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Searching for Outer Membrane Proteins Typical of Serum-Sensitive and Serum-Resistant Phenotypes of *Salmonella*

Bozena Futoma-Koloch[1], Gabriela Bugla-Ploskonska[1] and Jolanta Sarowska[2]
[1]*Institute of Genetics and Microbiology*
University of Wroclaw, Przybyszewskiego, Wroclaw
[2]*Department of Basic Sciences*
Wroclaw Medical University, Chalubinskiego
Poland

1. Introduction

The pathogenesis of serum-resistance *Salmonella* infections seems to be connected with a variety of their surface structures. *Salmonella* resistance to innate immune factors aids the despersal of bacteria in host tissues and body fluids. This paper shows the natural resistance of clinical *S*. Enteritidis, *S*. Typhimurium, and *S*. Hadar strains to the antibacterial activity of human serum. Curiously, some of the pathogens modify their lipopolysaccharide (LPS) to escape host surveillance. A well-known strategy developed by bacteria is sialylation with sialic acid (NeuAc) of surface structures to mimic host tissues. It is very interesting, that even though LPS of the same chemical structure covers *Salmonella* O48, these bacteria differ in their susceptibility to the antibacterial activity of serum. Previous results indicate that the presence of sialylated LPS do not protect *Salmonella* O48 against the bactericidal activity of human and animal serum, and the presence of NeuAc in the LPS structure is not sufficient to block activation of the alternative pathway of complement in serum. Because outer membrane proteins (OMPs) are also surface virulence factors and have a significant role in pathobiology and bacterial adaptation to environmental conditions, researchers have directed their investigations through the analysis of *Salmonella* OMPs patterns and have attempted to identify among them key molecular targets of the protective immune response against *Salmonella*. This work also highlights the importance of OMPs as candidates for vaccine targets. In this review, we have collected and discussed published results, as well as new ones, shown for the first time.

2. Salmonellosis – An emerging problem

Salmonella enterica is a main ethological factor for infectious diseases worldwide (Hohmann, 2001; Rabsch et al., 2001). Serotypes, which cause disease, are divided into the following groups: typhoid species (TS) that are human specific pathogens (Typhi and Paratyphi serotypes) and non-typhoid species (NTS) spread to humans from animal sources. The most

common non-typhoid *Salmonella spp*. serovars have a potential to cause two basic kinds of infections: gastroenteritis and extraintestinal infections. The host environment varies from the ubiquitous (non-host-adapted) serovars for example Typhimurium and Enteritidis, to host restricted - *S.* Dublin, *S.* Choleraesuis. Both can cause infection in cattle, pigs or humans, and host-specific ones – e.g. *S.* Pullorum, which is found in chickens only. About 5% of patients with gastrointestinal illness of non-typhoid *Salmonella* spp. serotypes develop bacteremia (Hohmann, 2001). The survival of *S.* Enteritidis in poultry products has been linked to its remarkable ability to quick respond to environmental signals and adapt to its surroundings. *S.* Enteritidis may exist naturally in poultry at low incidence but it seems to have another reservoir, rodents. Human cases of *S*. Enteritidis rapidly increased through the 1980s and 1990s. For the last few decades, *S.* Hadar has been one of the main common serotypes isolated from foodborne disease in Europe. In humans, *S.* Hadar usually causes gastroenteritis, characterized by non-bloody-diarrhea, vomiting, nausea and fever (Rowe et al., 1980). Non-typhoid *Salmonella spp.* (NTS) are broadly dispersed in the environment as well as in the gastrointestinal tracts of both domesticated and wild animals. Up to 90% of *Salmonella* infections in the United States are food-borne in origin (CDC, 2009). Regardless of the fact that non-typhoid *Salmonella spp.* gastroenteritis is most often self-limited, these bacteria cause the most food-borne disease worldwide. Among the conditions for salmonellosis to develop are: gastric hypoacidity, extremes of age, alteration of the endogenous flora, diabetes, rheumatological disorders, sickle cell disease, malaria and immunosuppression (Bronzan et al., 2007).

In developed countries, the main risk factor for acquisition of typhoid *Salmonella* bacteraemia is travel to an endemic region, however, non-typhoid *Salmonella* may more often lead to food-borne diseases in non-endemic countries (Simonsen et al., 2010; Scallan et al., 2011). Invasive NTS is endemic in sub-Saharan Africa where it is a leading cause of fatal bacteremia among African children and HIV-infected adults. Increasing levels of antibiotic resistance among African strains of NTS indicate that a vaccine is urgently needed (Siggins et al., 2011). In Malawi, MacLennan et al. (2008) recently found that NTS bacteriemia particularly affects African children between 4 months and 2 years of age, the period in which immunoglobulin levels to NTS are low or absent. Mortality for nontyphoid *Salmonella* is reported to be as high as 60% in African patients with HIV (Boyle et al., 2007).

The local inflammation of specific tissues or organs also called focal infections can cause diseases such as: pneumonia, meningitis, endocarditis, or infections of the urinary tract (Ekman et al., 2000; Tena et al., 2007; Kedzierska et al., 2008). Gastroinestinal infection due to *Salmonella* may also lead to reactive arthritis (ReA) (Yu, 1999). It has been suggested that the persistence of bacterial antigens is typical of ReA, and is a result of the ineffective elimination of microbes in patients with post infection complications (Granfors et al., 1998). Data presented by Locht et al. (1993) and Thomson et al. (1995) indicated that 1–15% of individuals infected with *Salmonella* gastroenteritis develop a postinfection ReA. Despite the fact that most *Salmonella* strains are pathogenic to humans and animals, their virulence seems to be different depending on the serovar (Threlfall, 2005). Serovars of *Salmonella* belonging to somatic antigen group O48 are clinically important bacteria causing intestinal dysfunction and diarrhoea in animals, infants and children. Table 1 includes epidemiologic data (1984-2008) of the distribution of *S. enterica* and *S. bongori* strains in animals world-wide. Note that reptiles are the hosts for the greatest number of *Salmonella* serovars producing diseases especially in children.

***Salmonella* isolates**	**Origin**	**Biological source**	**Source of occurrence in human after contact with animals/infections symptoms**	**References**
Salmonella enterica subsp. *diarizonae*	Ireland	turtles, iguanas, snakes	*Salmonella* was detected in a 6-month-old boy who had diarrhoea and respiratory symptoms	O'Byrne and Mahon, 2008
	Japan	gecko, boa garden, python	salmonellosis	Nakadai et al., 2005
Salmonella enterica subsp. *houtenae*	Canada	turtles, iguanas, snakes	*Salmonella* was detected in blood and urine of the 11-year-old boy after contact with family pets	Woodward, 1997
Salmonella enterica subsp. *enterica* sv. Minnesota	Ireland	iguana, Persian cats, rabbits	bloody diarrhoea, fever, abdominal pain, haematuria	O'Byrne and Mahon, 2008
Salmonella enterica subsp. *enterica* sv. Enteritidis	Ireland	fish, dog, terrapin	diarrhoea, fever, abdominal pain	O'Byrne and Mahon, 2008
	Japan	gecko	salmonellosis	Nakadai et al., 2005
Salmonella enterica subsp. *enterica* sv. Pomona	Ireland	terrapins	bloody diarrhoea, vomiting	O'Byrne and Mahon, 2008
Salmonella enterica subsp. *enterica* sv. Poona	Belgium	turtles	4-month-old girl/ septicaemia	Bertrand et al, 2008
	Canada, United States (Indiana, Pensylvania)	iguana	*Salmonella* was detected in an 3-year-old boy, 3-week-old boy who died; 21-day-old girl	Woodward et al., 1997
Salmonella enterica subsp. *enterica* sv. Typhimurium	France	snakes, iguana	infection imported from China to young children	Bertrand et al., 2008
Salmonella enterica subsp. *arizonae*	Ireland	snakes, fowls, goats, swine, minks	diarrhoea	O'Byrne and Mahon, 2008
	United Kingdom	snakes	*Salmonella* was detected in the stool/gastroenteritidis with reactive arthritis	Foster and Kerr, 2005
	Japan	lizards, iguana	salmonellosis	Nakadai et al., 2005
	Italy	chameleons	----------	Corrente et al., 2004
Salmonella bongori	Italy	----------	*Salmonella* was detected in children/acute enteritidis	Giammanco et al., 2002
Salmonella enterica subsp. *houtenae* serovar Marina	Canada	iguanas	*Salmonella* was detected in an 11-year-old boy, twin baby brothers, and a baby boy after contact with a pet iguana	Woodward et al., 1997
	Germany	snakes	----------	Schröter et al., 2004

Table 1. The distribution of *Salmonella* isolates in animals, selected cases in period 1984-2008

In Poland, a total of 13,362 salmonellosis cases were reported in 2006, and 11,934 in 2007. In 2007 the incidence rate was 30.7 per 100.000 population. The most common type of outbreaks (251 recorded cases) was household outbreaks. Over seventy percent of patients were hospitalized. As in previous years, the seasonal peak of outbreak in Poland was observed in July and August. *S.* Enteritidis was the most frequently isolated serotype of *Salmonella* and constituted over 80% of cases. Two-year old children were the most affected age group (Lazinska et al., 2005; Sarowska et al., 2005). The relative high burden of *S.* Enteritidis in Europe accounts for 67% of salmonellosis, and of *S.* Typhimurium accounts for 9% of the cases (de Jong and Ekdahl, 2006). However, the proportion of *S.* Enteritidis cases from different countries varied from 25% (Iceland) to 98% (Latvia). *S.* Enteritidis is currently the second most frequently isolated serovar in the United States - accounting for nearly 15% of reported human salmonellosis cases (Callaway et al. 2008).

3. Susceptibility of *Salmonella* spp. to the bactericidal activity of serum

It is known that complement and bactericidal activity of serum may protect healthy hosts from invasion of serum sensitive microorganisms (Gondwe et al., 2010). Complement (C) is a part of the innate adaptive immune system and it consists of at least 35 proteins, mainly in pre-activated enzymatic forms. Bacterial invasion activates this defense host system in a few seconds. The complement system mainly recognizes and promotes the clearance of invading microorganisms and host cells damaged by phagocytosis. The following three mechanisms lead to complement activation: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP), which results in membrane attack complex (MAC, C5b-9) formation. It has been suggested that complement is necessary for protection against microbial infection. Immunoglobulins (Ab) can potentially protect against salmonellosis. Ab can protect in a cell-independent manner through complement-dependent bactericidal activity and by opsonizing bacteria for uptake and killing by phagocytic cells (Würzner, 1999; Morgan, 1999).

Resistance to complement-mediated killing is a key virulence property of microbial pathogens, such as *Salmonella* strains. Long-chain LPS has been shown to confer resistance by promoting the deposition of C components at a distance from the outer membrane (OM), thus preventing membrane disruption with MAC (Bravo et al., 2008). The surface-exposed protein, PagC has been shown to confer resistance when present in *S.* Choleraesuis (Nishio et al., 2005). The surface protease PgtE also mediates serum resistance presumably *via* its ability to cleave C components C3, C4, and C5 (Ramu et al., 2007). TraT, an OMP has been found to inhibit complement at the MAC formation stage (Pramoonjago et al., 1992). Rck is a 17-kDa protein structurally related to PagC and has been shown to inhibit MAC function. Rck belongs to a family of 17-19 kDa OMP including PagC, OmpX and Ail. Nevertheless, these proteins have been shown to confer resistance in Gram-negative bacteria to C-mediated killing. Ho and co-workers (2010) presented evidence that Rck of *S.* Typhimurium and *S.* Enteritidis expressed in *E. coli* BL21 with a defective *galE* gene binds the regulator of the AP factor H. fH binding is accociated with resistance to the AP and reduced deposition of C3b, Bb and MAC. Biedzka-Sarek and co-workers (2008) observed that Ail was not masked by distal region of LPS, such as O-antigen. Further investigations are required to determine if LPS length plays a role in fH binding to Rck. LPS with a sialic acid moiety within an O-specific chain also regulates AP amplification by binding fH, hence preventing activation of the C system through the alternative pathway.

Sialic acids present in the surface antigens of bacteria may contribute to the pathogenicity of the microorganisms by mimicking host tissue components (Vimr and Lichtensteiger, 2002). Serovars Enteritidis, Typhimurium, and Hadar do not possess sialylated O-antigens. Results presented by Cisowska et al. (2004) indicate that encapsulated *E. coli* K1 strains have different degrees of susceptibility to the bactericidal action of normal human serum (NHS). These results confirm previous observations (Mielnik et al., 2001) concerning sialylated LPS of *Salmonella spp., Escherichia spp., Citrobacter spp., and Hafnia spp*. Lipooligosaccharide sialylation in *Neisseria meningitidis* serogroup C is a critical determinant of MBL binding. Data provided by Jack et al. (2001) idicate that sialylation down-regulates the rate of MBL-mediated C activation. In nonsialylated microorganisms, MBL increased the rate of acquisition of C5b-9.

Long-term investigations carried out on *Salmonella* O48 shown that strains differ in the susceptibility to various sera, human or animal (Bugla-Ploskonska et al., 2011, 2010a, 2010b, 2009b, 2009c; Futoma et al. 2005). These authors suggest that the presense of sialylated LPSs do not play a decisive role in determining bacterial resistance to the bactericidal activity of serum, and that the presence of NeuAc in the LPS structure is not sufficient to block activation of the alternative pathway of complement in serum. Curiously, even though LPS of the same chemical structure cover *Salmonella* O48, they differ in the susceptibility to the antibacterial activity of serum. Hence we can place a question „Which feature of bacteria determines their behaviour in the serum as an environment to live?". Because OMPs are also surface virulence factors and have a significant role in pathobiology of Gram-negative bacteria and bacterial adaptation to environmental conditions, many authors have directed their investigations through the analysis of *Salmonella* OMP patterns using various molecular methods to investigate these antigens (see below). OMPs are of special interest to researchers as they can modify the susceptibility of bacteria to the bactericidal activity of serum (Alberti et al., 1993; Kustos et al., 2007).

The above-mentioned authors (Bugla-Ploskonska et al., 2011, 2010a, 2010b, 2009b, 2009c; Futoma et al. 2005) and Sarowska and co-workers (2010) described the results of *Salmonella* susceptibility to human and bovine serum. Bactericidal activity of serum was determined as described previously (Doroszkiewicz, 1997). In short, adequately prepared bacteria were incubated with diluted serum (12.5%, 25% or 30%, 50% and 75% in physiological saline) in a water bath at 37°C. The number of colony-forming units per milliliter (CFU/ml) at time 0 was taken as 100%. Strains with survival rates greater than 100% in serum after 180 min of incubation were considered resistant, and those with survival rates less than 100% were considered susceptible to the bactericidal action of the serum. The results of *Salmonella* O48 susceptibility to serum are presented in Table 2. The last column of Table 2 lists the molecular weights of OMPs, which are characteristic only for a given serovar and are characteristic for only resistant or sensitive strains.

The studies were carried out on seventeen *Salmonella* strains, which contain sialic acids in the O-specific side chains of LPSs (O48 somatic antigen group):

S. enterica subsp. *arizonae* PCM 2543, *S. enterica* subsp. *arizonae* PCM 2544, *S. enterica* subsp. *salamae* sv. Ngozi PCM 2514, *S. bongori* sv. Balboa PCM 2552, *S. enterica* subsp. *salamae* sv. Hammonia PCM 2535, *S. enterica* subsp. *salamae* sv. Hagenbeck PCM 2534, *S. enterica*

subsp. *enterica* sv. Dahlem PCM 2512, *S. enterica* subsp. *enterica* sv. Djakarta PCM 2513, *S. enterica* subsp. *enterica* sv. Toucra PCM 2515, *S. enterica* subsp. *enterica* sv. Hisingen PCM 2536, *S. enterica* subsp. *salamae* sv. Sakaraha PCM 2538, *S.* sp. PCM 2548, *S. enterica* subsp. *diarizonae* PCM 2511, *S. bongori* sv. Bongori PCM 2547, *S. enterica* subsp. *salamae* sv. Erlangen PCM 2533, *S. enterica* subsp. *houtenae* sv. Marina PCM 2546, *S. enterica* subsp. *enterica* sv. Sydney PCM 2551. The strains were obtained from the Polish Collection of Microorganisms (PCM), Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland. The rest of the tested strains, shown in Table 2) had been isolated from fecal samples from children with gastroenteritis at the Lower Silesian Pediatric Center in Wroclaw during 2009-2010, and they were *S. enterica* subsp. *enterica* sv. Typhimurium (O4), *S. enterica* subsp. *enterica* sv. Enteritidis (O9), *S. enterica* subsp. *enterica* sv. Hadar (O8). Susceptibility of these serovars to 50% normal human serum was established by Sarowska et al. (2010).

Eleven strains of *Salmonella* O48 were sensitive to the bactericidal effect of human, bovine or cord serum, whereas six *Salmonella* O48 strains, and serotypes of somatic antigen groups, O4, O9, and O8 demonstrated higher resistance to the bactericidal activity of the serum. In other experiments, aimed at analyzing the mechanism of serum complement activation, serovars, which were sensitive to the bactericidal action of serum, were used. Bugla-Ploskonska et al. (2010a, 2010b, 2009b) examined serum in which AP or CP/LP pathways of C were inhibited. Authors established the following mechanisms of complement activation by *Salmonella* O48.

- CP - only the classical/lectin pathways were important in the bactericidal mechanism of complement activation,
- AP - only the alternative pathway was important in the bactericidal mechanism of complement activation,
- CP/AP - independent activation of the classical/lectin pathways and enhancing the alternative pathway in the bactericidal mechanism of complement activation,
- CL + AP - parralel activation of the classical/lectin and enhancing the alternative pathway in the antibacterial activity of complement system.

It is interesting and new information that the strains possessing the same somatic antigen group O48 present diverse susceptibility to serum and different patterns of C activation.

Sarowska and co-workers (2010) examined that *Salmonella* ESBL+ transconjugants belonging to three serovars: Enteritidis, Typhimurium, and Hadar were more sensitive to NHS than before conjugation process. It seems that the acquisition of new plasmids from *Klebsiella pneumoniae* (donor) might have unfavourable consequences for these bacteria and increased their susceptibility to serum activity. A probable explanation of this could be the remodeling of the envelope of the bacterial cells, e.g. OM composition.

It was shown, that the complex bacterial stress response may be conducted with the releasing of the outer membrane vesicles. McBroom and Kuehn (2007) demonstrated that the quantity of vesicle release correlates with the level of toxic misfolded protein accumulation in the cell envelope. Accumulation of material occurs when cells are exposured to damaging stressors such as temperature, nutrient availability, toxic agents. This process can act to selectively eliminate unwanted material. Further work is required to identify how envelope stress translates into bacterial resistance in serum. Native vesicles

contain OM and periplasmic material, and they are released from the bacterial surface without loss of membrane integrity. More surprisingly, vesicles from some species contain DNA (Nikaido, 2003). These vesicles, however, have not been well characterized in most studies. For example, the reported protein composition of vesicles from *P. aeruginosa* is strikingly different from that of the OM (Kadurugamuwa and Beveridge, 1995). Vesicles were shown to be enriched in monomeric OM proteins, OmpA, OmpX, and OmpW, but contain less of the trimeric porins OmpF and OmpC (Horstman and Kuehn, 2000). Hellman et al. (2000) showed that bacterial OMPs are released into serum in complexes that also contain LPS. Release of 18-kDa OMP from *E. coli* J5 into serum was greater for bacteria in early logarithmic than in late logarithmic growth and was increased by antibiotics *in vitro* and *in vivo*. These data raise the question as to whether released OMPs, such as the 18-kDa OMP, could play a role in the pathogenesis of Gram-negative sepsis.

Strain no	Sera	Concentration of serum				OMPs (kDa)[1]
		12.5 %	25% or 30%	50%	75%	
SENSITIVE STRAINS						
S. arizonae PCM 2543	NHS NBS	NT[2]	sensitive sensitive	sensitive sensitive	sensitive sensitive	60, 48, 45[3]
S. arizonae PCM 2544	NHS NBS	NT	sensitive sensitive	sensitive sensitive	sensitive sensitive	60, 31, 24, 12[3]
S. Ngozi PCM 2514	NHS NBS	NT	sensitive sensitive	sensitive sensitive	sensitive sensitive	58, 45, 42[3]
S. Balboa PCM 2552	NHS NBS	NT	sensitive sensitive	NT sensitive	sensitive sensitive	49, 46, 39[3]
S. Hammonia PCM 2535	NHS NBS	NT	sensitive sensitive	NT sensitive	sensitive sensitive	57[3]
S. Hagenbeck PCM 2534	NHS NBS	NT	sensitive sensitive	NT sensitive	sensitive sensitive	47, 31[3]
S. Dahlem PCM 2512	NHS NCS NBS	NT sensitive NT	NT sensitive sensitive	sensitive sensitive sensitive	sensitive sensitive sensitive	50
S. Djakarta PCM 2513	NHS NCS NBS	NT sensitive NT	NT sensitive sensitive	sensitive sensitive sensitive	sensitive sensitive sensitive	56
S. Toucra PCM 2515	NHS NCS NBS	NT **resistant** NT	NT sensitive sensitive	sensitive sensitive sensitive	sensitive sensitive sensitive	none[4]
S. Hisingen PCM 2536	NHS NCS NBS	NT **resistant** NT	NT **resistant** sensitive	sensitive sensitive sensitive	sensitive sensitive sensitive	56
S. Sakaraha PCM 2538	NHS NCS NBS	NT **resistant** NT	NT **resistant** **resistant**	sensitive sensitive sensitive	sensitive sensitive sensitive	none[4]

RESISTANT STRAINS[5]						
S. sp. PCM 2548	NHS NCS NBS	NT NT NT	NT NT sensitive	**resistant** NT **resistant**	**resistant** NT **resistant**	93, 62, 30, 25, 17[3]
S. diarizonae PCM 2511	NHS NCS NBS	NT **resistant** NT	NT **resistant** sensitive	sensitive **resistant** sensitive	sensitive **resistant** sensitive	194, 84, 66, 8
S. Bongori PCM 2547	NHS NCS NBS	sensitive **resistant** NT	sensitive **resistant** sensitive	sensitive **resistant** sensitive	sensitive **resistant** sensitive	183, 99
S. Erlangen PCM 2533	NHS NCS NBS	sensitive **resistant** NT	sensitive **resistant** **resistant**	sensitive **resistant** sensitive	sensitive **resistant** sensitive	69, 54, 8
S. Marina PCM 2546	NHS NCS NBS	NT **resistant** NT	NT **resistant** **resistant**	NT **resistant** **resistant**	**resistant** **resistant** **resistant**	41
S. Sydney PCM 2551	NHS NCS NBS	**resistant** **resistant** NT	sensitive **resistant** **resistant**	sensitive **resistant** **resistant**	sensitive **resistant** **resistant**	74
S. Typhimurium	NHS NCS NBS	NT	NT	**resistant** NT NT	NT NT NT	82, 66, 65, 51, 41, 27, 25, 17
S. Enteritidis	NHS NCS NBS	NT	NT	**resistant** NT NT	NT NT NT	79, 66, 65, 53, 41, 29, 27, 17
S. Hadar	NHS NCS NBS	NT	NT	**resistant** NT NT	NT NT NT	82, 66, 65, 51, 41, 27, 25, 17

References: Bugla-Ploskonska et al. (2011, 2010a, 2010b, 2009b, 2009c); Futoma et al. (2005); Sarowska et al. (2010)
NHS – normal human serum; NCS – normal cord serum; NBS – normal bovine serum
[1] OMPs characteristic for only serum- sensitive strains (not present in serum-resistant strains) or for only serum-resistant strains (not present in serum-sensitive strains)
[2] NT – not tested
[3] data presented only in this paper
[4] there are any individual OMPs in this strain when compare to others
[5] strains were regarded as resistant when their survival was above 100% in the highest concentrations of serum

Table 2. The susceptibility of *Salmonella* strains to NHS, NCS, and NBS put together with OMPs

3.1 The role of sialylated lipopolysaccharide

Bacteria have evolved mechanisms for evading recognition by the immune system. Sialylation of LPS or LOS mediates serum resistance of *N. gonorrhoeae* (Ram et al., 1998). Sialic acids are important constituents of glycoconjugates in animal tissues, which regulate

innate immunity. Covering bacterial surfaces with sialylated oligosaccharides mimic those of the host (molecular mimicry). Incorporation of NeuAc into the surface components of the cell envelope of some pathogenic bacteria inhibits the direct activation of the alternative complement pathway (Rautemaa and Meri, 1999). Activation of AP is regulated by an interaction between C3b and factor H. It is known that NeuAc can enhance the binding of factor H to C3b on the cell surface, which blocks the amplification of the AP of C activation. fH is a critical regulator of the C system, and acts a a cofactor for factor I-mediated cleavage of C3b. fH also inactivates AP convertase by dissociating Bb from the C3bBb complex (Pangburn et al., 1977). Interestingly, the positive effect of LPS sialylation on fH binding requires the presence of gonococcal porin PorB, suggesting that sialylation may cause a conformational change in the LPS that unmasks novel sites in PorB (Severi et al., 2007).

Smooth strains of *S*. Typhimurium (nonsialylated) were usually resistant to the action of serum, whereas strains of the Ra chemotype by sera of some piglets were killed (Dlabac, 1968). It was shown that the LPS O-antigen (O-Ag) plays an important role in resistance to complement-mediated serum killing in several Gram-negative bacteria (Bengoechea et al., 2004, Grossman et al., 1987, Joiner, 1988). It was shown that LPS O-antigen and the outer core of *Y. enterocolitica* do not contribute directly to complement resistance. Instead, the major *Y. enterocolitica* serum resistance determinants include OMPs such as YadA and Ail (Kirjavainen et al., 2008).

It was reported that the amount of O-Ag and its chain length distribution are important factors in protecting bacteria from serum complement. Bravo et al. (2008) demonstrated that increased amounts of length distribution produced by *S*. Typhi grown to stationary phase confered higher levels of bacterial resistance to human serum. They suggest that O-Ag is more important for survival of *S*. Typhi in serum than the Vi antigen, and that non-typhoidal serovars are more resistant than serovar Typhi to human serum.

3.2 The role of OMPs

The sensitivity of bacteria to the bactericidal activity of serum depends on the structure and organization of their outer membrane. About 50% of OM consists of proteins, so their role in the pathogenicity of Gram-negative bacteria cannot be dismissed (Koebnik et al., 2000). The resistance of bacteria to serum's lytic activity may be one of the virulence factors essential in the development of sepsis. Changes in OMPs' expression can result in Gram-negative bacteria developing resistance to the bactericidal activity of serum (Bugla and Doroszkiewicz, 2006; Weiser and Gotschlich, 1991; White et al., 2005; Attia et al., 2005; Nishio et al., 2005). Studies have shown that porins from several bacteria stimulate cells to produce and secrete cytokines (Galdiero et al., 1993). Whole bacterial cells, which express on their surface LPS and porins both use CD14 when interact with leukocydes. Galdiero and others (2001) showed that CD14 and CD11a/18 are involved in cytokine responses to LPS, but only CD11a/18 is involved in those of porins. Their previous studies (Galdiero et al., 1990) showed that S. Typhimurium porins inhibit phagocytosis by activating the adenylate cyclase system.

Some OMPs amplify the sensitivity of bacteria (Zollinger et al., 1987, Merino et al., 1998, Alberti et al., 1993) to serum. Binding of OmpK36 of the serum sensitive strain *K. pneumoniae* to C1q leads to activation of the complement classical pathway and the subsequent deposition of C components C3b and C5b-9 on the porin (Alberti et al., 1996). *Y. enterocolitica* serum resistance is dependent of the presence of proteins YadA and Ail, which bind C4b-binding

protein, an inhibitor of both the classical and lectin pathways of C (Kirjavainen et al., 2008). Another example of the serum resistance phenotype of bacteria is *Actinobacillus actinomycetemcomitans* expressing surface protein Omp100, which traps factor H, an inhibitor for C3 (Asakawa et al., 2003). Futoma-Koloch et al. (2006) investigated the serial passage of *Proteus mirabilis* O18 in 90% bovine serum what contributed to over-expression of some classes of OMPs. It was also found a large heterogeneity of the OMPs' profile among *Salmonella spp*. after their passage in serum (Skwara et al., 2011). Studies of Kroll and co-authors (1983) have shown that treating *E. coli* cells with sera also generates changes in their OMPs composition.

Many years ago, some authors (Joiner et al., 1982; Taylor, 1983) suggested that the nature of bacterial resistance to the action of sera is quite complex. Galdiero et al. (1984) showed that the C system could be activated by porins isolated from *S.* Typhimurium. Chaffer et al. (1999) showed that *E. coli* O2 isolates that possess 35 kDa OmpA are higly resistant to the bactericidal effect of serum. Others (Cirillo et al., 1996) pointed out that Rck (17-19 kDa) protein in *S.* Typhimurium is associated with high–level serum resistance. This OMP probably inhibits lysis of the bacterial cell that occurs with the MAC complex. *S.* Choleraesuis' resistance to serum may be enhanced by the presence of the outer membrane protein PagC (17 kDa) (Nishio et al., 2005). Rck is a member of a family of OMP including PagC of *S*. Typhimurium (Gunn et al., 1995) and Ail of *Y. enterocolitica* (Miller et al., 1990). Site-directed mutagenesis and "domain-swapping" experiments done with Rck show that loop 3 is required for serum resistance and invasion in *E. coli* (Cirillo et al., 1996).

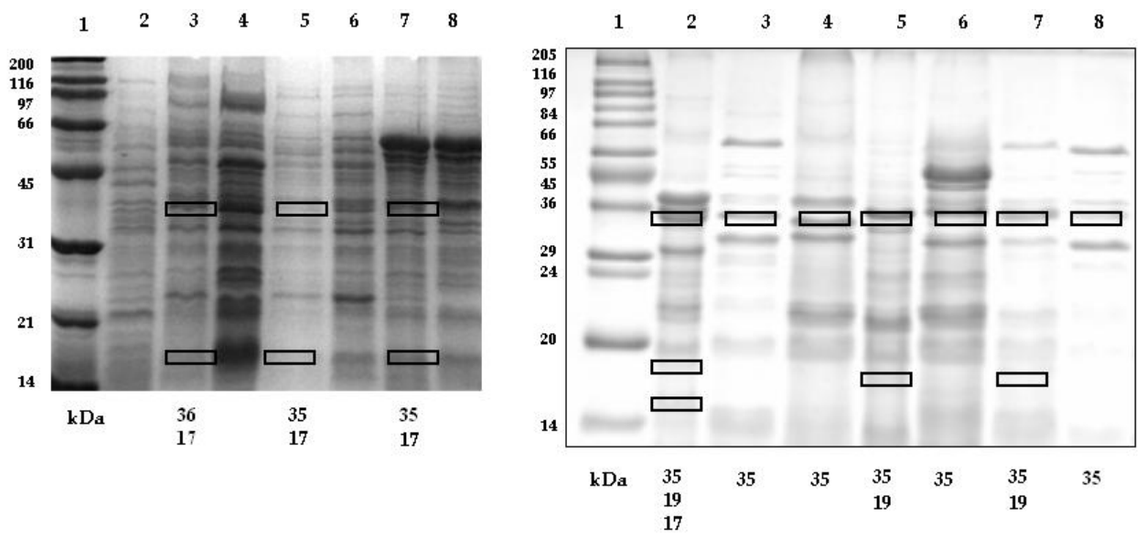
Bugla-Ploskonska and co-workers (2011) confirmed that certain OMPs are characteristic of the serum-resistant and serum-sensitive phenotypes of *Salmonella* O48 to the bactericidal action of cord serum (see Table 2.). But the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analysis of the OMPs isolated from: *S*. Hisingen PCM 2536, *S*. Dahlem PCM 2512, *S*. Djakarta PCM 2513, *S*. Toucra PCM 2515, *S*. Sakaraha PCM 2538, *S. diarizonae* PCM 2511, *S*. Bongori PCM 2547, *S*. Erlangen PCM 2533, *S*. Marina PCM 2546, and *S*. Sydney PCM 2551, showed no correlation between numbers of colored stripes within the same path and the susceptibility of the tested strains to cord serum. The strain, which was most sensitive to the lytic activity of cord serum S. Dahlem PCM 2515 had 32 OMPs with distinct molecular weights, while serum-resistant S. Marina PCM 2546 had only 20 OMPs.

When comparing the results of Bugla-Ploskonska et al. (2011) (data not shown in Fig. 1.), Sarowska et al. (2010) (Fig. 1.A) and Futoma-Koloch et al. (data published in this paper) (Fig. 1.B) one may note different electrophoretic band patterns for OMPs of tested strains of *Salmonella*. The patterns of OMPs (Fig. 1.) are similar to those reported in the literature, and allow the identification of the major proteins, including the 36- to 41-kDa proteins known as porins (Kamio and Nikaido, 1977). These proteins, which were common in *S.* Typhimurium, *S.* Enteritidis, and *S*. Hadar strains, are 66-, 65-, 41-, 27-, and 17-kDa. These results are similar to that obtained for *S*. Typhi by Ortiz et al. (1989), which had 17-, 28-, and 36- to 41-kDa proteins. While studying the composition of OM of *S*. Typhimurium, Smit and Nikaido (1978) observed that these bacteria contain three porins of 34-, 35-, and 36-kDa.

As show in Fig 1., the tested strains share the same OMP peptide band located at 35 kDa, the region containing the OmpA. This protein has been shown to contribute to the increased resistance of *E. coli* to the bactericidal effect of serum by classical pathway activation. All the serum-resistant strains *S.* sp. PCM 2548 (Fig. 1.B, line 2), *S. diarizonae* PCM 2511, *S.* Bongori

PCM 2547, *S.* Erlangen PCM 2533, *S.* Marina PCM 2546, *S.* Sydney PCM 2551 (Bugla-Ploskonska et al., 2011), *S.* Enteritidis, *S.* Typhimurium, and *S.* Hadar (Sarowska et al., 2010) (Fig. 1.A, lines 3, 5, 7) possess additional proteins, not present in the strains, which were susceptible to serum. This suggests, some OMPs are characteristic of sensitive strains (group 1), as well as associated with resistant strains (group 2):

- Group 1, consisting of eleven serovars **sensitive** to the bactericidal action of serum (see Table 2.). The OMPs characteristic of this group of bacteria were not detected in the outer membranes of the strains resistant to the bactericidal action of serum: 60-, 58-, 57-, 56-, 50-, 49-, 48-, 47-, 46-, 45-, 42-, 39-, 31-, 24-, 12-kDa;
- Group 2, consisting of nine serovars **resistant** to the bactericidal action of serum (see Table 2.) The OMPs characteristic of this group of bacteria were not detected in the outer membranes of the strains sensitive to the bactericidal action of serum: 194-, 183-, 99-, 93-, 84-, 82-, 79-, 74-, 69-, 66-, 65-, 62-, 54-, 53-, 51-, 41-, 30-, 29-, 27-, 25-, 17-, 8-kDa.



(A) (B)

Fig. 1. The SDS-PAGE OMPs isolated with Zwittergent 3-14®[1]

The results presented by Sarowska et al. (2010) show that the ESBL conjugative plasmid donor strain *K. pneumoniae*, and recipient strains (*S.* Enteritidis, *S.* Typhimurium and *S.* Hadar) were resistant to the bactericidal action of NHS, whereas the three *Salmonella* transconjugants identified as ESBL producers (*S.* Enteritidis ESBL$^+$, *S.* Typhimurium ESBL$^+$, and *S.* Hadar ESBL$^+$) demonstrated sensitivity to serum. SDS-PAGE analysis of the OMPs of *Salmonella* transconjugants revealed that the parental strains (Fig. 1.A, lines: 3-*S.* Enteritidis; 5-*S.* Typhimurium, 7-*S.* Hadar) and transconjugants (Fig. 1.A, lines 4, 6, 8) exhibited slightly

[1] Lane 1 (A, B): Molecular Weight Standards (Sigma); (A) 2: *K. pneumoniae*; 3: *S.* Enteritidis; 4: *S.* Enteritidis ESBL$^+$; 5: *S.* Typhimurium; 6: *S.* Typhimurium ESBL$^+$; Lane 7: *S.* Hadar; Lane 8: *S.* Hadar ESBL$^+$ (Sarowska et al., 2010); with permission of Editor-In-Chief of *Advances in Clinical and Experimental Medicine* (B) 2: *S.* sp. PCM 2548; 3: *S.* Balboa PCM 2552; 4: *S. arizonae* PCM 2543; 5: *S. arizonae* PCM 2544; 6: *S.* Ngozi PCM 2514; 7: *S.* Hammonia PCM 2535; 8: *S.* Hagenbeck PCM 2534 (data shown only in this paper).

different outer-membrane banding patterns. These changes included the presence or absence of particular OMPs. New OMPs (absent before conjugation) with molecular masses of 25-kDa and 44-kDa were observed in *S.* Enteritidis ESBL$^+$ isolate, and 43-kDa in *S.* Typhimurium ESBL$^+$ strain. The acquisition of ESBL plasmids also resulted in the loss of some OMPs in the transconjugants compared with the parental strains. In the case of the *S.* Enteritidis ESBL$^+$ isolate, no bands for the 15-, 22-, 53-, 58- and 78-kDa protens were noted. For the remaining transconjugants no bands were observed for the 16-, 38-, 44-, 55-, and 82-kDa proteins (*S.* Typhimurium ESBL$^+$) and 27-, 65- and 66-kDa (*S.* Hadar ESBL$^+$)..

In our study, all the *Salmonella* strains tested, regardless of their susceptibility to serum, possessed the 35-kDa peptide band (Fig. 1.). Note that Rck of *S.* Typhimurium and *S.* Enteritidis, binds fH, which is accociated with resistance to the alternative pathway of complement (Ho et al., 2010), allowing to analyse OMPs patterns on this basis. In the studies of *Salmonella* O48 strains, it was shown that alternative pathway was important in the bactericidal mechanisms of C activation, in spite of the fact that some strains had 17- or 19-kDa proteins, as can be seen in Fig. 1. It seems that any identified agent masks the inhibitory properties of 17-19-kDa proteins in *Salmonella* O48 against the AP pathway.

Bugla-Ploskonska et al. (2009a, 2011), Futoma-Koloch et al. (2009), and Sarowska et al. (2010) used a method of isolating OMPs from bacteria during exponential growth using the Zwittergent Z 3-14® detergent (Calbiochem-Behring), primarily used for enrichment of proteins from cell lysates. Sulfobetaine 3-14 has a strongly basic quaternary ammonium ion and acidic sulfonate with equivalent strength and its zwitterionic character is maintained over a wide range of pH. This method was adapted for *Salmonella* strains using methods developed for isolating of OMPs of *Branhamella catarrhalis* (presently named *Moraxella catarrhalis*) (Murphy and Bartos, 1989, Faden et al., 1992), and *Haemophilus influenzae* (Murphy and Bartos, 1988). There were two modifications introduced into Murphy and Bartos's original method. The first one was that the bacteria were cultured overnight at 37°C for 18 h within liquid Brain Heart Infusion medium (not on solidified medium, similar as in bactericidal assay (YP broth)), secondly, OMPs in a buffer Z, were kept overnight at 4°C, before they were centrifuged at 8700 rpm at 4°C for 10 min. It's important to emphasize that culturing bacteria in liquid or solidified medium may influence the molecular pattern of the surface antigens. Harvesting bacteria from plates provides large numbers of cells without the need for centrifugation. In this manner, the cells are relatively free from components of the growth medium, which could interfere with subsequent procedures. Zwitterionic detergents like non-ionic detergents do not possess a net charge; they lack conductivity and electrophoretic mobility, and do not bind to ion exchange resins. Like ionic detergents, they are also suited for breaking protein-protein interactions (Srirama, 2001).

Hundreds of articles have been published with the words "*Salmonella* OMP" or "*Salmonella* porin". Of note is the review by Nikaido (2003), who has been working in that field during 30 years of his scientific carrier. He exhaustively described the molecular basis of bacterial OM permeability, taking into concideration i.a. protein channels, and LPS. Since the OMPs are surface-exposed antigens, they provide attractive targets for the development of vaccines, thus various techniques have been developed for their characterization. OMPs isolated with Zwittergent Z 3-14® can be also separated with two-dimensional electrophoresis (2-DE). Preliminary studies by Futoma-Koloch et al. (2009), Bugla-Ploskonska et al. (2009a) have

reported that using the zwitterionic detergent Zwittergent Z 3-14® is suitable to isolate OMPs from *S. arizonae, S.* Dahlem, and other Gram-negative rods such as *E. coli* O56. Samples of these peptides may then be separated by 2-DE in a capillary tube system (Futoma-Koloch et al., 2009) (Fig. 2.). It was also shown that the use of the same detergent to isolate OMPs from *E. coli* O56 enables their separation by 2-DE using pH 3-10 immobilized pH gradient IPG strips (Bugla-Ploskonska et al., 2009a). Detection of hydrophobic OMPs in 2-D gels is associated with certain limitations (Fountoulakis, 2005). The poor solubility of hydrophobic OMP accounts for their absence from the 2D gel map, but the addition of zwitterionic detergents can improve protein solubilization (Shaw and Riederer, 2006).

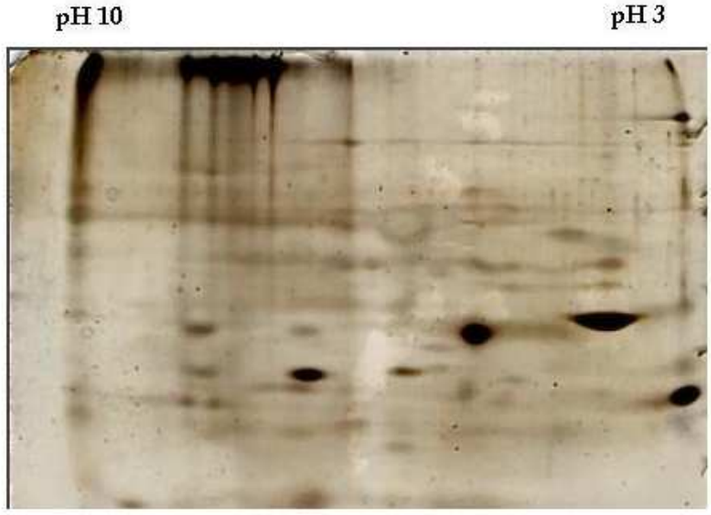


Fig. 2. 2-DE profile of OMPs isolated with Zwittergent Z 3-14®: *S. arizonae* PCM 2543 (Futoma-Koloch et al. 2009)[2]

2-DE was carried out with the Mini-PROTEAN® 3 System (Bio-Rad) and Protean IEF cell and Mini-Protean Tetra Cell (BioRad) respectively (Fig. 2.). Spots of OMPs of *S. arizonae* PCM 2543 were visualized by silver staining. Some modifications were introduced into to the original method described by O'Farrell in 1975 (O'Farrell, 1975) in order to improve the resolution of OMPs isolated with Zwittergent 3-14®. It was necessary to reduce the molar concentration of urea and replace Bio-Lyte 5/7 ampholyte with ddH_2O in three reagents: the first-dimension sample buffer, the first-dimension sample overlay buffer, and the first-dimension gel monomer solution. Tricine was used instead of glycine in the electrophoresis buffer (second dimension). The tube gels were pre-electrophoresed (electrode preparation) by running at 200 V for 10 min, 300 V for 15 min, and 400 V for 15 min (power supply adaptor - Apelex PS 900S TX) then appropriate prepared samples were loaded on the top surfaces of the gels in capillary tubes and separated by IEF on pH 3.0-10.0 at 300 V for 16 h, 400 V for 2 h, and 800 V for 1 h (Apelex PS 900S TX). Three tube gels per sample were run. After the IEF (isoelectring focusing) run was completed, gels were electrophoresed according to Laemmli (1970) for 2 h (20 mA/gel) and stained with a Silver Stain Plus™ kit (Bio-Rad). Fig. 2. contains a total of 35 spots. The major proteins were found in the acidic

[2] With permission of Editor-In-Chief of *Polish Journal of Microbiology*

regions of the gels. This procedure was described in details in paper autorship Futoma-Koloch et al. (2009).

The next issue for consideration concerns various methods developed for isolation, separation and identification of OMPs. The power of 2-DE in the OMPs analysis was demonstrated by Hamid and Jain (2008), who confirmed that OMPs provide promising targets for the development of a candidate vaccine against typhoid. 2-DE methodology in conjunction with the Western Blot has a potential for the rapid development of specific, safe, and highly efficacious vaccines against salmonellosis in human and livestock. Non-detergent sulphobetaines were also used by Blisnick and co-workers (1998) to enhance the recovery of membrane proteins and active proteases from erythrocytes infected by *Plasmodium falciparum*, a parasite. Protein extracts of parasites obtained with NDSB195 (non-detergent sulfobetaine) were separated by IEF, as well as by SDS-PAGE. Proteins were then identified with western blotting using specific antibodies. *S.* Typhimurium served as a model for isolating OMPs by the method of Foulaki et al. (1989). From the urea extract a 55-kDa protein was isolated by ion-exchange chromatography and gel filtration free of LPS. All steps in the isolation of this protein were carried out without detergents.

Huang et al. (2004) presented a newly developed method combining sucrose density centrifugation and aqueous two-phase partitioning for the isolation of pure OM from *Synechocystis* sp. The purity of the membrane fractions was verified by immunoblot analysis using Ab against specific membrane marker proteins. They have examined the protein composition by 2-DE followed by matrix-assisted laser desorption/ionization time–of-flight (MALDI-TOF) analysis. Using a combination of two nonionic detergents (CHAPS and ASB-14), proteins were solubilized and resolved within a range of pH 4.0-7.0. The studies of Ortiz et al. (1989) were undertaken to assess the ability of the OMPs of *S.* Typhi to induce a humoral immune response in human with typhoid fever. OMPs in this case were isolated with the nonionic detergent, Triton X-100. Proteins were contaminated with approximately 4% LPS. SDS-PAGE patterns showed protein bands with molecular size ranges from 17 to 70 kDa. The major group of proteins corresponded to the OmpA. Isibasi et al. (1988) isolated OMPs from *S.* Typhi also with Triton X-100, as described by Schnaitman (1971). Seleim and others (2002) performed preparation of the *Salmonella* OMPs with 2% sarkosyl in 10 mM HEPES buffer. Chooneea et al. (2010) attempted to characterize the surface proteome of *S.* Typhimurium using lipid-based protein immobilization technology in the form of LPI™ FlowCell. No detergents were required and no sample clean up was needed prior to downstream analysis. The proteins were then characterized by liquid chromatography – tandem mass spectrometry (LC-MS/MS). In these studies 54 OMPs were identified.

Proteomic analysis of the *E. coli* OM performed by Molloy and co-workers (2000) included isolation of cell membranes by carbonate extraction according to the method of Fujiki and co-workers (1982). Bolla et al. (2000) developed a method for purification of the major outer membrane protein (MOMP) of *Campylobacter*, involving outer-membrane preparation followed by specific detergent extraction and chromatography. In that study, to identify poorly expressed porin proteins, they analysed a large amount of outer membrane detergent extract by ion-exchange chromatography. This method allowed them to identify and characterize a novel porin protein Omp50 of *C. jejuni*. Extraction of the porin from the membrane was carried out in six steps. Two extractions with 0.1% sodium lauryl sarcosinate in Tris buffer to solubilize the inner membrane were followed by four successive extractions with

n-octyl- polyoxyethylene (octyl-POE; Bachem AG-Switzerland) in 20 mM NaPi (pH 7.6), leading to the specific recovery of the outer membrane porin associated with octyl-POE micelles. In turn, OMPs of *Brucella* spp. were extracted with Triton X-114 (Tibor et al., 1999), and confirmed to be lipoproteins.

The preparations of OMPs in the soluble fraction in buffer Z (in a method with Zwittergent Z-14®) were assayed for the enzymatic activity of succinic dehydrogenase, a marker for the cytoplasmic membranes, according to Rockwood et al. (1987). To make certain that the extracts of proteins were free of membrane fraction contaminations, Futoma-Koloch and others (data shown only in this paper) employed transmission electron microscopy (TEM) for imaging OMPs isolated from *S. arizonae* PCM 2544.

For electron microscopy investigation:

a. The isolated material was negatively contrasted with 2 % uranyl acetate in a conventional procedure (Fig. 3.A);
b. For thin-sections isolated material was fixed in 1% osmium tetroxide, dehydrated in graded ethanol and embedded in Epon 812. Ultrathinsections were cut with a Reychert Ultracut E ultramicrotome and stained with lead citrate (Fig. 3.B).

The specimens were examined in Tesla BS 540 electron microscope. The proteins appeared bright against the dark background. Scale markers correspond to 500 nm. Note, that membrane contarrdinants are not present. If they were, they would be visible as white linear trails of phospholipid bilayers. Proteins or peptides released from the membranes tend to aggregate to assume globular or filamentous clusters of the diameter from 40 nm to about 200 nm.

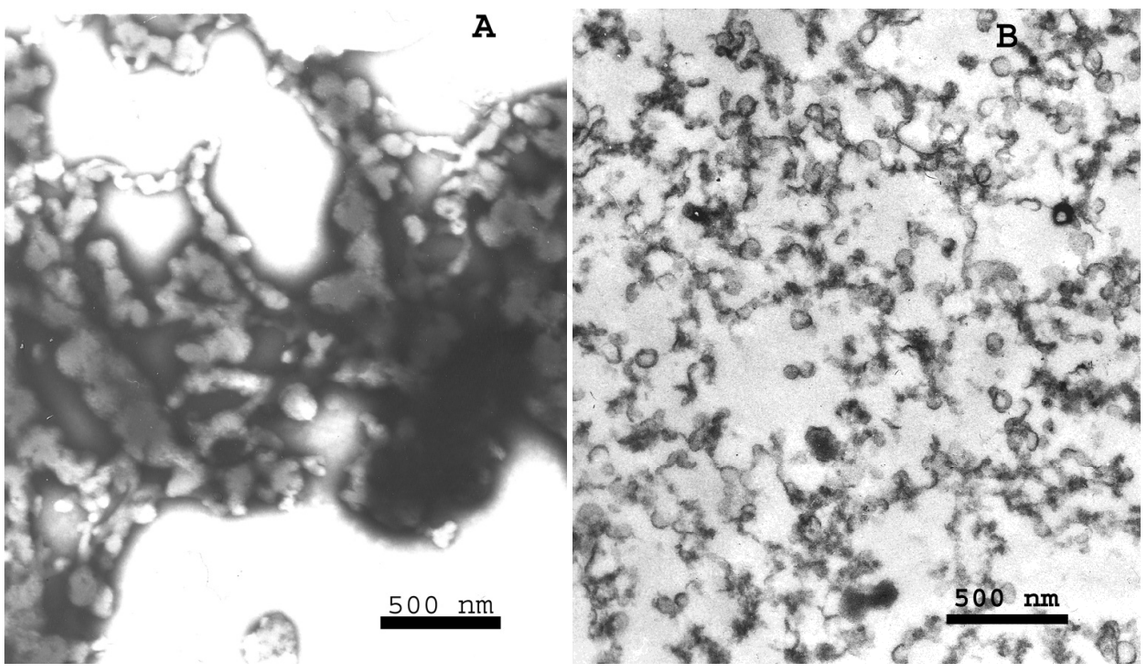


Fig. 3. Transmission electron micrographs of isolated OMPs released with Zwittergent 3-14® from *S. arizonae* PCM 2544, bar=500nm, 25 000x

4. *Salmonella*'s OMPs as potential vaccines

Another aspect of studying OMPs of Gram-negative bacteria is the potential for finding molecular candidates for vaccines. Vaccination is an effective tool for the prevention of *Salmonella* infections (Mastroeni et al., 2011). The currently available vaccines against salmonellosis can be divided into three major classes: whole-cell killed vaccines, subunit vaccines, and live attenuated vaccines. Subunit vaccines such as the ones based on the Vi polysaccharides are safe, immunogenic, and are currently licenced for human use. Other subunit vaccines such as those based on detoxified LPS, cell extracts, porins, O-polysaccharides, and O-conjugates have been tested in experimental models (Mastroeni and Menager, 2003). Recognition and neutralization of OMPs by the immune system is of great importance. When porins are used as immunogens they can ablate bacteremia and provide equivalent protection against salmonellosis. Available vaccines containing either acetone or heat-killed *S.* Typhi are of limited value, because they confer short-lived protection and they produce unacceptable side effects, due mainly to the presence of endotoxin, which prevents their use in children. At the moment there are also several vaccines prepared from Vi antigen.

The trimeric porins show stability to temperature and denaturants, and are also resistant to proteolysis. These properties make them good candidates for industrial applications towards the development of oral vaccines (Galdiero et al., 1990). Data delivered by Salazar-Gonzales et al. (2004) showed that *S.* Typhi porins-based candidate vaccine is safe and immunogenic and healthy. They observed that side effects after vaccination were mild and transient. Ortiz et al. (1989) established that sera from patients with typhiod fever contained M antibodies, which reacted with a protein of 28-kDa. Singh and others (1995) were interested in OmpC and OmpD porins from *S.* Typhimurium because of their potential role in diagnostic assays, in antibiotic resistance, and as immunogens for vaccination.

In animal models, it was shown that mouse monoclonal antibodies were raised against recombinant *S.* Typhi 36-kDa porin monomer (Kissel et al., 1994). Hamid and Jain (Hamid and Jain, 2008) investigated OMPs of *S.* Typhimurium as potential vaccine candidates for conferring protection against typhoid. They showed that OMP with an apparent molecular mass of 49-kDa were highly immunogenic, evoke humoral and cell-mediated immune responses and confer 100% protection to immunized rats. Seleim and co-workers (2002) compared patterns of OMPs bands in SDS-electrophoretic analysis, and found common protein bands in the range from 20-45 kDa in four serovars of *Salmonella* (Typhimurium, Dublin, Enteritidis, and Anatum) collected from calves' fecal samples. The OMPs of serovars Dublin and Enteritidis reacted similar in the Western Blot method with the antisera collected from infected calves. Two protein bands were characteristic, one at, 14.4- and the other at 24-kDa. The authors conclude that *Salmonella* OMPs can be employed as effective vaccine candidates. Vaccination could confer active immunity and reduce the expenses associated with treating *Salmonella*-infected calves. Studies of Maripandi and Al-Salamah (2010) were conducted on *S.* Enteritidis isolates from chicken meat samples for OMPs analysis. The immunoblotting results showed that 14.4- and 24-kDa proteins were good chicken immunogens. The authors conclude that these proteins can be used for vaccine preparation in the future. Outer membrane proteins 82- and 76-kDa are potentially involved in the attachment of *S.* Enteritidis to the intestinal mucosa. They could be used as vaccines to block or reduce *S.* Enteritidis colonization in chickens (Fadl et al., 2002). Ochoa-Reparaz et al.

(2004) state that the immunogenicity of *S.* Enteritidis porins in infected birds may serve as components of an effective subcellular vaccine for poultry salmonellosis.

VonSpecht and co-workers (1996) tested the ability of the recombinant OprI of *P. aeruginosa* to serve as a protective vaccine against this Gram-negative pathogen. Oral immunization of mice with recombinat OprI expressing *S.* Dublin, induced s-IgA antibodies in the gut mucosa against OprI. Recombinant *Lactobacillus casei* may also express SipC and OmpC antigens for vaccination against infections caused by *S.* Enteritidis (Kajikawa and Shizunobu, 2007).

Attenuated *Salmonella* species expressing heterologous antigens are promising candidates for the development of mucosal vaccines. This strategy is based on the ability of *Salmonella* bacteria to persist in the antigen-presenting cells (APC) during its migration to the lyphatic organs of the mucosal immune system. Oral live vaccines based on recombinant *Salmonella* strains were successfully developed to induce a specific immune response against human immunodeficiecy virus, *Helicobacter pylori, Clostridium difficile*, and human papilloma virus in mice and humans (Heinz et al., 2004).

The ability of OMPs to induce a protective immunity against infection caused by diverse Gram-negative bacteria, such as *H. influenzae* type b (Granoff and Cates, 1986), *N. meningitidis* group B (Wang, 1984), *P. aeruginosa* (Gilleland et al., 1984), *S.* Typhimurium (Kuusi et al., 1979) and *Borrelia bronchiseptica* (Montaraz et al., 1985), has been demonstrated previously. Witkowska and others (2006) showed that a 38-kDa OMP present in most *Enterobacteriaceae* species is a protein that generates immunological response in human organisms and is a good candidate for creating a vaccine against such species as *E. coli*, *Shigella flexneri, K. pneumoniae*, and *Proteus vulgaris.*

5. Conclusions

The phenomenon of serum resistance of bacteria has a multifactorial basis. Alteration of the Gram-negative bacterial envelope, including altered protein and LPS composition, may be considered as before general mechanism for survival within the host. Further studies are required to better understand all the molecular mechanisms of microbial surface remodeling and the process of recognizing of the outer membrane vesicles from the bacteria. The role of OMPs, LPSs, and LOSs in determining serum-sensitivity or serum-resistance in *Salmonella* is not entirely clear. It is important to determine which OMPs from *Salmonella* are immunogenic in animals as well as in humans, in order to improve subunit vaccines against salmonellosis.

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