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Original Article

Quantification of *Proteus mirabilis* virulence factors and modulation by acylated homoserine lactones

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Background and Purpose: Measurement of the main *Proteus mirabilis* virulence factors would increase our understanding of how the organism infects and colonizes the urinary tract. The purpose of this study was to quantify the virulence factors of twelve *P. mirabilis* laboratory strains and to determine whether expression of virulence factors of *P. mirabilis* depends on the presence of homoserine-lactone derivatives.
Methods: Twelve *P. mirabilis* strains with defined lipopolysaccharide structures were used. The activity levels of urease, proteases, and hemolysins and the swarming abilities of *P. mirabilis* rods were tested by qualitative and quantitative methods. The effect of addition of acylated homoserine lactones (acyl-HSLs) was evaluated in order to determine their influence on the pathogenic features of the *P. mirabilis* strains.
Results: The ureolytic, proteolytic, and hemolytic abilities and the swarming motility of *P. mirabilis* rods were strain-specific. The *P. mirabilis* strains which possessed a negatively charged O-polysaccharide demonstrated strong ureolytic and proteolytic properties and faster migration speed on solid media. There was no influence of acyl-HSLs on the process of urea decomposition. The acyl-HSLs inhibited the protease activity of five *P. mirabilis* strains. *N*-butanoyl-L-homoserine lactone accelerated the migration speed of the tested *P. mirabilis* strains.
Conclusions: The levels of tested virulence factors were strain-specific. The acetylated homoserine lactone derivatives modified the expression of some virulence factors of the *P. mirabilis* strains.

Key words: Acyl-butyrolactones; *Proteus mirabilis*; Urinary tract infections; Virulence

Introduction

Urinary tract infections (UTIs) are among the most frequently occurring human bacterial infections, accounting for about 20% of all infections acquired outside the hospital. Almost 90% of UTIs are ascending, with bacteria gaining access to the urinary tract via the urethra to the bladder and then to the upper part of the urinary tract [1,2]. The organism causing a UTI usually originates from the patient's own bowel flora. The most frequent etiological agents causing UTIs are Gram-negative bacteria belonging to the *Enterobacteriaceae* family [3-5]. Bacterial virulence factors are regulated by quorum-sensing molecules which are derivatives of serine substituted by fatty acid, i.e., acylated homoserine lactones (acyl-HSLs) [6-11]. *Proteus mirabilis* is one of the most common causes of UTI in individuals with long-term indwelling catheters, or complicated UTIs, and of bacteremia among the elderly [5,12,13].

Pathogenic bacteria have developed numerous virulence factors to adapt to their host environment. Quantification of the main *P. mirabilis* virulence factors would increase our understanding of how a microorganism from normal flora infects and colonizes the urinary tract and establishes infection. The main pathogenic features of *P. mirabilis* rods are their ureolytic, proteolytic, and hemolytic abilities, swarming motility, and lipopolysaccharide (LPS) presence. LPS, the major surface molecule of Gram-negative bacteria, interacts with the host and, depending on the dose, induces an inflammatory response [14,15].

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Among the hundreds of known *Enterobacteriaceae* species, only selected strains with defined O-antigens are considered to be human or animal pathogens. The *Escherichia coli* O157:H7 serotype and the pig *E. coli* O9 serotype [16,17] are described as human enteropathogenic strains. *E. coli* strains that often cause UTIs belong to the O1, O2, O4, O6, O16, and O18 serogroups [18]. *P. mirabilis* strains originating from UTIs were most often classified to the O3, O6, O10, O11, O13, O23, O24, O26, O27, O28, O29, and O30 serotypes [19,20].

In our previous study, we demonstrated that differences in the structure of the O-polysaccharide part of the LPS influences the biological activities of *P. mirabilis* strains [21]. *P. mirabilis* O18 was biologically more active than *P. mirabilis* O3 (S1959) LPS. *P. mirabilis* O18 LPS is characterized by a phosphocholine substituent on the O-polysaccharide part, whereas *P. mirabilis* S1959 possesses a lysine residue. Apart from the biological activity of LPS, other virulence factors of *P. mirabilis* O3 and *P. mirabilis* O18 strains were not examined [21].

In the present study, we examined twelve laboratory *P. mirabilis* strains (including the O3 and O18 strains) with different O-polysaccharide LPS structures. The aim was to determine the levels of expression of the virulence factors urease, proteases and hemolysins and the swarming abilities of the *P. mirabilis* strains. Stable *P. mirabilis* laboratory strains were used in order to define the basic variability of virulence factors. Correlation between the structures of *P. mirabilis* O-antigen and some virulence factors were also examined. The second goal was to discover whether the expressions of the above virulence factors of *P. mirabilis* are modulated by the presence of acyl-HSL derivatives.

Methods

Strains, culture conditions, and acyl-HSLs

The *P. mirabilis* laboratory strains recruited for the study, i.e., O7 (Prk 15/57), O9 (Prk 18/57), O18 (Prk 34/57), O20 (Prk 38/57), O35 (Prk 61/57), O36 (Prk 62/57), O38 (Prk 63/57), O40 (Prk 66/57) and O49 (Prk 75/57), were from the Czech National Collection of Type Cultures. The smooth *P. mirabilis* O3 (S1959) strain and its Ra type mutant strain R110 were from the Institute of Microbiology and Immunology, University of Lodz, Poland. A clinical isolate of the UTI *P. mirabilis* 1748 strain was from the hospital of the Swietokrzyskie Center of Oncology, Kielce, Poland. The strains were cultured at 37°C in Mueller-Hinton broth (pH ≈ 7.4) or in M9 Minimal Medium supplemented with 0.2% glucose, 0.01% casamino acids, biotin (0.15 μg/mL), 1.5 μM thiamine and 25 mM urea.

The following acyl-HSLs were used: *N*-butanoyl-L-homoserine lactone (BHL), *N*-hexanoyl-L-homoserine lactone (HHL), *N*-octanoyl-L-homoserine lactone (OHL), and *N*-decanoyl-L-homoserine lactone (DHL), *N*-dodecanoyl-L-homoserine lactone (dDHL), *N*-tetradecanoyl-L-homoserine lactone (tDHL), purchased from Fluka (Buchs, Switzerland).

Measurement of ureolytic activity

The level of urea was determined by the plate method, based on the method described by Christensen with Hormache and Munill's modifications [22], which allows the measurement (in mm) and observation of changes in the color of the medium from yellow to red caused by alkalization of the surroundings of the bacterial colonies. Moreover, Weatherburn's method [23] using phenol and hypochlorite allowed color measurement of the tested samples treated and untreated with acyl-HSL, where the color intensity depends on the amount of ammonia. Applying Weatherburn's method in relation to the amount of protein calculated by the Lowry test, we calculated the urease activity from the equation: number of units [U] of urease = nmol urea/mg protein/min reaction.

Measurement of proteolytic activity

Bacteria were cultivated at 37°C for 18 h in Müller Hinton broth. The bacteria were then seeded on plates containing 2% skim milk, 1% gelatine, 1% casein on 3.6% w/v cystine-lactose-electrolyte-deficient agar (Sigma, St. Louis, MO, USA), and 50 mM Tris-hydrochloride, pH 8.0. After 18 h in culture, the degree of agar transparency was evaluated [24].

The release of azo dye from azocasein was used to measure extracellular protease in the broth culture supernatants of the *Proteus* strains treated and untreated with acyl-HSLs and the proteolytic activity was assayed according to the protocol of Loomes et al [25]. The amount of hydrolyzed azocasein was determined at 440 nm using a Pharmacia Ultrospec 2000 spectrophotometer (Biotech, Sweden). One unit of protease was defined as that capable of hydrolyzing 1 mg of azocasein/60 min at 37°C and was calculated from the equation: number of units [U] of protease = A_{440} of reaction × 60/1.65 × reaction time (min).

Measurement of hemolytic activity

The hemolytic activity of the *Proteus* strains was tested by two methods. The first was the blood plate method, where the zone of hemolysis produced by the colony of tested bacteria was observed. We measured (in mm) the zones surrounding single colonies of growing bacteria and evaluated the degree of clearing. Using the spectrophotometric method, we evaluated the level of cell-associated and extracellular hemolysins released by *P. mirabilis* treated and untreated with acyl-HSL in comparison with a 100% hemolysin-positive control [26].

Measurement of swarming motility

Assessment of changes in the swarming growth of *P. mirabilis* strains was made by the plate method, where measurements were taken every 1 h. The speed of migration was measured (in mm) from the centre of the inoculated bacteria (5 μL of inoculum contained 1-1.5 × 10^8 cells/mL) to the last zone of swarming growth. The number of zones formed was also compared between samples varying in the acyl-HSL used. The speed of migration (mm/h) of *P. mirabilis* through urological catheters in the presence of acyl-HSL was performed according to the method described by Sabbuba et al [27] with modifications.

Protein determination

Overnight cultures in M9 media of each of the *Proteus* strains were sonicated (3 × 3 min) on ice and centrifuged at 14,000 rpm for 5 min and the resulting supernatant was used for total protein determination. Proteins were quantified according to the method of Lowry et al [28].

Detection of genes

Polymerase chain reaction (PCR) was used to detect the *ureC* and *zapA* genes in the genomes of the bacterial strains. Matrix DNA was prepared by warming bacterial cells at 100°C for 5 min and removing solid debris by centrifugation. The *ureC* primers used were forward: 5'-GTTATTCGTGATGGTATGGG and reverse: 5'-ATAAAGGTGGTTACGCCAGA. The PCR cycles were: denaturation at 95°C for 1 min, annealing at 52°C for 1 min, and extension at 72°C for 1 min, repeated 35 times. For *zapA* the primers were forward: 5'-ACCGCAGGAAAACATATAGCCC and reverse: 5'-GCGACTATCTTCCGCATAATCA. The PCR cycles were: denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, repeated 35 times. The products were separated in 2% agarose gel in Tris-acetate ethylenediamine tetra-acetic acid, stained with ethidium bromide, and photographed in ultraviolet (305 nm) light.

Plasmid DNA was extracted from the *Proteus* strains by the method described by Kado and Liu [29]. The extracted plasmids were electrophoresed on 1% agarose gel with Tris-borate buffer (pH ≈ 8.6).

Statistical analysis

Statistical tests were performed using the SigmaStat (Version 2.03; SPSS Inc., Chicago, IL, USA) software. Data were expressed as mean ± standard error of the mean. To compare means following one-way analysis of variance (ANOVA), the Tukey honestly significant difference or Student-Newman-Keuls method was used. In some cases, one-way ANOVA without post-tests was used. To assess whether there was a relationship among the five variables, the Pearson product-moment correlation was used. p Values <0.05 were considered statistically significant.

Results

Comparison of *P. mirabilis* O-polysaccharide structures

The pathogenic properties of twelve *P. mirabilis* strains that differ in the O-specific part of their LPS structures were studied. Eleven of them were laboratory strains and one a clinical strain belonging to serogroup O18 (*P. mirabilis* 1784) [Fig. 1]. Most of the presented O-parts of LPS have a negative charge of the polysaccharide. The exceptions are *P. mirabilis* O3 LPS, possessing a positive charge, and *P. mirabilis* O20 LPS, which has no charge. Some of these LPS structures are characterized by unique components. For example, *P. mirabilis* O9 possesses ribose, *P. mirabilis* O18 phosphocholine, *P. mirabilis* O38 2-acetamidoethylphosphate, and *P. mirabilis* O40 lactate in the R configuration.

Ureolytic activity of *P. mirabilis* laboratory strains

All tested virulence factors of *P. mirabilis* were determined by two methods: qualitatively on plates, and quantitatively by spectrophotometric methods. Applying the qualitative plate method with 20% urea, the *P. mirabilis* strains were put into two groups with low and high ureolytic activity. The first group consisted of the strains belonging to the O7, O9, O35, O36, O49, and O18 (1784) serogroups. The second group of strains was characterized by high ureolytic activity

Serogroup	Structures of O-polysaccharide	Charge
O3 (S1959)	α-D-GalpA6(L-Lys) 1 ↓ 4 →3)-β-D-GalpNAc-(1→6)-β-D-GalpNAc-(1→4)-β-D-GlcpA-(1→ ; α-D-Glcp 1 ↓ 2	+
R110	Ra mutant of *P. mirabilis* O3 – O-specific polysaccharide not present	
O7	Quip4NMal 1 ↓ 6 →3)-β-D-GlcpNAc-(1→2)-β-D-Galp-(1→4)-β-D-Glcp-(1→	–
O9	→4)-β-D-GalpA-(1→2)-β-D-Ribf3Ac-(1→4)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→	–
O18	α-D-Glcp 1 ↓ 4 →6)-β-D-Glcp-(1→3)-β-D-Galp-(1→3)-β-D-GlcpNAc-(1→3)-α-D-GlcpNAc-1-P-(O→ ; ChoP | 4	–
1784 (O18)	Serologically classified into O18 serogroup, see above	–
O20	α-D-Glcp-(1→2)-β-D-Galp 1 ↓ 4 →3)-β-D-GlcpNAc-(1→3)-α-D-GlcpNAc-(1→4)-β-D-Glcp-(1→	N
O35 (O17)	→2)-β-D-Fucp3N(R3HOBu)4Ac-(1→6)-α-D-Glcp3Ac-(1→4)-β-D-GlcpA-(1→3)--α-D-GlcpNAc-(1→	–
O36	Structure under current investigation	–
O38	AcEtnP | 6 4)-α-D-GlcpNAc-(1→3)-β-D-Quip4N(Ac-D-Asp)-(1→6)-α-D-Glcp-(1→3)--α-D-GalpA-(1→	–
O40	3)-β-D-GlcpNAc-(1→3)-β-D-GlcpNAc4(R-Lac)-(1→3)-α-D-Galp-(1→3)-Gro-1-P-(O→	–
O49	α-D-Quip4NSuc 1 ↓ 4 →3)-β-D-GlcpNAc-(1→2)-α-D-GalpA-(1→3)-α-L-Rhap-(1→4)-α-D-Glcp-(1→2)-α-L-Rhap-(1→	–

Fig. 1. Chemical structures of the O-polysaccharide part of the lipopolysaccharide part of *Proteus mirabilis* strains. α = alpha; β = beta; + = positive charge; – = negative charge; N = neutral molecule.

(data not shown). Fig. 2 shows the ureolytic activity determined by the quantitative spectrophotometric method.

In general, there was agreement in the results of the plate and the spectrophotometric methods. The lowest ureolytic activity (in nmol urea/mg protein/min) was detected in the *P. mirabilis* O36 strain, whereas the O40, O18, and O38 strains possessed around 100% greater ureolytic activity than the O36 strain. Based on the ureolytic activity of the strains, four major groups could be determined ($p<0.05$ between groups): strain O36; strains R110 and O49; strains O35, O7, 1784, O9, O20, and S1959; and strains O40, O18, and O38.

We performed PCR to confirm the presence of urease genes in the studied strains. The specific primers for urease *ureC* gene identification were used. As expected, the twelve tested *P. mirabilis* strains possessed *ureC* gene (Fig. 3). Much less PCR product was visible in the *P. mirabilis* O35 strain. In the same strain, one additional band of the PCR was detected. (Fig. 3, lane 5). This may suggest mutation of the *ureC* gene in these strains. Differences in PCR products were detected in the strains with reduced urease activities.

The *P. mirabilis* O38 and O18 strains produced LPS with the non-carbohydrate components AcEtnP and ChP, respectively. These are rods classified in the

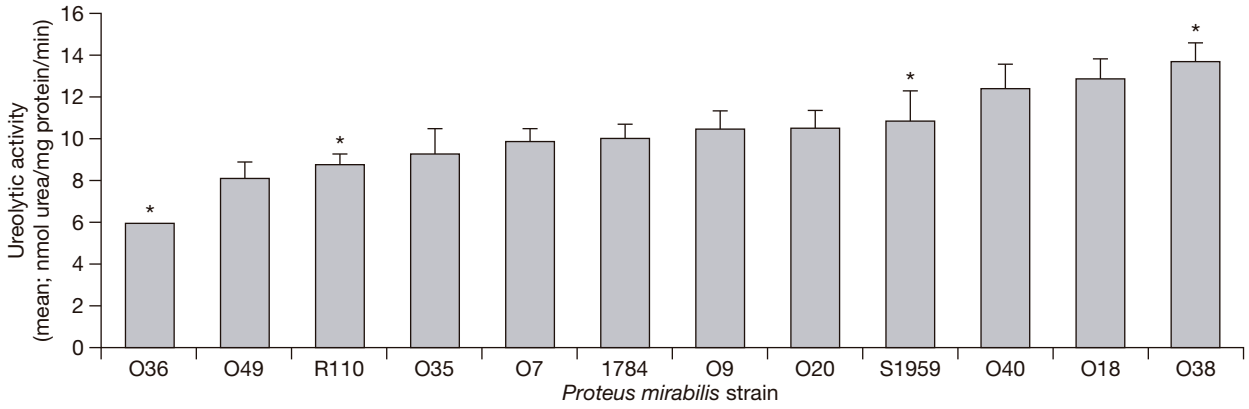

Fig. 2. Ureolytic activity of *Proteus mirabilis* strains. *Differences statistically significant ($p<0.05$, analysis of variance test). Bars indicate standard deviation.

group with the highest ureolytic activity. There were statistical differences in ureolytic activity between the smooth *P. mirabilis* S1959 and its Ra mutant strain R110 ($p<0.001$, Tukey test). This may indicate that the presence of long, hydrophilic O-polysaccharide chains in LPS facilitated ureolytic activity.

Proteolytic activity of *P. mirabilis* strains

Metalloproteases are a major group of proteolytic enzymes in *Proteus* strains [30]. Using PCR, we found that in all strains the genes for protease *zapA* were presented (Fig. 4). For unknown reasons, the expected PCR products were present in reduced amounts in the O18 and O35 strains.

The plate method on milk, gelatine, and casein media (Table 1) showed different abilities of the strains to decay the tested supplements. Six of the twelve *P. mirabilis* strains revealed a strong capacity for degrading the milk, gelatine, and casein. To differentiate *Proteus* strains with the ability of casein proteolysis, the quantitative azocasein test was used (Fig. 5). The most highly active were the O9, 1784 and O49 strains, with supernatant casein proteases content of 4.9 to 6.8 U/mL. Those strains also showed

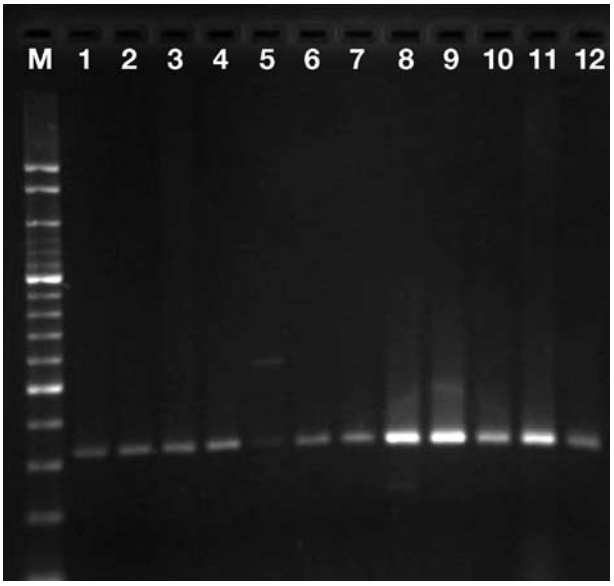

Fig. 3. Products of polymerase chain reaction performed with *ureC* primers and the specified *Proteus mirabilis* DNA. Lane M, length marker 100 bp; lane 1, *P. mirabilis* O7; lane 2, O9; lane 3, O18; lane 4, O20; lane 5, O35; lane 6, O36; lane 7, O38; lane 8, O40; lane 9, O49; lane 10, S1959 (O3); lane 11, R110; lane 12, 1784 (clinical isolate, serotype O18).

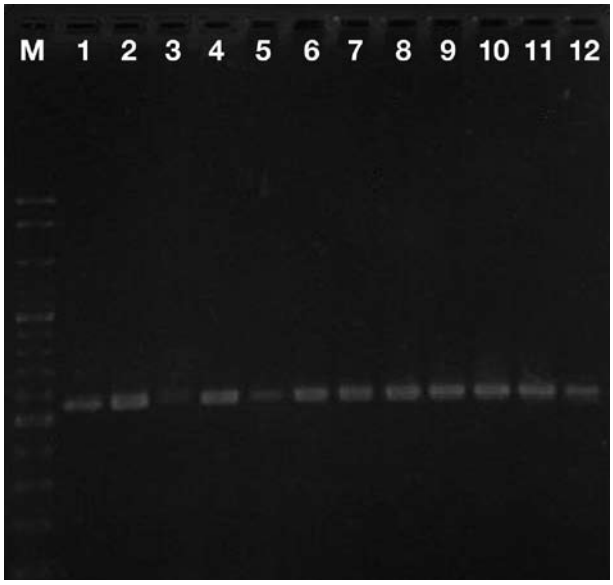

Fig. 4. Products of polymerase chain reaction performed with *zapA* primers and the specified *Proteus mirabilis* DNA. Lane M, length marker 100 bp; lane 1, *P. mirabilis* O7; lane 2, O9; lane 3, O18; lane 4, O20; lane 5, O35; lane 6, O36; lane 7, O38; lane 8, O40; lane 9, O49; lane 10, 1784 (clinical isolate, serotype O18); lane 11, R110; lane 12, S1959 (O3).

Table 1. Proteolytic activity of *Proteus mirabilis* strains evaluated on cystine-lactose-electrolyte-deficient agar supplemented with skim milk, gelatine and casein

Strain	Supplement		
	2% skim milk	1% gelatine	1% casein
O7	LT	LT	LT
O38	LT	LT	LT
R110	LT	LT	LT
O40	LT	LT	WTA/LT
S1959	LT	LT	WTA/LT
O18	LT	LT	PTA
O36	CTA	CTA	WTA
O20	PTA	CTA	PTA
1784	CTA	PTA	CTA
O9	CTA	CTA	PTA
O35	CTA	CTA	PTA
O49	CTA	CTA	CTA

Abbreviations: LT = lack of transparency; CTA = complete transparency of agar; PTA = partial transparencies of agar; WTA = weak transparencies of agar

strong proteolytic activities on plate methods. The *P. mirabilis* O35 strain was active with the plate method, but not in the azocasein test. Interestingly, there were statistical differences in proteolytic activity between the two O18 strains: *P. mirabilis* O18 had low activity, in contrast to the clinical isolate of the 1784 strain ($p<0.001$, Tukey test). The S strain and its R mutant (*P. mirabilis* S1959 and R110) had similar proteolytic activities in both methods.

Hemolytic activity of *P. mirabilis* laboratory strains

In the blood plate method, only two *P. mirabilis* strains, O38 and R110, did not demonstrate hemolytic activity. The O9 and O49 strains possessed the strongest activities. The spectrophotometric assay of hemoglobin release allowed quantitative measurements of cell-free and cell-bound hemolysis of *P. mirabilis* rods (Fig. 6). The *P. mirabilis* R110 strain showed very weak hemolytic activity, as opposed to the smooth *P. mirabilis* S1959 strain. The high hemolytic activity of the *P. mirabilis* S1959 strain, in contrast to its Ra mutant (R110), may indicate the role of the O-polysaccharide chain in the export of soluble hemolysin out of the bacterial cell membrane. There were statistical differences in the hemolytic properties between the *P. mirabilis* pair S1959 and R110 ($p<0.001$, Tukey test). The hemolytic activities of the two strains of serogroup O18, *P. mirabilis* O18 and 1784, were not statistically different. A discrepancy was observed for the *P. mirabilis* O38 strain, which was not active in the plate method but revealed hemolytic activity in the spectophotometric assay. This may be due to different hemolysin production in solid and liquid surroundings.

Swarming growth of *P. mirabilis* laboratory strains

Swarming of *P. mirabilis* was measured (in mm) from the centre of the inoculated bacteria to the last zone of swarming growth. Fig. 7 presents a comparison of the speed of migration captured at 10 h of *P. mirabilis* strain growth. As expected, the rough *P. mirabilis* R110 strain had no swarming motility and it was used as a negative control for migration. The *P. mirabilis* O20 and O38 strains had a specific swarming pattern (not shown). The *P. mirabilis* O18 (1784), O35, and O9 strains were characterized by the fastest rates of migration on agar plate media. The *P. mirabilis* strains with a negative charge on the O-polysaccharide showed the highest speeds of migration on solid media. There were statistical differences in the velocity of swarming

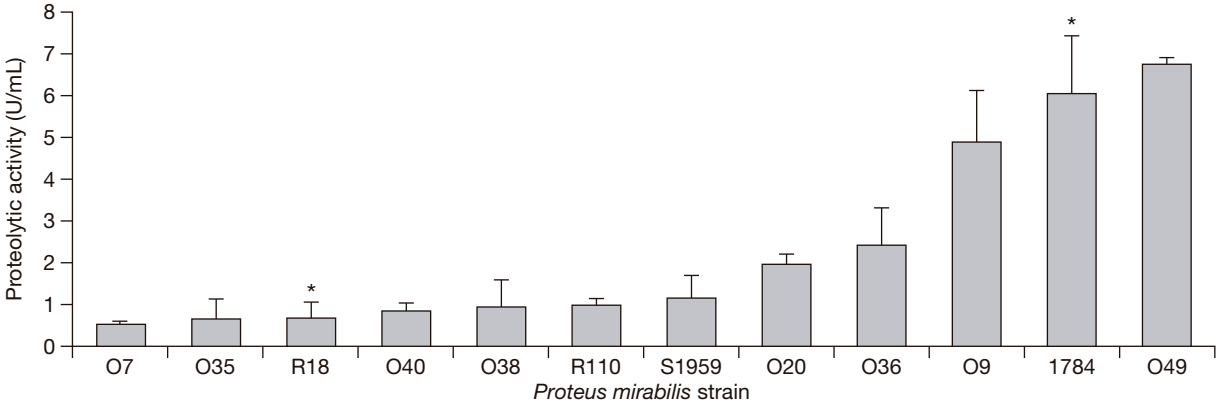

Fig. 5. Proteolytic activity of *Proteus mirabilis* strains, determined using azocasein assay. *Activity statistically different ($p<0.001$, Tukey test). Bars indicate standard deviation.

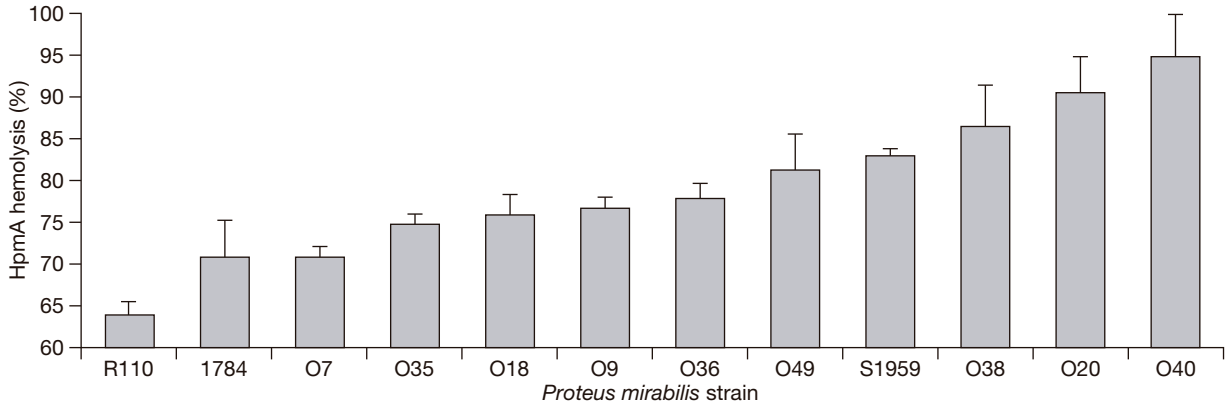

Fig. 6. Hemolytic activity of *Proteus mirabilis* strains, determined by spectrophotometric assay of released hemoglobin. Bars indicate standard deviation.

between the two *P. mirabilis* strains O18 and 1784 ($p<0.001$, Tukey test).

Acyl-HSL modulation of the pathogenic features of *P. mirabilis*

Six acyl-HSL derivatives were used to moderate *P. mirabilis* virulence factors. The ureolytic activity of the *P. mirabilis* strains tested, presented in Fig. 2, was unaffected by the presence of acyl-HSL. The quantitative azocasein assay for *P. mirabilis* proteases was applied in the presence of a series of 1 μM acyl-HSL derivatives: BHL, HHL, OHL, DHL, dDHL, and tDHL. Depending on the acyl-HSL type, significant inhibition of protease activity of five *P. mirabilis* strains was observed ($p<0.05$, one-way analysis of variance) [Table 2]. The exception was tDHL, which enhanced the proteolytic abilities of the O7 and S1959 *P. mirabilis* strains. The quantitative assay of hemolytic activity of *P. mirabilis* culture of ≈ 10^8 cells/mL after overnight growth at 37°C in the presence of the acyl-HSL derivatives showed no statistically significant changes in hemolytic activity. The exception was *P. mirabilis* O18, where dDHL increased the production of hemolysins in comparison with the control ($p<0.004$, Student-Newman-Keuls method).

The changes in swarming motility under the influence of acyl-HSL (10 nM) were dependent on the strain and the type of acyl-HSL (Table 3). BHL accelerated the speed of migration in most of the tested *P. mirabilis* strains. The results in Table 3 demonstrate that the addition of acyl-HSL modulated the swarming motility of *P. mirabilis* on solid media. To test if that effect would also be observed in urological catheters, two strains from the O18 serogroup were used. The differences in speed of migration of *P. mirabilis* strains O18 and 1784 through urological catheters in artificial urine are presented in Table 4. As in solid media, the observed speed of migration was strain- and HSL-type-specific.

Detection of plasmids in *P. mirabilis* laboratory strains

Gram-negative bacteria virulence factors are often present in the plasmids. Only in two analyzed strains,

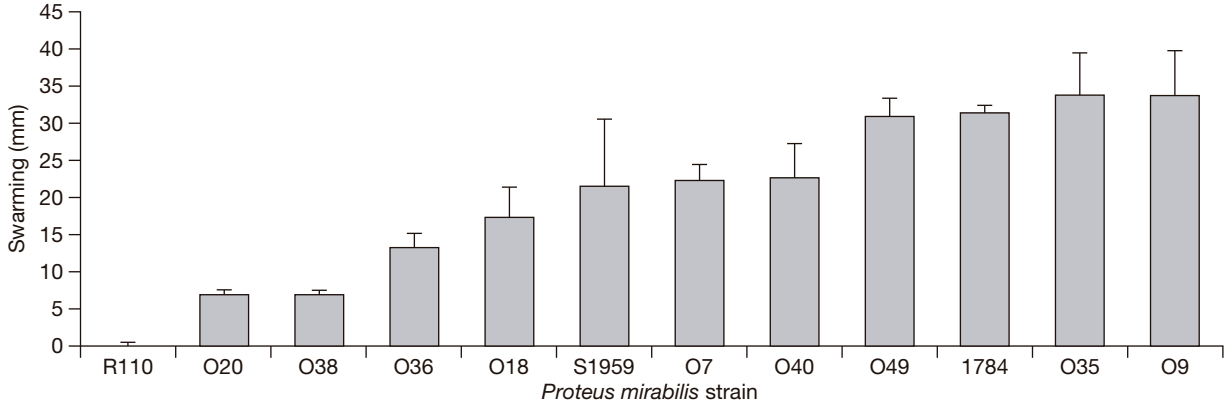

Fig. 7. Swarming motility of *Proteus mirabilis* strains, calculated after 10 h of culturing. Bars indicate standard deviation.

Table 2. Proteolytic activity of *Proteus mirabilis* strains in the presence of acylated homoserine lactone (acyl-HSL) derivatives

Strain	Acyl-HSL derivative (1 μM)					
	BHL	HHL	OHL	DHL	dDHL	tDHL
O7	NI	NI	NI	NI	NI	IPA
O20	NI	RPA	NI	NI	NI	NI
O35	NI	RPA	NI	NI	RPA	RPA
O49	RPA	RPA	RPA	RPA	RPA	NI
1784	NI	NI	NI	NI	RPA	NI
S1959	NI	NI	NI	NI	NI	IPA

Abbreviations: BHL = *N*-butanoyl-L-homoserine lactone; HHL = *N*-hexanoyl-L-homoserine lactone; OHL = *N*-octanoyl-L-homoserine lactone; DHL = *N*-decanoyl-L-homoserine lactone; dDHL = *N*-dodecanoyl-L-homoserine lactone; tDHL = *N*-tetradecanoyl-L-homoserine lactone; NI = no influence; RPA = reduction of proteolytic activity; IPA = increase of proteolytic activity

P. mirabilis O38 and the clinical isolate 1784 plasmids (of about 6 kb and 93 kb, respectively) were detected (data not shown). This may suggest that major *Proteus* virulence factors are not coded on plasmids.

Discussion

Pathogenic bacteria have developed numerous means of adapting to their host environment [31]. Quantification of the main *P. mirabilis* virulence factors would increase our understanding of their role in the infection and colonization of the human urinary tract [32]. In the present study, we showed that the ureolytic, proteolytic, and hemolytic abilities and the swarming motility of *P. mirabilis* laboratory strains differ significantly. The results of the multifactor Pearson's correlation test indicated a significant relationship between the ureolytic and hemolytic activities of the *P. mirabilis* O36 (r = –0.76), O38 (r = –0.73), and O49 (r = –0.87) strains. Also, the ureolytic and swarming motility were correlated in the cases of *P. mirabilis* O7 (r = –0.83) and 1784 (r = –0.89). *P. mirabilis* strains with a negative charge of the O-polysaccharide part of their LPS demonstrated stronger ureolytic, proteolytic, and swarming properties than strains with a positive or neutral O-polysaccharide part. In contrast, hemolytic activity did not reveal correlation with O-polysaccharide structure and charge. The five virulence factors of the twelve *P. mirabilis* strains were strain-specific at the quantitative level.

To compare the virulence factors of each strain, the relative virulence index was calculated (Fig. 8). As expected, the *P. mirabilis* R110 strain (Ra mutant) had lowest relative virulence index. The highest pathogenic activities were noted in the clinical 1784 strain and two laboratory strains, O9 and O49. The rest of the tested strains were situated between the above two groups. The results presented in Fig. 8 seems to indicate the relative stability of virulence factors, even in laboratory strains that were passaged for years. We suggest that the relative virulence index may also be useful in characterizing clinical strains.

Table 3. Swarming of *Proteus mirabilis* strains under the influence of acylated homoserine lactone (acyl-HSL) derivatives

Strain	Acyl-HSL derivative (10 nM)					
	BHL	HHL	OHL	DHL	dDHL	tDHL
O7	SM	RM	RM	NI	NI	SM
O9	SM	NI	SM	SM	NI	RM
O18	SM	SM	SM	NI	RM	RM
O20	NS	NS	NS	NS	NS	NS
O35	SM	SM	SM	SM	RM	SM
O38	NS	NS	NS	NS	NS	NS
O36	NI	SM	NI	NI	SM	RM
O40	SM	NT	NT	SM	NT	SM
O49	SM	NT	NT	SM	NT	SM
1784	NI	SM	NI	SM	RM	SM
S1959	SM	SM	NI	SM	NI	SM
R110	M	M	M	M	M	M

Abbreviations: BHL = *N*-butanoyl-L-homoserine lactone; HHL = *N*-hexanoyl-L-homoserine lactone; OHL = *N*-octanoyl-L-homoserine lactone; DHL = *N*-decanoyl-L-homoserine lactone; dDHL = *N*-dodecanoyl-L-homoserine lactone; tDHL = *N*-tetradecanoyl-L-homoserine lactone; SM = enhanced migration; NS = varied swarming strain, not swarming in 3 out of 5 experiment; NI = no influence; M = non-swarming mutant; RM = reduced migration; NT = not tested

Table 4. Migration of *Proteus mirabilis* 1784 and O18 strains through urological catheters with artificial urine in the presence of acylated homoserine lactone (acyl-HSL) derivatives (3-h data)

Acyl-HSL derivative (10 nM)	*Proteus mirabilis* O18	*Proteus mirabilis* 1784
BHL	NI	NI
HHL	RM	EM
OHL	NI	NI
DHL	EM	RM
dDHL	RM	EM
tDHL	EM	NI

Abbreviations: BHL = *N*-butanoyl-L-homoserine lactone; HHL = *N*-hexanoyl-L-homoserine lactone; OHL = *N*-octanoyl-L-homoserine lactone; DHL = *N*-decanoyl-L-homoserine lactone; dDHL = *N*-dodecanoyl-L-homoserine lactone; tDHL = *N*-tetradecanoyl-L-homoserine lactone; NI = no influence; RM = reduced migration; EM = enhanced migration

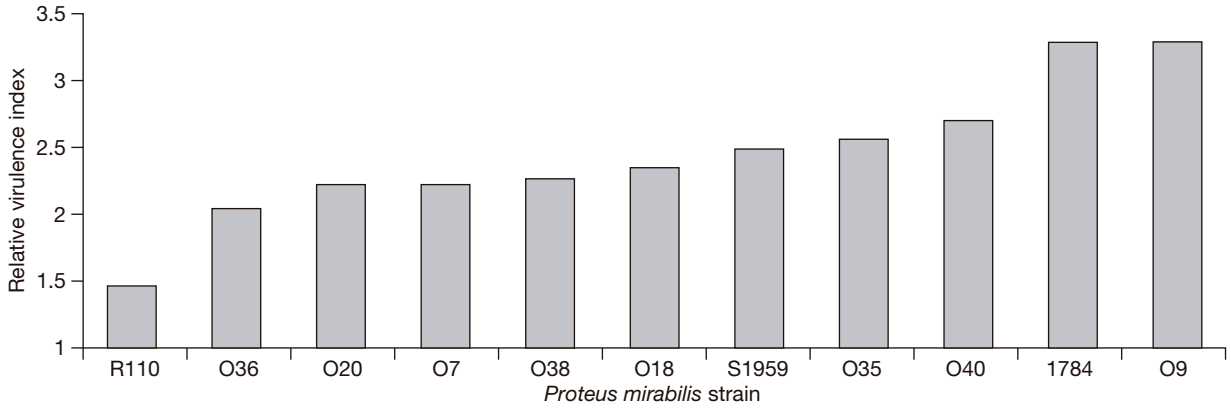

Fig. 8. Relative virulence index of *Proteus mirabilis* laboratory strains based on ureolytic, proteolytic, hemolytic and swarming abilities, calculated by summing scores for these activities (in relative units).

The pathogenic features of bacteria can be regulated by different signals from the environment [33]. The common signaling molecules of Gram-negative bacteria [11], acyl-HSL derivatives, were used to modulate the virulence factors of twelve *P. mirabilis* laboratory strains. The ureolytic activities of the twelve *P. mirabilis* strains were not influenced by acyl-HSL presence. In contrast to *P. mirabilis* ureases, the protease activities are regulated with acyl-HSL quorum-sensing molecules. The majority of the acyl-HSL derivatives inhibited the protease activity of five *P. mirabilis* strains, with the exception of tDHL, which enhanced the proteolytic abilities of the the *P. mirabilis* O7 and S1959 strains. We demonstrated that only one of the twelve strains, *P. mirabilis* O18, statistically significantly increased the production of hemolysins under the influence of dDHL. These observations are in agreement with studies of *Pseudomonas aeruginosa*, where 3-oxo-dDHL enhanced heat-stable hemolysin production [33].

Strain-dependent changes in swarming motility under the influence of acyl-HSL were observed in our study. BHL, DHL, and tDHL accelerated the speed of migration in the tested *P. mirabilis* strains. dDHL enhanced the swarming motility through urological silicone catheters of one clinical and one laboratory isolate of *P. mirabilis* belonging to the O18 serogroup. Not only the speed of migration, but also the pattern of swarming of the *P. mirabilis* O20 and O38 strains might be modified by the presence of acyl-HSL. The modulation of swarming and virulence factors of a *P. mirabilis* strain by single fatty acid residues has been reported [13]. One can speculate that acyl-HSL derivatives may act in a manner similar to fatty acids. This is because fatty acid residues are components of acyl-HSL and they may act as part of the RsbA signaling system [13]. Uropathogens use quorum sensing to regulate the genes involved in virulence, such as motility and protease production [34].

The varied, strain-dependent level of virulence factors of twelve *P. mirabilis* strains is worth emphasizing. The complete structures of the unique O-polysaccharide part of *P. mirabilis* LPS were established by us previously [35-40]. Understanding of the relationship between the quorum-sensing, virulence factors of *P. mirabilis* and their unique O-antigen structures may lead to new targets for antibacterial drugs.

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