Polish Journal of Microbiology
2006, Vol. 55, No 2, 153–156

Survival of *Proteus mirabilis* O3 (S1959), O9 and O18 Strains in Normal Human Serum (NHS) Correlates with the Diversity of their Outer Membrane Proteins (OMPs)

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Received 27 October 2005, revised 25 March 2006, accepted 28 March 2006

This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

Abstract

Urinary tract infections are frequently caused by *Proteus mirabilis* strains. In the previous studies there were defined the complete structures of O-polysaccharide parts of lipopolysaccharides from strains: *P. mirabilis* O3 (S1959), *P. mirabilis* O9 and *P. mirabilis* O18. In the present study it was investigated bactericidal effect of normal human serum (NHS) to *P. mirabilis* strains. We also focused on the diversity of outer membrane proteins (OMPs) being separated on a gel isolated from tested strains. Serial passage of *P. mirabilis* O18 in 90% normal bovine serum (NBS) contributed to over-expressing some classes of OMPs.

Key words: *Proteus mirabilis,* serum, the complement system, outer membrane proteins (OMPs)

Proteus mirabilis strains are a common cause of urinary tract infections and also have been described as opportunistic etiological agents in infections of the respiratory tract and of wounds, burns, skin, eyes, nose and throat diseases (Różalski *et al*., 1997). Pathogenic bacteria have worked out many different ways to overcome the host defense system for example proteolytic degradation of secretory immunoglobulin or steric shielding or modification of exposed pathogen-associated molecular patterns (PAMPs) (Hornef *et al*., 2002). One of the mechanisms which protect the macroorganism from infections is the bactericidal activity of serum. The complement system is a complex of serum proteins which interact in a cascade. It recognizes and promotes clearance, by phagocytosis, of invading microorganisms and also causes lysis of Gram-negative bacteria itself. Activation of the complement system is achieved through three major pathways: the classical pathway (CP), which is activated by certain isotypes of antibodies bound to antigens (immune complexes); the alternative pathway (AP), which is activated on microbial cell surfaces in the absence of antibodies and the lectin pathway (LP), which is activated by a mannose binding lectin – MBL, that binds to mannose residues on microbes (Sim and Tsiftsoglou, 2004). The mechanism of the bacterial resistance to the bactericidal effect of the serum is still not fully understood, but it is known that some outer membrane proteins (OMPs) and lipopolysaccharides (LPSs) are the factors determining the resistance (Pilz *et al*., 1992; Prasadarao *et al*., 2002; White *et al*., 2005) or the sensitivity (Alberti *et al*., 1993; Merino *et al*., 1998; Weber *et al*., 1992) of bacteria to the bactericidal action of the serum.

Lipopolysaccharide (LPS, bacterial endotoxin), the major component of the outer membrane is one of the virulence factors of *Proteus*, preventing the *Proteus* rods against bactericidal effects of the complement proteins deposition. There are different mechanisms for LPS mediated complement activation (Vukajlovich *et al*., 1992). The antibody-independent classical pathway (CP) is mediated only by the lipid A portion of the molecule (Vukajlovich *et al*., 1987). Activation of the alternative pathway (AP) requires the polysaccharide moieties of LPS, core oligosaccharide and/or O-antigen polysaccharides (Vukajlovich *et al.*, 1992; Vukajlovich *et al*., 1987). Previous studies tested complement activation by LPSs isolated from *P. mirabilis* O10, O23, O30, and O43 strains, which differ in the number of negative COO-groups on their polysaccharide

components. Four *P. mirabilis* strains studied were resistant to complement-mediated killing, despite of the complement binding by their LPSs (Kaca *et al*., 2000). In the other studies of two smooth *P. mirabilis* strains O9 and O49 bacterial resistance to the bactericidal effect of the serum was found to be depended on the chemical structure and polysaccharide lenght of O9 and O49 LPSs (Fudała and Kaca, 2004). The complete structures of O-polysaccharide parts of lipopolysaccharides of studied strains were established. *P. mirabilis* O3 (S1959), O9 and O18 LPSs have the unique structures with lysine, furanosic-form of ribose and phosphocholine residues, respectively (Fudała *et al*., 2003; Kondakova *et al*., 2003; Ziółkowski *et al*., 1997). In the above mentioned studies the role of the outer membrane proteins in the bacterial complement mediated-resistance has not been investigated yet.

Below, we focused on electrophoretic patterns of OMPs isolated from *P. mirabilis* O3 (S1959), O9 and O18 strains. A correlation between the presence of some OMPs and the susceptibility of bacteria to the bactericidal activity of serum was observed.

Three strains: *P. mirabilis* O9, *P. mirabilis* O18 and *P. mirabilis* O3 (S1959) obtained from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague, Chech Republic) and Institute of Microbiology and Immunology, University of Łódź, Poland (strain *P. mirabilis* O3 (S1959)) were used. Normal human serum (NHS) was obtained from three healthy volunteers which had not been previously treated with any antimicrobial drug. The serum samples were collected and kept frozen (–70°C) for a period no longer than 4 months. A suitable volume of serum was thawed immediately before use. Each portion was used only once. Normal bovine serum (NBS) was obtained from five healthy animals, prepared and stored in the same way like NHS. To define bacterial sensitivity to the bactericidal serum activity the modified Doroszkiewicz method (Doroszkiewicz, 1997) was used. Briefly, the strains were grown overnight, and then bacterial cells of early exponential growth phase were transferred to a fresh nutrient broth and incubated at 37°C for 1 hour. After incubation the bacterial cells were centrifuged (4000 × g, 20 min, 4°C). The bacteria were then added to 50% and 75% NHS or 90% NBS (serum was diluted with 0.1 M NaCl) and incubated in a water bath at 37°C. After 0, 60 and 180 min samples were collected, diluted and cultured on nutrient agar plates for 18 h at 37°C. The number of colony forming units (CFU) at the time zero was taken as 100%. Strains with a survival ratio not less than 50% after 180 min of incubation in NHS or NBS were regarded as resistant. The control samples of NBS and NHS were decomplemented by heating at 56°C for 30 min (Doroszkiewicz *et al*., 1995; Eidinger *et al*., 1977; Mielnik *et al*., 2001). The level of C3 and C4 components in NHS was determined using monospecific polyclonal antibodies anti-C3 and anti-C4 proteins (Roitt and Male, 2000). Outer membrane proteins (OMPs) were isolated (Murphy and Bartos, 1989) from the bacteria grown in 100 ml of Brain Heart Infusion (BHI) broth (Difco) at 37°C for 18 h. *P. mirabilis* O18 strain after serial passage in 90% NBS was also grown in BHI broth at 37°C for 18 h before the isolation procedure. After incubation the bacterial cells were centrifuged (4000 × g, 15 min, 4°C) and the pellet was suspended in 2.5 ml of buffer β (1 M sodium acetate, 0.001 M β-mercaptoethanol, pH 4.0). Then 22.5 ml of water solution containing 5% (w/v) Zwittergent 3–14 (Calbiochem-Behring) and 0.5 M $CaCl_2$ was added. That mixture was stirred at room temperature for 1 h. To precipitate nucleic acids a 6.25 ml volume of 96% (v/v) cold ethanol was added very slowly. The mixture was then centrifuged at 17000 × g at 4°C for 10 min. The remaining proteins were precipitated by addition of 96% (v/v) cold ethanol and were centrifuged at 17000 × g for 20 min at 4°C. The pellet was then suspended in buffer Z (0.05% (w/v) Zwittergent, 0.05 M Tris and 0.01 M EDTA, pH 8.0) and stirred at room temperature for 1 h. The solution was kept at 4°C overnight and centrifuged at 12000 × g for 10 min at 4°C. OMPs were present in the buffer Z soluble fraction after centrifugation. To determine a concentration of outer membrane proteins in the samples the Bradford protein assay (Bradford, 1976) was used. Discontinuous sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) was carried out on slabs with 12.5% acrylamide according to Laemmli (Laemmli, 1970). The Wide Molecular Weight Range Sigma Marker protein standard was used for molecular mass calibration. OMPs prepared in the sample buffer were loaded in each of the wells in the same volume of 10 µl. The samples consisted of almost the same amounts of protein in the range of 0.9–1.2 mg/ml. After electrophoresis the gels were kept for 1h in a solution containing 25% (v/v) methanol, 10% (v/v) acetic acid and 0.05% (w/v) Coomassie Brilliant Blue and then destained in 10% (v/v) acetic acid for 3–5 h. For the molecular analysis of OMPs the BIO-CAPT v. 99 software as well as the BIO-1D++ v.99 (Vilber Lourmat, France) were used.

The data concerning the determination of the level of C3 and C4 in NHS showed that the level of C3 was 78 mg/dl (standard for a normal human serum is 55–120 mg/dl) and C4 was 37 mg/dl (standard for a normal human serum is 20–50 mg/dl).

To determine the killing effect of the serum on bacteria, we studied the survival of bacteria after three hours of incubation in serum. The results concerning the sensitivity of three *P. mirabilis* strains to NHS after

Table I
Bactericidal activity of NHS against *P. mirabilis* O3 (S1959), *P. mirabilis* O9 and *P. mirabilis* O18

Strain	CFU/ml[1] Serum solution % (v/v)					
	50%		75%		50%[2]	
	Time of incubation (min)					
	T_0	T_{180}	T_0	T_{180}	T_0	T_{180}
P. mirabilis O3 (S1959)	3.7×10^6	1.2×10^8	2.1×10^6	1.1×10^7	4.4×10^6	5.3×10^7
P. mirabilis O9	3.3×10^6	3.6×10^3	2.3×10^6	3.4×10^2	3.6×10^6	3.4×10^7
P. mirabilis O18 (not passaged in 90% NBS)	1.8×10^5	7.2×10^3	6.1×10^6	4.9×10^5	4.3×10^5	2.4×10^7
P. mirabilis O18 (23 times passaged in 90% NBS)	3.0×10^6	2.5×10^5	3.5×10^6	7.3×10^4	4.1×10^6	4.0×10^7

[1] CFU/ml – colony forming units, [2] 56°C/30 min – serum decomplement by the heating

180 min of incubation are given in Table I. The results presented in the Table I indicate that the strains *P. mirabilis* O9 and *P. mirabilis* O18 were more sensitive to the bactericidal action of NHS than *P. mirabilis* O3 (S1959) strain. The latter one was resistant to the lytic action of the serum and the number of cells increased in 50% and 75% human serum dilution. When NHS decomplemented by heating at 56°C for 30 min was used in the experiment, the bacteria proliferated very intensively. It confirmed that the complement was responsible for the killing action of NHS. The serial passage (23 times in 90% NBS) of *P. mirabilis* O18 strain increased the level of the resistance of bacteria to the bactericidal action of 50% NHS (Table I).

Also some differences appeared in SDS-PAGE patterns of the outer membrane proteins. *P. mirabilis* O18 treated by the sequencial passage in 90% NBS (data not shown) over-expressed some of the outer membrane proteins compare to *P. mirabilis* O18 before the passage (Fig. 1). It was observed, that OMPs with the molecular masses: 36 kDa, 35 kDa, 34 kDa, 33 kDa, 22 kDa, 21 kDa, 19 kDa, 18 kDa were present in all tested strains of *P. mirabilis*. Particular analysis of bands on 12.5% polyacrylamide gel have shown, that *P. mirabilis* O18 after passage in 90% NBS produced some excess of proteins which were unexpectedly related in their quantity to these isolated from the resistant strain *P. mirabilis* O3 (S1959) and the main were: 22kDa, 19 kDa, 18 kDa, 17 kDa. As a result of the passage of *P. mirabilis* O18 in 90% NBS there appeared OMPs with the molecular masses 110 kDa, 67 kDa, 62 kDa and 34 kDa which were not produced by *P. mirabilis* O18 before the passage in 90% NBS. Densitometric analysis of OMPs (data not shown) was helpful to the preliminary defining of the dependence of the survival of bacteria in NHS and the presence and ratio of some outer membrane proteins. The following investigations will comprise protein elution and determining the proportional contents of particular OMPs in the samples.

In the previously published papers the participation of OMPs in the bacterial serum resistance phenomena (Cisowska *et al*., 2005; Futoma *et al*., 2004; Murphy *et al*., 2000; Pilz *et al*., 1992; Prasadarao *et al*., 2002) was discussed. It has been suggested that outer membrane protein A (OmpA) of *Escherichia coli* contributes to serum resistance by binding to C4b binding protein (C4bp), a complement fluid phase regulator (Prasadarao *et al*., 2002). In other bactericidal assays, the mutants of *Moraxella catarrhalis* were more readily killed by normal human serum compared to the isogenic parent strains which possessed outer membrane protein E (OmpE). Those results indicated that OmpE was involved in the expression of serum resistance (Murphy *et al*., 2000). Some OMPs can inhibit complement activation on the C3, C9 and C5b-9 level (Pilz *et al*., 1992). It has been shown that porin (OmpK36) from *Klebsiella pneumoniae* activated the classical pathway of the complement system by forming complexes with C1q, a component of the complement. Together with an activated complement in the alternative pathway it came to effective elimination of serum-sensitive *K. pneumoniae* cells (Alberti *et al*., 1993).

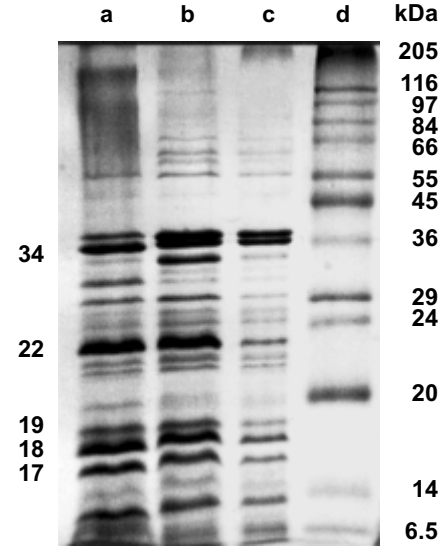

Fig.1. The SDS-PAGE patterns of outer membrane proteins (OMPs).

Lane a: outer membrane proteins isolated from *P. mirabilis* O3 (S1959); Lane b: *P. mirabilis* O18 (23 times of serial passage in 90% NBS); Lane c: *P. mirabilis* O18 (not passaged in 90% NBS); Lane d: Molecular Size Marker M4038 (Sigma).

The presented data indicate that the serum resistance of *P. mirabilis* rods may be mediated by outer membrane proteins patterns in a concert with O-polysaccharides LPSs structures.

Acknowledgments. This research was partially supported by grant 2 P04C 028 28 from the State Committee for Scientific Research (KBN), Poland.

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