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Proteus sp. – an opportunistic bacterial pathogen – classification, swarming growth, clinical significance and virulence factors

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ABSTRACT

The genus *Proteus* belongs to the *Enterobacteriaceae* family, where it is placed in the tribe *Proteeae,* together with the genera *Morganella* and *Providencia*. Currently, the genus *Proteus* consists of five species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, as well as three unnamed *Proteus* genomospecies. The most defining characteristic of *Proteus* bacteria is a swarming phenomenon, a multicellular differentiation process of short rods to elongated swarmer cells. It allows population of bacteria to migrate on solid surface. *Proteus* bacteria inhabit the environment and are also present in the intestines of humans and animals. These microorganisms under favorable conditions cause a number of infections including urinary tract infections (UTIs), wound infections, meningitis in neonates or infants and rheumatoid arthritis. Therefore, *Proteus* is known as a bacterial opportunistic pathogen. It causes complicated UTIs with a higher frequency, compared to other uropathogens. *Proteus* infections are accompanied by a formation of urinary stones, containing struvite and carbonate apatite. The virulence of *Proteus* rods has been related to several factors including fimbriae, flagella, enzymes (urease - hydrolyzing urea to CO_2 and NH_3, proteases degrading antibodies, tissue matrix proteins and proteins of the complement system), iron acqusition systems and toxins: hemolysins, *Proteus* toxin agglutinin (Pta), as well as an endotoxin - lipopolysaccharide (LPS). *Proteus* rods form biofilm, particularly on the surface of urinary catheters, which can lead to serious consequences for patients. In this review we present factors involved in the regulation of swarming phenomenon, discuss the role of particular pathogenic features of *Proteus* spp., and characterize biofilm formation by these bacteria.

KEY WORDS: *Proteus*, pathogenicity, mechanisms of virulence

1. Classification and environmental distribution of *Proteus* rods

The genus *Proteus*, which was described for the first time by Hauser in 1885, belongs to the *Enterobacteriaceae* family. In this family it is placed in the tribe *Proteeae,* together with the genera *Morganella* and *Providencia*. *Proteus* rods are distinguishable from most other genera by their ability to swarm across agar surfaces of solid media. The differentiation of *Proteus* rods according to the results of biochemical tests is shown in

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the table 1. The genus *Proteus* currently consists of five species: *P. mirabilis*, *P. vulgaris*, *P. penneri*, *P. hauseri* and *P. myxofaciens*, as well as three unnamed *Proteus* genomospecies. *Proteus myxofaciens* is the only *Proteus* species without any significance in the pathogenicity of humans, it has been isolated from living and dead larvae of the gypsy moth *Porteria dispar* (O'HARA *et al.* 2000, JANDA & ABBOT 2006). Recently described results of the analysis of partial sequences of the *rpoB* gene encoding the β-subunit of RNA polymerase, led to the conclusion that a genus-level genetic distance exists between *P. myxofaciens* and the species of the *Proteus-Providencia* group. Classification of *P. myxofaciens* in a new genus *Cosenzaea* gen. nov. has therefore been proposed (GIAMMANCO *et al.* 2011).

TABLE 1. Biochemical tests used in differentiation within the genus *Proteus* (RÓŻALSKI & STĄCZEK 2010, 2011). + 90–100 % positive, - 0 – 9.9 % positive; S – susceptible, R – resistant, V – variable.

Test	*P. mirabilis*	*P. vulgaris*	*P. penneri*	*P. hauseri*	*P. myxofaciens*
Salicin fermentation	-	+	-	-	-
Maltose fermentation	-	+	+	+	+
D-Xylose fermentation	+	+	+	+	-
Esculin hydrolysis	-	+	-	-	-
Ornithine decarboxylase	+	-	-	-	-
Indole production	-	+	-	+	-
Chloramphenicol susceptibility	*S*	*V*	*R*	*S*	*S*

Proteus microorganisms are widely distributed in the natural environment, including polluted water, soil, and manure. Due to their proteolytic activity, the ability to hydrolyze urea to ammonia and carbon dioxide, as well as the oxidative deamination of amino acids, these bacteria are involved in the decomposing of the organic matter of the animal origin. They are also present in the intestines of humans and animals. *P. mirabilis* was most frequently isolated from dogs, cows and birds, whereas *P. vulgaris* was most frequently isolated from pigs and cold-blooded vertebrates (JANDA & ABBOT 2006).

2. Swarming phenomenon

Swarming is a multicellular differentiation phenomenon that allows a population of bacteria to migrate on a solid surface in a coordinate manner. It is important in movement of *Proteus* rods to new locations and most probably helps them in the colonization macroorganisms. It involves cell-to-cell signaling and multicellular interactions and is connected with the possibility of morphological differentiation of bacteria depending on growth media. *Proteus* are dimorphic bacteria, which in liquid media are motile, peritrichously flagellated short rods (1.0 to 2.0 μm in length with 6–10 flagella). These bacteria are called swimmer cells. However, when transferred onto solid media these short rods change into elongated (20–80 μm in length), hyperflagellated, multinucleated, nonseptated swarmer cells. The latter migrate out from the inoculation site as long as the population of swarmer cells is reduced on solid surfaces. Then, the

consolidation process takes place. In this period of swarming growth, the long rods disintegrate to short bacteria. The processes of differentiation and dedifferentiation of *Proteus* bacteria are cyclic. It results in the formation of characteristic rings of bacterial growth on the agar plate (fig. 1) (VERSTRAETEN *et al*. 2008, MORGENSTEIN *et al*. 2010, RÓŻALSKI & STĄCZEK 2010).

Differentiation of swimmer cells into swarmer cells is induced by the contact of bacteria with a solid surface and the inhibition of flagellar rotation. It was found that the addition of thickening agents to liquid media resulted in differentiation of short rods to swarmer cells. A similar effect was obtained when antiflagellar antibodies were added to liquid medium (BELAS & SUVANASUTHI 2005). Mutations in gene *flaA* encoding flagellin, as well in genes involved in flagellum assembly led to the inhibition of *Proteus* short rods differentiation (BELAS 1994). Lipopolysaccharide (LPS), a surface component of these bacteria, seems to be important in the swarming phenomenon, however, its exact role has not been described. Mutation in the *wzz* region encoding O-antigen chain, as well as in genes *waaD* and *waaC* required for the core region of LPS are impaired in swarming growth (MORGENSTEIN *et al.* 2010). Swarmer cells translocation is facilitated by an extracellular acidic polysaccharide designated as Cmf (colony migration factor), which acts as a lubricant, reducing surface friction (GYGI *et al*. 1995). This capsular polysaccharide is structurally identical with the O-specific part of *Proteus* LPS serogroup O6 (KNIREL *et al*. 2011). Most likely, *Proteus* O-specific polysaccharides are important for the swarming. It was found that the Re mutant of *P. mirabilis* R45 producing LPS having only the lipid A and Kdo region was unable to swarm. The Ra mutant of *P. mirabilis* R110 containing lipid A and a complete core region expressed only a limited ability for migration on the solid medium, whereas most of S-forms of *P. mirabilis*, *P. vulgaris* and *P. penneri* could swarm vigorously (BABICKA 2001, KWIL 2003).

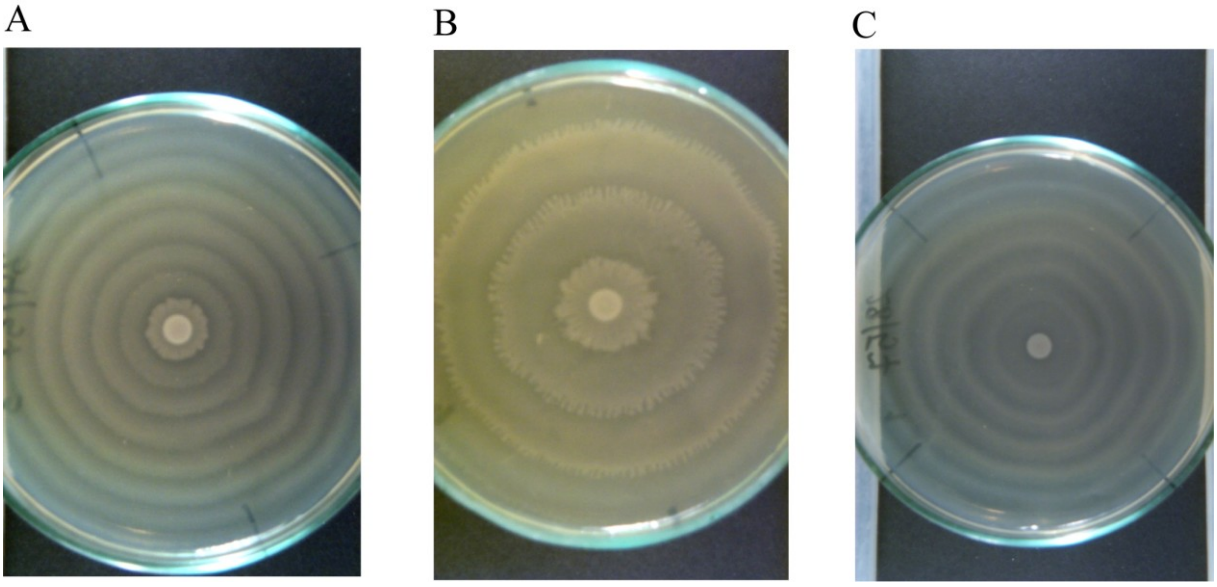

Figure 1. Swarming growth of three strains *P. mirabilis* strains 34/57 (A), 14/57 (B) and 26/57 (C) on a agar plate (KWIL data not published).

Swarming growth is characteristic for population of bacteria, thus it is dependent on multicellular interactions and cell to cell signaling in the phenomenon described as quorum sensing. It was found that extracellular signal AI-2 (autoinducer-2) does not play a role in swarming. Cyclic dipeptides were also shown as *P. mirabilis* signaling molecules out of importance in swarming (RATHER 2005). Other signal molecules

N-acyl homoserine lactones crucial in quorum sensing are not produced by *P. mirabilis* (MORGENSTEIN *et al*. 2010). Nevertheless, it was shown that *N*-acyl homoserine lactones added to the media are able to accelerate migration of some *P. mirabilis* strains (STAŃKOWSKA *et al*. 2008). In earlier studies, glutamine was found to be able to initiate swarming on minimal media (ALLISON *et al*. 1993). Swarming growth is also influenced by fatty acids. Oleic acid stimulates, whereas lauric and myristic acids inhibit this phenomenon, respectively (LIAW *et al*. 2004). The mechanism by which glutamine and fatty acids play a role in the swarming process as signals for cell to cell communication is currently unknown. In swarmer cell differentiation putrescine was described as a biochemical signal, however, the mechanism by which this amine regulates swarming is not clear. Most probably putrescine can form a complex with galacturonic acid which is component of CPS. This suggests a possibility that putrescine complexed with cell surface polysaccharide acts as a signal factor or putrescine regulates expression of CPS (RATHER 2005, MORGENSTEIN *et al*. 2010).

A number of factors playing a role in swarming regulations have been recognized (DANIELS *et al*. 2004, RATHER 2005, VERSTRAETEN *et al*. 2008, MORGENSTEIN *et al*. 2010). The most important gene involved in upregulation of flagellin production is *flhDC*, class 1 gene of flagellar regulon. This gene encodes FlhD2C2 complex, a heterotetrameric transcriptional regulator, which also regulates the expression of additional genes required for swarmer cells differentiation. The expression of *flhDC* increases 10-fold during initiation of swarmer cells differentiation and it is influenced by a variety of environmental factors and regulating genes. FlhD2C2 activates class 2 genes coding basal body and hook proteins of flagella, as well as σ28 factor, which activates class 3 genes required for synthesis of flagellin and flagellum assembly. FlhD2C2 activity is regulated by two identified factors DisA (decarboxylase inhibitor of swarming) and Lon protease (ATP-dependent protease). DisA most probably inhibits the assembly of heterotetramer or its binding to DNA (MORGENSTEIN *et al*. 2010).

Proteus has 16 predicted two-component systems – TCS (PEARSON *et al*. 2008). TCS consists of sensor kinase, which activates response regulator DNA – a binding protein controlling gene expression. Two of TCS - Rcs and Rpp are involved in swarming regulation (MORGENSTEIN *et al*. 2010). Rcs system contains RcsC sensor kinase, RcsB response regulator, RcsD intermediate transferring phosphate to the RcsB and RcsF which is an outer membrane lipoprotein. Mutation in the genes coding Rcs system in *P. mirabilis* lead to the hyperswarming phenotype, most probably due to an increased expression of the *flhDC*. *P. mirabilis* Rcs mutant grows in liquid media as elongated cells. The Rpp system consists of RppA response regulator and RppB histidine sensor kinase like protein. Mutation in the Rpp system in *P. mirabilis* also results in a hyperswarming phenotype.

flhDC expression is also regulated by several proteins including Umo, MrpJ, WosA, Lrp and RsmA. Umo A-D proteins (umo – upregulated expression of the *flhDC* master operon) are located in the cell membrane and periplasm. Mutant defective in production of UmoD protein is not able to swarm, however, such effect was not noticed in the case of UmoA and UmoC (DUFOUR *et al*. 1998).

mrpJ gene is located in the *mrp* operon encoding MR/P fimbriae, which are required for sessile lifestyle of bacteria (NIELUBOWICZ & MOBLEY 2010). It is opposite to the swimming or swarming phenotype of bacteria. The existence of bacteria in one of two forms is regulated by *mrpJ* gene product – MrpJ protein, a transcriptional regulator. It binds the *flhDC* promoter, which causes its repression and results in loss of motility of bacteria (PEARSON & MOBLEY 2008).

wosA gene coding WosA protein, is overexpressed in strains exhibiting hyperswarming phenotype (wos – wild type onset superswarming). WosA overexpression increases the expression of *fhlDC* and leads to a differentiation of swarmer cells in liquid

media. The hyperswarming *wosA* bacteria move quickly and spend less time in the consolidation phase. *wosA* expression is growth phase dependent, as well as it is partially dependent upon the expression of *flaA* gene encoding the flagellar filament (HATT & RATHER 2008).

Leucine responsive protein (Lrp) is a transcriptional global regulator involved in regulating different processes including amino acids synthesis, peptide transport, and pilin biogenesis (RATHER 2005, MORGENSTEIN *et al*. 2010). In *P. mirabilis* strains Lrp plays a role in the regulation of the swarming phenomenon, most probably its action leads to the inhibition of hyperflagellation. Mutation in *lrp* results in a decrease in the *flhDC*, *flaA* and *hpm* (hemolysin) expression and, in consequence, a nonswarming phenotype (HAY *et al*. 1997). Lrp is well known to respond to leucine and to a lesser extent to alanine. Recently, it was found that it also responds to methionine and is sensitive to isoleucine, histidine and thyrosine (HART & BLUMENTHAL 2010). In additional studies HART *et al*. (2011) showed that recognition of DNA by the helix-turn-helix motif of Lrp is modulated by the N-terminal tail.

RsmA – repressor of secondary metabolites is a member of regulatory systems involved in the expression control of genes which code factors commited to stationary growth processes. This protein stabilizes mRNA. In *P. mirabilis* RsmA is 6.8 kDa protein containing 62 amino acids. Increasing its expression results in the inhibition of swarmer cells differentiation and production of hemolysin, protease, urease and flagellin (LIAW *et al*. 2003). Overexpressing of *rsmA* led to repression of *flhDC* (MORGENSTEIN *et al*. 2010).

The role of the swarming phenomenon in the pathogenicity of *Proteus* bacteria (see below) is till now unclear. It was shown that swarmer cells demonstrate higher production of urease, HpmA hemolysin and IgA metaloprotease ZapA, as well as flagellin synthesis, as compared to swimmer cells (RATHER 2005, JACOBSEN *et al*. 2008). There is some controversy concerning invasiveness of swarmer cells. ALLISON *et al*. 1992 showed *in vitro* that this morphotype could invade uroepithelial cells, whereas nonflagellated and nonswarming forms were noninvasive. Other authors (RÓŻALSKI *et al*. 1986, CHIPPENDALE *et al*. 1994) showed the short rods as invasive forms, rather than swarmer cells. These results were confirmed by JANSEN *et al*. (2003) in a mouse model of ascending UTI. These authors have found that the predominant cell type in the urinary tract are short swimmer cells but not elongated swarmer cells. Recently, FUJIHARA *et al*. (2011) suggested that *P. mirabilis* cells differentiate into hyperflagellated and multinucleated swarmer cells in acidic pH of the host's urine and differentiate back into swimmer forms, when the urinary pH is increased and it is alkaline after urease action (see below). In acidic condition swarmer cells exhibit higher cytotoxicity against T24 line. In alkaline condition *P. mirabilis* showed few elongated cells with high number of flagella and cytotoxic activity.

3. Pathogenicity

Proteus rods are opportunistic bacterial pathogens which under favorable conditions cause urinary tract infections (UTIs), wound infections, meningitis in neonates or infants and rheumatoid arthritis (O'HARA *et al*. 2000, JANDA & ABBOT 2006). KALRA *et al*. (2011) reviewed endocarditis due to *Proteus* species and OKIMOTO *et al*. (2010) reported *P. mirabilis* pneumonia. Brain abscesses during *P. vulgaris* bacteremia were described by BLOCH *et al*. (2010). However, it should be stressed that *Proteus* bacteria cause UTIs with higher frequency. This type of infections is classified as uncomplicated or complicated. Uncomplicated infections occur in patients who are otherwise considered healthy, whereas complicated infections usually take place in patients with an urinary catheter in place or with structural and/or functional abnormalities in the urinary tract, suffering

from another illness, immunocompromised, as well as after surgical intervention in the urogenital system. It was found that *Escherichia coli* is a common cause of uncomplicated infections. Complicated UTIs might be polymicrobial and are usually caused by Gram-negative bacteria *Proteus* spp., *Providencia stuartii*, *Morganella morganii*, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* as well as some Gram-positive bacteria (NIELUBOWICZ & MOBLEY 2010). *Proteus* rods can cause hematogenous infections and ascending infections, however, the latter are more common for these microorganisms. The ascending infections comprise colonization subsequently urethra, bladder, ureters and kidneys. *P. mirabilis* causes UTIs with the highest frequency among all *Proteus* species. These bacteria are a common cause of complicated UTIs with the frequency of 12 % and catheter associated bacteriuria (WARREN 1996, JACOBSEN *et al*. 2008). They also cause uncomplicated lower urinary tract infections, however less frequently. CHLABICZ *et al*. (2011) have found in Poland uncomplicated *P. mirabilis* infections with the frequency of 3.4 %.

The virulence of *Proteus* rods has been related to several factors including fimbriae; flagella; enzymes: urease, hydrolyzing urea to CO_2 and NH_3; proteases degrading antibodies, tissue matrix proteins and proteins of complement system; iron acqusition systems and toxins: hemolysins, *Proteus* toxin agglutinin (Pta), as well as endotoxin - lipopolysaccharide (LPS) (table 2) (COKER *et al*. 2000, MOBLEY 1996, RÓŻALSKI *et al*. 1997, RÓŻALSKI 2002, NIELUBOWICZ & MOBLEY 2010). BEVERIDGE (1999) postulated the possible role of outer membrane vesicles (OMVs) released from the *P. mirabilis* surface, as an alternative route of delivery of some enzymes, as well as some virulence factors.

PEARSON *et al*. (2011) using microarrays analyzed *P. mirabilis* gene expression *in vivo* from experimentally infected mice and showed 471 gene upregulated and 82 downregulated in comparison to the *in vitro* broth culture. Upregulated genes encoded among others mannose-resistant *Proteus*-like fimbriae, urease, and iron uptake systems. Genes encoding flagella were downregulated. Earlier studies (FLANNERY *et al*. 200) showed the identification of 94-kb PAI (pathogenicity island) designated ICE*Pm1* (integrative and conjugative elements *P. mirabilis* 1). This PAI is common to *P. mirabilis*, *P. stuartii* and *M. morganii* and carries virulence factors including *Proteus* toxic agglutinin (see below) and the high pathogenicity island of *Yersinia* spp.

The *P. mirabilis* genome predicts 17 distinct fimbrial operons and 13 additional orphan fimbrial genes not associated with complete operons (PEARSON *et al*. 2008, NIELUBOWICZ & MOBLEY 2010). The biogenesis and/or the biological role were studied for six types of fimbriae and agglutinins: MR/P (mannose resistant *Proteus* like fimbriae), MR/K (mannose resistant *Klebsiella* hemagglutinins), PMF (*Proteus mirabilis* fimbriae), NAF (nonagglutinating fimbriae), ATF (ambient temperature fimbriae) and *P. mirabilis* P-like fimbriae (fimbriae, which are similar to *E. coli* P pili) (COKER *et al*. 2000, ROCHA *et al*. 2007b, JACOBSEN *et al*. 2008).

The most important *P. mirabilis* fimbriae seem to be MR/P. This type of fimbriae is encoded by *mrp* operon containing 10 genes located on bacterial chromosome. The main structural subunit of these fimbriae is the MrpA protein. The MrpC protein is an outer membrane platform, MrpD plays a role of a chaperon protein and MrpH is an adhesin located at the fimbrial tip (BAHRANI *et al*. 1991, LI *et al*. 1999, COKER *et al*. 2000). MR/P/ fimbriae undergo phase variation. It was found that the MrpI protein acts as *P. mirabilis* recombinase controlling "on/off" orientation of the *mrp* promoter. The fimbrial genes are transcribed and MR/P fimbriae are synthesized when the promoter is in "on" orientation (LI *et al*. 2002a, NIELUBOWICZ & MOBLEY 2010). In the "off" orientation the production of fimbriae is stopped. Expression of MR/P fimbriae is increased under oxygen limitation (LANE *et al*. 2009). These fimbriae contribute to biofilm formation and facilitate

colonization of upper urinary tract and were found more often on bacterial strains which cause pyelonephritis (JANSEN *et al*. 2004). MR/P fimbriae are involved in aggregative adherence to HEp-2 cells (ROCHA *et al*. 2007a). MR/P fimbriae elicit a strong immune response during infection. It is directed against the MrpA protein. It was found that immunization of mice with the attenuated strain of *Salmonella* Typhimurium expressing MrpA-TetC fusion protein led to an increase in serum level of IgG antibodies against MrpA and significantly reduced *P. mirabilis* colonization of bladder and kidney. TetC is a non-toxic highly immunogenic fragment of the tetanus toxin (SCAVONE *et al*. 2011). The presence of *mrp* operon was shown not only in *P. mirabilis* strains but also in other species of the *Proteus* genus (BABICKA 2001, KWIL, 2002, 2003, KWIL *et al*. data not published).

Other than MR/P fimbriae are known to a lesser extent. MR/K hemagglutinins were found more frequently on *P. penneri* strains than on *P. mirabilis* strains and most probably they facilitated the adherence of bacteria to the urinary catheters (JACOBSEN *et al*. 2008). The exact virulence role of PMF fimbriae is unclear (NIELUBOWICZ & MOBLEY 2010). ZUNINO *et al*. (2007) by using *P. mirabilis pmfA/mrpA-D* mutant showed the specific and additive roles of MR/P and PMF fimbriae in UTI caused by these bacteria. NAF fimbriae, earlier known as UCA fimbriae, allow bacteria to colonize uroepithelial cells (WRAY *et al*. 1986). It was found that NAF/UCA binds the following receptors: asialo-GM_1, asialo-GM_3, lactosyl ceramide and galectin (LEE *et al*. 2000, ALTMAN *et al*. 2001). ATF fimbriae are not important in the pathogenicity of human beings (ZUNINO *et al*. 2000). PMP fimbriae were found on canine UTI isolates (BIJLSMA *et al*. 1995), however genes coding this type of fimbriae were found in human clinical isolates (PEARSON *et al*. 2008).

There are conflicting results concerning the importance of flagella in the infection. Since flagella mediate motility of bacteria, they seem to be required for virulence of uropathogens, particularly during ascending infection. Indeed, it was shown *in vivo* that flagella-negative mutant of *P. mirabilis* is less virulent, compared to the wild strain, which suggests an important role of these bacterial surface structures in the pathogenicity (MOBLEY *et al*. 1996). However, it must be stressed, that lack of flagella on *P. mirabilis* human isolate was found (ZUNINO *et al*. 1994). Flagella play a role of H antigen and are strongly immunogenic. Bacteria can avoid immune response of human organism due to the antigenic variation process. This phenomenon is based on flagellin genes rearrangement. Flagellar antigenic variations allow bacteria to evade the action of secretory IgA antibodies directed against these organelles (BELAS 1994).

P. mirabilis evades the immune system response during infection thanks to the production of sIgA protease. The best characterized is 54-kDa secreted metalloprotease ZapA, mirabilysin belonging to the serralysin family (LOOMES *et al*. 1990). ZapA possesses a broad spectrum of proteolytic specificity, including the activity against antibodies of sIgA, IgA2 and IgG, proteins of the complement system, cell matrix proteins, cytoskeletal proteins, as well as antimicrobial defense components of human innate system peptides hBD1 and cathelicidin LL-37 (BELAS *et al*. 2004, JACOBSEN *et al*. 2008). ZapA was found to be an important virulence factor in a rat model of *P. mirabilis* induced acute and chronic prostatitis (PHAN *et al*. 2008). Recently, CARSON *et al*. (2011) described *N*-alpha mercaptoamide dipeptides, which can be used as inhibitors of ZapA. KWIL *et al*. (2011) have shown the presence of the *zapA* operon in *P. vulgaris* and *P. penneri* strains.

It was shown that amino acid deaminase (Aad) produces α-keto acids which serves as siderophores involved in iron acquisition (DRECHSEL *et al*. 1993, MASSAD *et al*. 1995). Recently, HIMPSI *et al*. (2010) described proteobactin and yersiniabactin related siderophore function in *P. mirabilis*. Zinc, another biologically important element needed for bacterial growth, is utilized by *P. mirabilis* by use of ZnuABC high affinity

transport system (NIELUBOWICZ & MOBLEY 2010). *P. mirabilis* also posses high-affinity phosphate transporter system Pst. It is postulated that this system is important in *P. mirabilis* virulence (JACOBSEN *et al*. 2008).

Urease is an urea amidohydrolase which catalyses the hydrolysis of urea to ammonia and carbamate, then digested to carbone dioxide and second molecule of ammonia (MOBLEY *et al*. 1995). This process results in elevation of pH and non-physiological alkalization of urine, which in turn induces the precipitation of magnesium and calcium ions, normally soluble in slightly acidic urine, and the formation of urinary stones, containing struvite and carbonate apatite (RÓŻALSKI *et al*. 1997, JACOBSEN *et al*. 2008, NIELUBOWICZ & MOBLEY 2010). The carbonate apatite forms an amorphous precipitate opposite to struvite, which forms crystals of defined morphology. It was found that struvite exhibits polar properties (PRYWER & TORZEWSKA 2010, ROMANOWSKI *et al*. 2010).

Table 2. Virulence factors (VP), biological features (BF) and types of growth (TG) of *Proteus* rods in relation to the pathogenicity.

VP/BF/TG	Specific data	Contribution to the pathogenicity
Fimbriae	*P. mirabilis* genome predicts 17 distinct fimbrial operons. Six types of fimbriae/agglutinins were identified	Adherence of bacteria to the epithelial tissue Contribution to the biofilm formation
Flagella	H-antigen	Ascension of bacteria in urinary tract. Elicitation of immunoresponce which can be avoid due to the antigenic variation
Invassiveness	Studied *in vitro*	Penetration and internalization of bacteria into host cells
Proteases	IgA and IgG proteases, which are also able to degrade other types of biologically important proteins	Diminish or elimination of phagocytosis
Amino acid deaminase	Production of alpha-keto acids which serve as siderophores	Iron acquisition
Proteobactin and yersiniabactin - related siderophore		
ZnuABC	High affinity zinc transport system	Zinc utilization
Pst	High affinity phosphate transporter	Phosphate accumulation
Urease	Enzyme hydrolyzing urea to NH_3 and CO_2	Elevation of urine pH level, resulting in stones formation. Cytotoxicity against urinary epithelial cells
Protein toxins:		
HmpA	Cell-bound hemolysin	Pore forming toxins mediating cytotoxicity
HlyA	Cell free hemolysin, RTX protein	
Pta	Cytotoxin and agglutinin	Cytotoxicity and autoagglutination of bacteria
CPS	Most probably structurally identical with O-specific part of LPS	Formation of biofilm. Importance in formation of urinary stones. Facilitation of swarmer cells translocation
LPS	Endotoxin	Endotoxicity. Resistance against bactericidal action of the serum. Production of glycocalyx important in biofilm formation
OMVs	Outer membrane vesicles containing periplasmic constituents	Transport of virulence factors to the host tissues
Swarming growth	Differentiation of short rods to swarmer cells	Not fully understood
Biofilm formation	Crystalline biofilm, particularly formed on the surface of urinary catheters	Protection of bacteria against action of antibiotics and immune mechanisms

The structural and elastic properties of struvite crystals were studied by PIECHOTA *et al*. (2011). Formation and morphology of struvite crystals depend on the pH of urine. PRYWER and TORZEWSKA (2009) have found that for pH in the range 7.5–9.0 struvite crystals take single and hemimorphic morphology. At higher pH many twin and dendritic crystals have been shown. DUMANSKI *et al*. (1994) has revealed that *Proteus* capsular polysaccharide structurally identical to O-specific polysaccharide of LPS accelerates crystallization process. TORZEWSKA *et al*. (2003) confirmed this observations and showed that urinary stone formation due to the urease activity, may be modified by surface O-antigens being a part of lipopolysaccharides (LPS). The sugar composition of these polysaccharides may either enhance or inhibit the crystallization of struvite and carbone apatite depending on their chemical structure and ability to bind cations. Bacteria present within urinary stones are protected from host defense mechanisms and antibiotic treatment (LI *et al*. 2002b). Therefore, several phytotherapeutic compounds have been investigated to prevent urinary stone formation. For example curcumin an yellow-orange pigment non toxic to humans or animals extracted from the roots of turmeric (*Curcuma longa*) was shown to inhibit urease activity and consequently decrease the struvite growth. It was found *in vitro* experiment of struvite formation in artificial urine that the addition of curcumin increases the induction time and decreases the efficiency of formation of these crystals (TORZEWSKA *et al*. 2010, PRYWER & TORZEWSKA 2010, 2012). Moreover, curcumin also inhibits biofilm formation and block swarming growth (TORZEWSKA *et al*. 2010). Recently, MALIC *et al*. (2011) reported on the development of an early warning sensor for encrustation of urinary catheters in case of *Proteus* infection.

It was shown that the high level of ammonia, a product of urease action also results in damage of the urinary tract epithelium. Toxic ammonia damages bladder surface glycosaminoglycans important in the prevention of bacterial adherence to the uroepithelium (JACOBSEN *et al*. 2008). *In vitro* studies performed by TORZEWSKA and RÓŻALSKI (2009) showed that glycosaminoglycans have no inhibitory effect on infection-induced crystallization.

In a mouse model of ascending UTI it was found that the urease negative mutant of *P. mirabilis* was less virulent as compared to the wild strains, which suggests the important role of this enzyme in infection caused by this bacterium (JONES *et al*. 1990). Therefore, investigation of different substances which can be used as urease inhibitors is being performed. Recently, BERLICKI *et al*. (2011) described N-substituted amino-methane-phosphonic and aminomethane-P-methylphosphonic acids as effective inhibitors of *P. mirabilis* urease.

Proteus rods produce three types of cytolysins, HpmA and HlyA hemolysins and Pta cytotoxic agglutinin. HpmA is the 166 kDa protein, calcium–independent cell-bound hemolysin secreted by the bacteria and activated in the process mediated by HpmB (UPHOFF & WELCH 1990). This hemolysin belongs to the pore forming toxins family and it is cytotoxic against a number of cells (SWIHART & WELCH 1990). HlyA hemolysin is produced by *P. vulgaris*, *P. penneri* and *P. hauseri* strains (KORONAKIS *et al*. 1987, RÓŻALSKI & KOTEŁKO 1987, RÓŻALSKI & STĄCZEK 2011). It is a strongly cytotoxic, calcium dependent 110 kDa extracellular toxin belonging to the RTX family of proteins (RTX - repeat in toxin). Pta (*Proteus* toxic agglutinin), an autotransporter with subtilase-like serine protease activity exhibits bifunctional activity. It is both cytotoxin and agglutinin, which mediates autoagglutination of bacteria and cytotoxicity against human bladder and kidney cells. The vaccination of mice with Pta results in protection against *P. mirabilis* UTI (ALAMURI & MOBLEY 2008).

Lipopolysaccharide is composed of three genetically and structurally distinct regions: O-specific chain (O-antigen, O-specific polysaccharide), the core oligosaccharide and lipid A, which anchors the LPS molecule to the bacterial outer membrane (RAETZ &

WHITFIELD 2002). LPS containing all these three regions is produced by smooth forms of bacteria. Rough strains synthesize LPS containing lipid A and the whole core region or only its part. All three regions of *Proteus* LPS have been studied (RÓŻALSKI 2002, 2004, 2008, RÓŻALSKI *et al*. 2002). The differences in the structure of O-antigens serve as a basis for the serological classification of *Proteus* strains. The serological classification scheme currently consists of 78 serogroups (DRZEWIECKA *et al*. 2004, 2008, 2010, DRZEWIECKA & SIDORCZYK 2005, KNIREL *et al*. 2011). The prevalence of particular *Proteus* O-serogroups among clinical isolates has been changing over the time. Previously, clinical isolates were found to belong to serogroups O3, O10, O11, O13, O23, O27 and O30. Recently, KACA *et al*. (2011) studying 123 clinical isolates from Sweden and Poland showed that none of serogroups was predominant. DRZEWIECKA *et al*. 2010 described seven *P. mirabilis* strains representing serogroup O78 which can be classified in one bacterial clone widespread in hospitals, most probably due to the nosocomial infection and autoinfections. The results of detailed studies of *Proteus* O-specific polysaccharides are presented in the review by KNIREL *et al*. 2011. WANG *et al*. (2010) identified five putative *Proteus* O-antigen gene clusters and *Proteus* specific genes of strains classified to five serogroups.

A

α-L-Alt*p*A-(1→
3
→4)-α-D-Gal*p*NAc-(1→3)-α-D-Gal*p*A-(1→3)-α-D-Glc*p*NAc-(1→

B

β-GalA*p*(1→7)-α-Hep β-Ara4N
1 1
↓ ↓
7 8

β-GalA*p*Lys-(1→3)-β-Glc*p*NAc-(1→6)-α-Glc*p*N-(1→4)-GalA*p*-(1→3)-Hep6*P*EtN-(1→3)-α-Hep-(1→5)-Kdo
2 4
↓ ↑
7 1
DD-Hep β-Glc*p*

Figure 2. Structure of the O-specific repeating unit (A) and core oligosaccharide obtained by mild acid degradation (B) of lipopolysaccharide of *Proteus mirabilis* genomic strain HI 4320. Alt - altruronic acid (KNIREL *et al*. 2011, VINOGRADOV 2011).

The core region of *Proteus* lipopolysaccharides was studied by use of rough mutants or smooth forms classified into different serogroups (RADZIEJEWSKA-LEBRECHT *et al*. 1989, VINOGRADOV *et al*. 1994, VINOGRADOV *et al*. 2002). The structural diversity of the core is characteristic for *Proteus* spp. and makes it different from *E. coli* and *Salmonella* (HOLST 1999, VINOGRADOV *et al*. 2002). The *Proteus* core region is composed of two parts – an inner part, common for several number of strains and second, an outer part, which is characterized by structural variability from strain to strain. The common part is not identical in all *Proteus* strains and is

subdivided into three forms known as glycoforms I-III. The outer part of the core region (outer core) contains an oligosaccharide characteristic for particular *Proteus* strains (VINOGRADOV *et. al* 2002, RÓŻALSKI 2008). The structure of the core part of *P. mirabilis* genomic strain HI4320 (formerly classified into serogroup O10) was shown by VINOGRADOV (2011) (fig. 2). The epitope specificity of the core region of *P. penneri* strains were studied by PALUSIAK and SIDORCZYK (2010). They have identified seven epitopes responsible for cross reactivity of LPS of these bacteria with appropriate antisera. The identification of genes required for the biosynthesis of the core region of *P. mirabilis* were described by AQUILINI *et al*. (2010).

LPS, released from pathogenic bacteria during infection induces a spectrum of biological activities. Therefore, LPS is also known as an endotoxin. The mechanism of biological action of the endotoxin is common to most bacteria. Its biological domain is lipid A (ŁUKASIEWICZ & ŁUGOWSKI 2003). *Proteus* lipid A contains glucosamine disaccharide substituted with phosphate residues and fatty acids. It also contains 4-amino-4-deoxy-L-arabinopyranose (L-Ar*ap*4N), which quantitatively substitutes the ester-linked phosphate residue of the glucosamine backbone (SIDORCZYK *et al*. 1983).

Proteus LPS is associated with a broad spectrum of biological activities, and with interactions with bacterial or eukaryotic cells (RÓŻALSKI 2008). LPS of S-forms of bacteria contributes to their resistance against bactericidal action of the serum (KACA & UJAZDA 1998, MIELNIK *et al*. 2004, BABICKA 2001, KWIL 2003, KACA *et al*. 2009). The O-specific polysaccharide chain exposed outside bacteria is involved in glycocalyx formation. Glycocalyx enables bacteria to grow in a form of biofilm on a solid surface.

Bacterial biofilm is defined as a matrix-enclosed bacterial population adhering to the surfaces. Bacteria enclosed in a glycocalyx capsule are protected against the action of antibodies, as well as against other immune mechanisms (DONLAN & COSTERTON 2002, STICKLER & FENLEY 2010). *Proteus* bacteria form a crystalline biofilm particularly on the surface of catheters (STICKLER 2008). It contains calcium phosphate and magnesium phosphate crystals formed due to the action of urease. JONES *et al*. (2007) reported that the structure of *P. mirabilis* biofilm depends on the environment (artificial urine, laboratory media) in which it occurs. Biofilms in Luria-Bertani (LB) broth formed a mushroom-like architecture, while biofilms grown in urine formed a flat layer. MORYL *et al*. (2008) studied the effect of environmental factors such as glucose, acidic pH, albumin and some antimicrobial agents: ciprofloxacin and methylene blue on the formation of *P. mirabilis* biofilm. The most inhibiting effect was observed after incubation of bacteria with albumin and in acidic growth media. KWIECINSKA-PIRÓG (2010) studied the formation of biofilm on different surfaces, and has found silicone latex surface to be better than polychloride vinyl and polypropylene surfaces for *P. mirabilis* growth in biofilm. Octenisept R an antiseptic products occured to be active against these bacteria growing in biofilm. Prevention and eradication of *P. mirabilis* biofilm by use of lytic bacteriophages was shown by CARSON *et al*. (2010). It was also found that two quorum sensing antagonists p-nitrophenyl glycerol (PNPG) and tannic acid inhibit the formation of *P. mirabilis* biofilm in artificial urine (JONES *et al*. 2009). *P. mirabilis* biofilm formation on glass and polystyrene can also be inhibited by extract of *Ibucella lutea*, a native plant of America (SOSA & ZUNINO 2009). MORYL (2010) observed inhibition of biofilm formation by *P. mirabilis* growing together with *Escherichia coli* or *Providencia stuartii*. STAŃKOWSKA *et al*. (2011) showed a significant influence of *N*-butanoyl homoserine lactone (BHL) on biofilm formation by *P. mirabilis* strain belonging to serogroup O18. Recently, SCHLAPP *et al*. (2011) excluded the contribution of swarmer cells in biofilm formation during the 7-day incubation of bacteria in LB broth on glass coverslips. These authors showed that

P. mirabilis biofilm formation is a five-stage process: i) reversible adhesion to the solid surface with slow growth of bacteria, presence of elongated cells and absence of extracellular matrix, ii) irreversible bacterial adhesion, beginning of production of extracellular materials and decreasing of elongation, iii) acceleration of bacterial growth, further decrease in elongation and halting production of extracellular material, iv) maturation of biofilm, v) detachment of bacterial cells and dispersion of biofilm.

4. Summary

Proteus bacteria inhabit the environment and they are also present in the intestines of humans and animals. These microorganisms under favorable conditions cause a number of infections including urinary tract infections (UTIs), wound infections, meningitis in neonates or infants and rheumatoid arthritis. *Proteus* opportunistic pathogens express virulence factors associated with adherence, motility, immunoavoidance, nutrient acquisition, host damage, biofilm formation and endotoxicity. The most defining characteristics of *Proteus* bacteria is the swarming phenomenon, a multicellular differentiation process of short rods to elongated swarmer cells. It allows the population of bacteria to migrate in a coordinate manner on a solid surface. The role of short rods and elongated swarmer cells in the pathogenicity of *Proteus* bacteria is still not established. The elongated morphotype seems to be in the minority during infection. *Proteus* bacteria form a crystalline biofilm, particularly on the surface of catheters present in the urinary tract of patients. It contains struvite and hydroxyapatite crystals formed due to the action of urease. The results of the study on *Proteus* virulence and fitness factors will lead to a better understanding of infectious processes and will subsequently allow developing new methods of prevention and clinical treatment.

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