In addition, the authors showed that the loss of the O-antigen capsule increases the sensitivity of *S.* Typhimurium to killing by human serum (Fig. 4, C). Increased serum sensitivity of the capsule deficient mutants (Δ*yshA-yihW*) correlated with increased C3 deposition on the bacterial surface (Marshall and Gunn, 2015).

5.2. Length control of the O-antigen by Wzz proteins

Due to the great influence of the length of the LPS molecule in protection against the complement system the question arose, how do the Wzz proteins control the length of the O-antigen? Closely related O-chain length regulators can be expressed in related bacteria, where they control the characteristics of length distribution of the O-antigen (Batchelor et al., 1992; Morona et al., 1995). The regulation of the length of the O-chain was found to be internally determined by the regulator itself (WzzB, WzzfepE) irrespective of huge differences in the chemical structure of individual substrates from which the O-antigen is formed in different bacterial species. The proteins regulating the length of the O-antigen belong to the superfamily of bacterial proteins known as polysaccharide co-polymers-1 (PCP-1), responsible for O-antigen processing (Cuthbertson et al., 2010; Morona et al., 1995; Tocilj et al., 2008). PCP-1 consists of two transmembrane helices and one periplasmic domain. Structural analysis of PCPs (WzzB from *S.* Typhimurium, FepE from *E. coli*) showed that the chain length regulators are assembled in oligomers of different composition (WzzB - pentamer, FepE - nonamer). They have similar three-dimensional structures despite significant differences in the sequence (identity of about 23 %). These observations may indicate the existence of a common mechanism used by these molecules to control the length of the polysaccharide chain (Tocilj et al., 2008).

So far, several models of interaction between the O-chain length regulators and the Wzy polymerase responsible for the addition of newly synthesized O-antigen repeating units to the lipid part of the LPS molecule have been proposed. A model by Carter and co-authors assumed that Wzz is a multi-protein complex, built of the flippase Wzx, the polymerase Wza and the ligase WaaL. However, the experimental data obtained from immunoprecipitation tests and chemical linking did not confirm the interactions between the WzzB length regulator and the Wza polymerase (Carter et al., 2009). Alternative in vitro studies carried out by Woodward and colleagues, with "reconstruction" of the O-antigen biosynthesis pathway using purified proteins, indicated that the length of mature antigen depends exclusively on the presence of the Wzy polymerase and the WzzB length regulator. However, neither in this case any clear interaction between these proteins was observed (Woodward et al., 2010). It is believed that the resulting O-antigen is elongated by the Wzy polymerase, attaching subsequent O-subunits to the non-reducing end of the growing molecule. Kalynych and colleagues (Kalynych et al., 2012) proposed a model of O-specific chain length regulation, where the Wzz proteins regulate the length of mature O-antigen by maintaining the growing O-specific chain in a conformation that enables further polymerization and prevents the growing polysaccharide from a premature release. When the O-antigen reaches the number of repeated subunits that can no longer be bound by the Wzz proteins, a change in conformation occurs and the length-regulating proteins dissociate. Then the mature O-antigen can be attached to the LPS lipid-core part by the WaaL ligase (Kalynych et al., 2012).

The control mechanism, responsible for the formation of LPS particles of a certain length, is not clear. Two hypotheses concerning regulation of the lipopolysaccharide molecule synthesis process are proposed: the "molecular clock", which regulates polymerization by reaction time (Bastin et al., 1993), or a "molecular ruler", where the assembly of the new O-specific chain stops upon reaching a certain length (Tocilj et al., 2008). Recent studies on the O-specific polysaccharide synthesis in *E. coli* O9a (King et al., 2014) show, however, that the length of the chain depends on the quantitative proportion of expression of two enzymes involved in the biosynthesis of O-antigen: WbdA responsible for the elongation of polysaccharide and WbdD responsible for the termination of the synthesis process.

Although there is some indication of mechanisms regulating the length of O-chains, there is little information on the proportions of LPS chains of different lengths on the bacterial cell surface. Palva and Mäkelä demonstrated that in *S.* Typhimurium the distribution of LPS molecules on the bacterial cell surface is uneven and is characterized by only a small percentage of molecules with 2–18 repeating O-specific subunits, while 77 % of LPS molecules have 19–34 RU (Palva and Mäkelä, 1980).

5.3. Salmonella O-antigen changes in response to external stimuli

In addition to regulation of the O-antigen length via *wzz* gene products, NTS strains have developed mechanisms for LPS O-antigen remodelling in response to external stimuli (i.e. growth phase, composition of medium, temperature, stress). Bravo et al. showed that the growth phases of *S.* Typhimurium and *S.* Enteritidis have an influence on the synthesis of the O-specific part of LPS. The authors demonstrated that these strains produce a higher amount of LMW-OAg LPS and L-OAg LPS in the stationary growth phase, compared to the logarithmic phase, while the synthesis of VL-OAg LPS remains at the same level during bacterial growth (Bravo et al., 2008). Additionally, the authors investigated the effect of growth phase on O-antigen production in deletion mutants *wzzST* and *wzzfepE*. *S.* Typhimurium LT2 *wzzfepE* has been shown to produce more LMW-OAg in the stationary phase compared to the logarithmic phase, while *S.* Typhimurium LT2 *wzzfepE* produces more L-OAg and LMW-OAg in the stationary phase compared to the logarithmic phase. *S.* Enteritidis PT4 *wzzSE* and *wzzfepE* strains were characterized by a similar length distribution under the same conditions (Bravo et al., 2008). Grossman et al. showed that the number of repeating units in the LPS molecule of *S.* Montevideo SL5222 is connected with the addition of mannose to the culture medium (Grossman et al., 1987). Delgado and co-authors demonstrated that in *S.* Typhimurium the expression of *wzzST* gene is induced in the culture medium containing Mg^{2+} and Fe^{3+} ions (Delgado et al., 2006). McConnell and Wright have shown that the length and distribution of the *S.* Anatum O-antigen are dependent on growth temperature. While the cells of *S.* Anatum synthesize smooth LPS at 37 °C, at lower temperatures

(20–25 °C) the synthesis of SR LPS also occurs (McConnell and Wright, 1979). Pawlak and co-authors demonstrated that a prolonged exposure of *Salmonella* O48 strains to human serum affects the average length of the resulting O-antigen (Pawlak et al., 2017). Bravo et al. tested also, whether LPS length regulation depending on the growth phase affects the resistance to human serum. The team demonstrated that *S.* Typhimurium LT2 strain and its mutant *wzzST* were resistant to 20 % human serum regardless of the growth phase. In contrast, *S.* Typhimurium LT2 *wzzfepE* was 100 % sensitive to 20 % human serum in the logarithmic growth phase, whereas in the stationary growth phase it showed a 30 % survival (Bravo et al., 2008). Despite numerous reports on the influence of environmental factors on the regulation of lipopolysaccharide length, the knowledge on how the microbial culture conditions modulate the length of the resulting O-specific chains is insufficient.

6. Contribution of outer membrane proteins to protection against complement

Salmonella bacteria show an exceptional ability to adapt into hostile environments, including human blood. Apart from LPS, OMPs also play a key role in protecting the bacteria against the lytic action of the complement system. To date, several surface proteins that determine serum resistance have been detected in NTS strains. These include TraT, Rck, PagC and PgtE (Fig. 5, A–D).

Proteins Rck and TraT are encoded on the virulence plasmid, while the PagC and PgtE are encoded on the chromosome. The *traT* gene is expressed on an F-like conjugative plasmid. TraT lipoprotein is a surface protein with a molecular weight of ca. 26 kDa, possessing 245 amino acid residues in the polypeptide chain. Lack of TraT protein is resulting in altered outer membrane permeability and decreased virulence (Rhen and Sukupolvi, 1988; Sukupolvi et al., 1990). It was shown by Rhen and Sukupolvi that TraT determines the resistance to serum in *E. coli* and *S.* Typhimurium (Rhen and Sukupolvi, 1988). The mechanism of action of TraT relies probably on inhibition of the formation of the C5b6 complex from the C5b and C6 precursor proteins or on formation of the non-functional complex, which in consequence may disrupt the proper action of the C5b-9 (Pramoonjago et al., 1992) (Fig. 5, A). The *rck* gene is encoded by a *pefI-srgC* operon carried by *Salmonella* virulence plasmids. Koczerka et al. (2021) demonstrated the presence of *rck* in 36 *Salmonella enterica* subspecies, highlighting an important role of Rck protein in virulence. The 17 kDa Rck is a protein (161 amino acid residues) of the outer membrane, composed of 8 transmembrane domains and 4 exposed loops (Cirillo et al., 1996). Hackett et al. (1987) have shown that a 2.4 kb fragment obtained from the cosmid DNA library of the virulent *S.* Typhimurium strain, after introduction into the rough (serum-sensitive) strain of *S.* Typhimurium, is responsible for the emergence of resistance to serum by producing 17 kDa protein on the cell surface. Heffernan et al. (1992) have suggested that the mechanism of serum resistance of Rck, is to inhibit the incorporation of the C5b-9 complex into the bacterial outer membrane (Fig. 5, C). Cirillo and al. demonstrated that a substitution of glycine to aspartic acid in the loop 3 of Rck reduced serum sensitivity and invasion of eukaryotic cells (Cirillo et al., 1996). The homology between Rck and other outer membrane proteins of Gram-negative bacteria described in the literature is referred to by Heffernan et al. as conditioning virulence. The Rck protein has 53 % similarity to PagC protein of *E. coli* and 48 % similarity to the Ail protein of *Y. enterocolitica*. Although these proteins have similar structural folds, the greatest sequence diversity is observed in the external regions, which most likely are responsible for the interactions with host cells (Heffernan et al., 1992). Despite differences in the structure, it has been shown that Rck, PagC and Ail help avoiding the host immune response in similar manner.

A mechanism commonly used by bacteria to avoid response from the human immune system is the recruitment of regulatory proteins of the complement system (Factor H (FH), C4b-binding protein (C4bp), vitronectin) (Kraiczy and Würzner, 2006; Lambris et al., 2008). Ho et al. have shown that the Rck proteins from *S.* Typhimurium and *S.* Enteritidis, when expressed in *E. coli* BL21, bind FH, a key regulator of the alternative complement pathway. The authors demonstrated that the expression of Rck protects the bacterium against the lytic action of the alternative complement pathway by limiting deposition of C3b and the C5b-9 complex on the cell surface. It has been shown that Rck binds domains 5–7 and 19–20 of F H (Ho et al., 2010). In further studies, Ho et al. have demonstrated also the ability of Rck to bind C4bp, a regulator of both the classical and lectin complement pathways. The authors have localized the Rck binding site in the 7th or 8th domain of C4bp (Ho et al., 2011) (Fig. 5, C). A similar mechanism of recruitment and utilization of FH and C4bp has been observed for the protein Ail from *Y. enterocolitica* (Ho et al., 2014, 2012).

PagC is an outer membrane protein of *E. coli* with a molecular weight of ca. 18 kDa (188 amino acid residues). Nishio et al. (2005) have shown that the *pagC* gene cloned into *E. coli* and *S.* Choleraesuis led to a high level of resistance to bactericidal action of porcine serum. The resistance to serum was dependent on the amino acid sequence (Glu-89, Val-90) at the C-terminus of the second domain of the PagC protein (Nishio et al., 2005). The authors also investigated the correlation between the presence of LPS, PagC expression and serum sensitivity. It has been demonstrated that the rough *S.* Choleraesuis mutant, lacking the O-specific antigen and the outer part of core oligosaccharide, had a reduced survival rate in porcine serum, with PagC expression level similar as in the wild-type strain. This indicated that in addition to PagC also LPS contributes to serum resistance. The two component system PhoP-PhoQ is responsible for regulation of genes involved in intracellular survival of *Salmonella* (Bader et al., 2005; Prost et al., 2007). Activation of PhoP-PhoQ leads to the modification of OM components like LPS, OMP and to the regulation of outer membrane vesicle (OMV) production (Bonnington and Kuehn, 2016; Dehinwal et al., 2021). Dehinwal et al. (2021) described a new PagC-depended mechanism of complement evasion in NTS strains (Dehinwal et al., 2021). The authors demonstrated that OMV enriched in PagC diverted and inactivated complement away from the cell surface. Additionally, the authors showed that OMV produced by *Salmonella* strains expressing protein PagC or Rck inactivated complement by FH recruitment (Fig. 5, C-D).

PgtE is a protein belonging to the family of enterobacterial aspartic proteases that activate human plasminogen (Kukkonen and Korhonen, 2004). PgtE is ca. 35 kDa outer membrane protein that requires short-chain LPS on the bacterial cell surface for full activity (Kukkonen et al., 2004; Lähteenmäki et al., 2005). *Salmonella* strains have been shown to produce a shortened form of LPS during infection in mouse macrophages and thereby to increase PgtE expression level. When released from mouse macrophages, *Salmonella* with a rough form of lipopolysaccharide are directly exposed to the activity of complement. However, an increased expression of PgtE on the cell surface protects them from lysis (Lähteenmäki et al., 2005). Ramu et al. have shown that the expression of PgtE increases the resistance of *S.* Typhimurium to human serum activity. The authors have demonstrated that PgtE cleaves the complement C3b component into partially similar fragments as Factor I (FI). Thus, the cleavage prevents formation of the alternative pathway C3 convertase and no further complement cascade is activated. The authors have shown that in addition to C3b, PgtE can also cleave C4b and C5 components (Ramu et al., 2007) (Fig. 5, B). Riva et al. (2015) have also demonstrated that the PgtE protease cleaves complement Factor B (FB) and the regulating protein FH, and thereby increases the survival of *Salmonella* strains after leaving the phagocytic cells. It is probable that FB after interaction with PgtE is no longer able to interact with C3b. Cleavage of FH in domains 1–4 may, however, cause uncontrolled activation of the complement system and lead to rapid consumption of any active C3b molecules (Riva et al., 2015).

Dudek et al. analyzed the lipopolysaccharide composition and outer membrane proteomes of *S.* Enteritidis strains differing in serum sensitivity. All the tested strains, regardless the level of sensitivity to human serum, produced VL-OAg LPS. However, mass spectrometric analysis

showed that serum-resistant strains produced higher levels of PgtE compared to serum sensitive strains (Dudek et al., 2016). It is very likely that proteins that determine serum resistance will be present also in other than *S*. Enteritidis or *S*. Typhimurium serovars. Futoma-Kołoch et al. showed that serum resistance of *S*. Hadar, *S*. Sydney, *S*. Marina and *S*. Erlangen can be connected to the presence of outer membrane proteins of 17-, 25-, 35- and 51–54 kDa (Futoma-Koloch et al., 2012; Futoma-Kołoch et al., 2019). The ability of *Salmonella* to modulate the OM composition is essential for survival in harsh environments. Thus, when combined, the above data shows that *Salmonella* have multiple mechanisms and layers of serum resistance. Often, more than one outer membrane structure is involved in the complement evasion mechanism and needed for the mechanism to work. Thus, the interplay between the individual components seems to be important. The activity of PgtE strongly depends on the LPS type presented on the cell surface. Ramu et al. (2007) showed that a wild type *S*. Typhimurium strain expressing a smooth type of LPS was not able to cleave C3b, C4b and C5 in comparison to the rough mutant. However, it should be emphasized that there is lack of information about the influence of different types of LPS varying in the RU number on the interplay with PgtE and other complement evasion proteins. There is a strong evidence for interplay between LPS and complement evasion proteins for other bacteria than *Salmonella*. It was also shown that Ail-mediated C4bp binding is connected with the LPS length. Only strains lacking the O-antigen and outer core region were able to actively bind the complement regulator C4bp (Kirjavainen et al., 2008).

7. Concluding remarks

It should be emphasized that there are many mechanisms that enable *Salmonella* to survive in an unfavorable environment and spread throughout the host organism. In this review, we have highlighted the main serum resistance mechanisms of NTS strains, showing that *Salmonella* bacteria can interact via LPS and/or OMP with complement components at different stages or use complement regulatory proteins to facilitate complement degradation. We described in detail the role of lipopolysaccharide O-antigen chain length in sequestration of complement proteins and pointed out the link between the exposition of a certain type of LPS on the outer membrane and the activation of bacterial proteases. This leads us to the conclusion that despite significant advances in understanding the biochemical and structural foundations of evasion mechanisms of NTS strains against the complement system, it seems essential for the future to study also the interactions occurring between various outer membrane structures to obtain a complete picture of the mechanisms that protect bacteria from the complement system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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