Influence of quorum sensing signal molecules on biofilm formation in *Proteus mirabilis* O18

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**Abstract** The influence of basis of quorum sensing molecules on *Proteus* strains is much less known as compared to *Pseudomonas* or *Escherichia*. We have previously shown that a series of acylated homoserine lactones (acyl-HSL) does not influence the ureolytic, proteolytic, or hemolytic abilities, and that the swarming motility of *Proteus mirabilis* rods is strain specific. The aim of the presented study was to find out if the presence of a series of acyl-HSL influences biofilm formation of *P. mirabilis* laboratory strain belonging to O18 serogroup. This serogroup is characterized by the presence of a unique non-carbohydrate component, namely phosphocholine. *Escherichia coli* and *P. mirabilis* O18 strains used in this work contains cloned plasmids encoding fluorescent protein genes with constitutive gene expression. In mixed biofilms in stationary and continuous flow conditions, *P. mirabilis* O18 overgrow whole culture. *P. mirabilis* O18 strain has genetically proved a presence of AI–2 quorum sensing system. Differences in biofilm structure were observed depending on the biofilm type and culture methods. From tested acylated homoserine lactones (BHL, HHL, OHL, DHL, dDHL, tDHL), a significant influence had BHL on thickness, structure, and the amount of exopolysaccharides produced by biofilms formed by *P. mirabilis* O18 pDsRed2.

**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 (Heydorn et al. 2000; Hryniewicz et al. 2001). 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 (Gupta et al. 2001; Stickler et al. 2003). *Proteus mirabilis* is one of the most common causes of UTIs in individuals with long-term indwelling catheters or complicated UTIs and of bacteremia among the elderly (Liaw et al. 2004; Sosa and Zunino 2009; Stickler et al. 2003). Bacterial virulence factors are regulated by quorum-sensing molecules which are derivatives of serine substituted by a fatty acid, i.e., acylated homoserine lactones, abbreviated as acyl-HSLs (Henke and Bassler 2004; Li et al. 2005; Lucas et al. 2000; Soto et al. 2002; Wang et al. 2004; Williams et al. 2000). The quorum sensing mechanism involves two types of autoinducers: AI–1 based on homoserine lactone and AI–2 based on other molecules. The majority of signal substances in Gram-negative bacteria are substituted by fatty acid derivatives of acyl-HSL (AI-1). There is no evidence that quorum sensing receptors and AI–1 signal molecules are associated with swarming motility in *P. mirabilis* (Belas et al. 1998). An essential enzyme for AI–2 type is the LuxS molecule...
featured by luxS gene, which has S-ribosylhomocysteine lyase activity. The product 4,5-dihydroxyptan-2,3-dione spontaneously cyclizes and combines with borate to form an AI-2 signal molecule (Schauder et al. 2001). Previous studies proved the presence of AI-2 type quorum sensing in P. mirabilis (Schneider et al. 2002). A luxS mutation in P. mirabilis showed no effect on growth, the production of urease, protease or hemolysin, swimming motility and behavior, swarmer cell differentiation, swarming behavior, and biofilm formation (Schneider et al. 2002). AI-2 quorum sensing is an important pathogenic factor present in species other than P. mirabilis. It is also widespread in the intestinal microflora of animals and humans (Schneider et al. 2002).

As of today, the complete sequences of two P. mirabilis strains, HI4320 and ATCC 29906, are known. Both strains contain the luxS gene encoding S-ribosylhomocysteine lyase, which takes part in the quorum sensing communication process. Also in another P. mirabilis strain, BB2000, RsbA membrane sensor proteins were found. They may play an important role in receiving density signals, similarly to the LuxQ protein in Vibrio harveyi (Schneider et al. 2002). The influence of basis of quorum sensing molecules on Proteus strains is much less known as compared to Pseudomonas or Escherichia. In our previous work, we showed that a series of HSL derivatives did not influence the ureolytic, proteolytic, or hemolytic abilities, and that the swarming motility of P. mirabilis rods was strain specific. P. mirabilis with a negatively charged O-polysaccharide demonstrated strong ureolytic and proteolytic properties and a greater migration speed on solid media. There was no influence of acyl-HSLs on the process of urea decomposition, but they inhibited protease activity in five P. mirabilis strains. N-butyl-N-l-homoserine lactone accelerated the migration speed of the tested P. mirabilis strains. Acetylated homoserine lactone derivatives modified the expression of only some virulence factors of P. mirabilis strains (Stankowska et al. 2008). In our previous study, we demonstrated that differences in the structure of the O-polysaccharide part of the LPS influences the biological activity of P. mirabilis strains (Chromek et al. 2005). P. mirabilis O18 was biologically more active than P. mirabilis O3 (S1959) LPS. P. mirabilis O18 LPS is characterized by a phosphocholine substituent in 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 (Chromek et al. 2005). Biofilm formation was not studied in our previous work. The aim of the presented study was to find out if the presence of a series of acyl-HSL would influence biofilm formation by P. mirabilis O18 and Escherichia coli laboratory strains. P. mirabilis strain belongs to O18 serogroup and is characterized by the presence of a unique non-carbohydrate component, namely phosphocholine.

### Materials and methods

#### Bacterial strains

P. mirabilis O18 laboratory strains PrK 34/57, O10 PrK 20/57 was obtained from the Czech National Collection of Type Cultures, P. mirabilis S1959 was obtained from the Institute of Microbiology and Immunology, University of Lodz, Poland, while the P. mirabilis 1785 (O18) clinical isolate was from the Swietokrzyskie Oncology Center in Kielce, Poland.

P. mirabilis O18 pDsRed2 and E. coli pCGJ strains

Marker genes such as gfp or dsRed coding fluorescent proteins (GFP, DsRed) are widely used for visualization bacterial cells (Wielbo et al. 2010). To obtain a P. mirabilis O18 strain expressing constitutively DsRed2 protein an ampicillin-sensitive P. mirabilis O18 was electroporated with a pDsRed2 prokaryotic expression vector that encodes DsRed2 containing pUC plasmid replication origin (Clontech). The transformants were screened on LB agar with ampicillin. Plasmids were isolated from randomly selected colonies. Isolated plasmids were checked on 1% agarose gel by electrophoresis with control pDsRed2 DNA. This strain was confirmed by appearance of red fluorescence. Ten passages of the obtained P. mirabilis O18 pDsRed2 strain showed stable plasmid replication. For the generation of the E. coli fluorescent strain expressing the GFP protein, the gfp gene was amplified by PCR using the primers with added restriction sites GFP-BamHI-F (5′-CGGGATCCCCAT GAGTAAAGGA GAAGAAC-3′) and GFP-EcoRI-R (5′-GGAATTCTATTGTATAGTTCATCC-3′) from pJFR8 plasmid. An ampiclon was eluted from agarose gel and ligated into pGEM-T (Promega) vector. E. coli TOP 10 (Invitrogen) was transformed with a ligation mixture. Transformants selection was performed on an LB medium supplemented with ampicillin, IPTG (isopropyl β-D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). A plasmid (named pGEM-GFP) was isolated from chosen transformants and was separated on 1% agarose gel where a plasmid DNA of pGEM-T was used as a control. The next step was a restriction analysis of obtained plasmid DNA with an EcoRI restriction enzyme. A selected clone (with proper lengths of restriction fragments) was digested with BamHI and EcoRI restriction enzymes. A 750-bp base pair (bp) fragment was eluted from gel and was ligated with the DNA of the pECFP vector previously digested with the same enzymes. E. coli TOP 10 was transformed with the ligation mixture. Transformant selection was performed on LB medium supplemented with ampicillin. A plasmid DNA was isolated from the selected transformants and was separated on 1% agarose.
gel where pGEM-GFP plasmid DNA was a control. The resulting plasmid was named pCG. The pCG plasmid DNA was isolated from the selected transformants and was separated by gel electrophoresis on 1% agarose gel, pJFR8 plasmid was used as a source of kanamycin-resistance cassette. A 2,116-bp ScaI–EcoRI fragment containing aph was PCR-amplified from E.coli-pJFR8 and was cloned into the ScaI–EcoRI sites of pCG to construct pCGJ. The E. coli strain showed stable plasmid replication and produced a GFP protein.

Plasmids used in work

- pJFR8 – modified pCR2.1TOPO vector (4.5 kilobase pair (kb)) coding GFP protein and kanamycin resistance gene. Obtained from the Institute of Medical Biology, Polish Academy of Sciences, Lodz, Poland.
- pECFP – 3.4 kb plasmid vector coding cyan variant of GFP tag and ampicillin resistance (Clonetech)

Acylated homoserine lactones

Acylated homoserine lactones (acyl-HSLs) used in this work are presented in Table 1. Acyl-HSLs were added to growing culture in a concentration of 10 nmol/L. Other concentrations of acyl-HSLs were examined in previous studies, and 10 nmol/L concentrations were selected as optimal for biofilm tests.

Identification of the quorum sensing marker genes

Identification of the luxS gene was performed by the amplification of DNA fragments and restriction analysis. Primers were designed with Primer-BLAST based on the genome sequence published for P. mirabilis HI4320. The sequence of the forward primer (luxsF) was 5′-GTATGTCTGACCTGCGGTA-3′ and that of the reverse primer (luxsR) was 5′-TTTGAGTTTGTCTTCTGGTAGTGC-3′. The length of the product was 464 bp with one restriction site for EcoRI. The template for PCR was chromosomal DNA isolated from P. mirabilis strains PrK 34/57 (O18) and PrK 20/57 (O10). PCR protocol contains steps: initial denaturation in 95°C for 3 min, 30× cycles; denaturation in 95°C for 90 s; annealing in 56.8°C for 60 s; and extending in 72°C for 60 s, the final extending step is done in 72°C which lasts for 10 min. PCR reaction was made to amplify a homolog of spnR and spnI genes from P. mirabilis strain PrK34/57 (O18), 1784 and S1959 genomic DNA by PCR using primers designed by Zhu et al. (2008). The sequence of primers for spnI gene was 5′-CTTGAACGTGTTGACGTCAGC-3′ and 5′-AGCGGCCAGGTAATAACTGA-3′ for the forward primer and reverse, respectively. And for the spnR gene, the expected product size was 627 and 744 bp for spnI and spnR, respectively.

In silico analysis

Calculations were performed with Vector NTI Suite 7 and the GenBank database.

Thin layer chromatography

Extraction of signal substances was performed with ethyl acetate both from supernatant and solid medium according to the method given by Shaw et al. (1997). Samples were separated on silica gel 60 F254 with acetone/methylene chloride (2:8, v/v) solvent or methanol/methylene chloride (1:9, v/v) for preparative thin layer chromatography (TLC) purpose. The identification of fractions was performed with UV light and dying with potassium permanganate. Desired spots were isolated by scraping from the TLC plate and extracted with subsequent washes of 10% methanol in H2O, dried, and re-suspended in ethyl acetate. Acyl-HSL mixture contained five selected lactones.

Biofilm formation

Biofilms were formed in 37°C in two different sets: in stationary conditions and in continuous flow chambers. Growth medium was artificial urine consisting of M9 minimal medium (Na2HPO4, 42.3 mmol/L; KH2PO4, 22 mmol/L; NH4Cl, 8.6 mmol/L; glucose, 12.2 mmol/L; casein hydrolysate, 0.005%; thiamine, 0.005%; MgCl2, 0.1 mmol/L; pH=8.0) supplemented with 0.01% urea. Biofilm formation process lasted 4 days. A continuous flow of the medium was performed at a speed of 0.2 mL/min on a peristaltic pump. Flow conditions assays were performed on FC 71-BST chambers (BioSurface Technologies Corp.). Biofilm

| Acronyms | Full name | R          |
|----------|-----------|------------|
| BHL      | N-butanoyl homoserine lactone | CH3(CH)2  |
| HHL      | N-hexanoyl homoserine lactone  | CH3(CH)4  |
| OHL      | N-octanoyl homoserine lactone  | CH3(CH)6  |
| DHL      | N-decanoyl homoserine lactone  | CH3(CH)8  |
| dDHL     | N-dodecanoyl homoserine lactone | CH3(CH)10 |
| tDHL     | N-tetradecanoyl homoserine lactone | CH3(CH)12 |
presence and parameters were calculated with the COM-STAT software by Heydorn et al. (2000). Statistical analyses were performed on a SigmaStat software (SPSS, Inc., IL). The features of the biofilms were analyzed by Tukey’s multiple comparisons test. A determination of the amount of exopolysaccharides in the biofilm was carried out in accordance with the method of Hamilton et al. (2003).

Results

In silico analysis of quorum sensing encoding molecules

In silico analysis with blast algorithms proved that there is no sequence similar to the luxP V. fischeri ES114 sequence in two sequenced P. mirabilis HI4320 and ATCC 29906. Also, the peptide sequence of LuxP has no analogs in the P. mirabilis databases. Similar results were obtained for the luxCDABE operon from Photobacterium luminescens subsp. laevis strain TT01 with P. mirabilis completed genomes showed no significant homology. A similar situation holds for two LuxI-type proteins EsaI and LasI which catalyze the synthesis of 3-oxo-C6-homoserine lactone (3OC6-HL) and 3-oxo-C12-homoserine lactone (3OC12-HL) (Nasser and Reverchon 2007). A comparison of the P. mirabilis HI4320 luxS sequence with the Megablast algorithm to the genomes of other bacteria revealed a high similarity to the Shewanella luxS sequence. Other genetic elements involved in the AI-1 quorum sensing mechanism were not found in the published genome sequences of P. mirabilis.

Identification of the luxS gene

The PCR reaction resulted in a 464-bp amplicon, which corresponds to the P. mirabilis HI4320 luxS gene. The cleavage of the amplified fragment resulted in 121- and 343-bp products and confirmed that the amplified products are luxS gene fragments (Fig. 1). Amplification of spnI and spnR genes did not succeed (data not shown).

Identification of signal substances in P. mirabilis strains

We attempted to identify acyl-HSL derivatives in P. mirabilis cultures. Twelve of the P. mirabilis strains used differ in their O-antigens structures. TLC was applied to investigate if acyl-HSLs were present in cell-free supernatants from P. mirabilis cultures. The TLC separation of acylated homoserine lactones (BHL, HHL, DHL, OHL, dDHL) from the mixture was successful. The separation was performed based on the differences in the chemical structure and polarity of the lactones. Extracts obtained with ethylene acetate were characterized with the presence of substances whose migration Rf was identical to that of the HHL control sample (Rf=0.46). To confirm that the obtained isolate from P. mirabilis O18 is the homoserine lactone derivative, the corresponding spot was extracted and analyzed by the GC-MS method. Retention times were 27.6 and 28.9 s—the same as in a parallel run of the HHL standard (data not shown). An analysis of the ion fragmentation revealed typically of a homoserine lactone ion 102.11 m/e similar to that observed by Shaw et al. (1997).

The influence of acyl-HSL derivatives on biofilm formation by P. mirabilis O18

Biofilm formation was assessed in the laboratory P. mirabilis strain expressing constitutively DsRed2 protein (P. mirabilis O18 pDsRed2). Biofilms formed by the laboratory strain P. mirabilis O18 pDsRed2 differ in structure depending on the type of culture method used for the biofilm formation. Bacterial cells grown in chambers without constant medium flow produced biofilms with a small amount of extracellular mucus. In contrast, cultures in chambers with continuous flow of artificial urine produced biofilms with significant amounts of extracellular mucus. To quantify biofilm composition, calculations were done with the aid of
P. mirabilis
cally different compared to single strain biofilms formed by
the control was also observed. The amount of polysacchar-
des in the biofilm by the P. mirabilis O18 pDsRed2 strain treated
with acyl-HSL, the following measurements were performed
every 12 h up to 96 h. Figure 2 shows graphs which present changes in biofilm thickness after 96 h of growth. The biofilm treated with BHL is characterized by very fast growth up to 24 h, after which fragments of the biofilm were dissociating BHL-treated biofilm, the thickest at every mea-
surement point and had an average thickness of 13.73±
3.58 μm. After 24 h, the BHL biofilm biomass was 34 times higher than that of the control. Differences observed in the
surface covered by biofilms were substantial after BHL
treatment biofilm covered an area that was six times greater
than that in the control. The volume of BHL-treated biofilm
was 5.3 times greater, too. However, differences after 96 h
were not so significant. Figure 3 shows that the control
biofilm was not aggregated compared to biofilms formed
under the influence of lactones, and especially BHL, DHL,
and tDHL. In the presence of acyl-HSLs, high aggregation
and increased amounts of mucus were noticed. Bacterial
biofilms contain extracellular polymeric polysaccharides
produced by bacterial cells grown in biofilm according to
the method of Hamilton et al. (2003). The highest amount of
polysaccharides was found in the biofilm produced after
treatment with BHL (p<0.002) and DHL (p<0.0033),
whereas treatment with OHL decreased the amount of exo-
polysaccharides in the biofilm.

Discussion

Biofilm is the most common mode of bacterial existence in
the environment (Costerton et al. 1999). Biofilms forming
on medical devices remain an unresolved medical problem
(Maczynska et al. 2010). It is well-known that in Gram-
negative bacteria signal molecules (quorum sensing) are an
important factor in biofilm formation and development
(Viana et al. 2009). The most common molecules involved
in quorum sensing are acylated homoserine lactones (acyl-
HSL). Their activity may influence many bacterial function,
including biofilm formation. Proteus strains are known uro-
pathogens capable of biofilm formation in catheters
(Broomfield et al. 2009). In order to find out if Proteus
strains are sensitive to a series of homoserine lactones, its
effect on biofilms was tested. The influence of acylated
homoserine lactones on mass, exopolysaccharide produc-
tion, and structure of biofilms formed by P. mirabilis O18
and E. coli pCGJ strains was observed. Statistics showed
that biofilms treated with BHL produced a higher amount of
exopolysaccharides compared to controls. Biofilm growth on
supplemented with BHL medium characterizes fast
growing and sloughing after 36 h (Fig. 2b). Similar results
gained by Rice and colleagues on their studies on Serratia
*marcescens* suggest that biofilm formation is a dynamic process that is controlled by the quorum-sensing system (Rice et al. 2005). We observed significant differences between the amount of exopolysaccharides in biofilms formed by *P. mirabilis* O18 pDsRed and in mixed biofilms (P. mirabilis O18 pDsRed2, E. coli pCGJ) after treatment with BHL (p<0.05, paired t test) and tDHL (p<0.05, paired t test). From tested acylated homoserine lactones (BHL, HHL, OHL, DHL, dDHL, tDHL), BHL had a significant influence on the thickness, structure, and the amount of

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**Fig. 2** The dynamics of biofilm formation for *P. mirabilis* O18 pDsRed under continuous flow conditions treated with 10 nm acyl-HSL. a Control, b BHL, c HHL, d OHL, e DHL, f dDHL, g tDHL. Error bars show standard deviation.
exopolysaccharides produced by biofilms formed by *P. mirabilis* O18 pDsRed2. Based on confocal microscopy observations, one may propose that acyl-HSLs influence biofilm formation and its structure. Under the influence of acyl-HSLs, the *E. coli* pCGJ biofilm significantly produced lower amounts of exopolysaccharides than the biofilm formed by *P. mirabilis* O18 pDsRed2 or mixed biofilm (*P. mirabilis* O18 pDsRed2, *E. coli* pCGJ). The different responses to different acyl-HSLs molecules by the two enteric strains may suggest that acyl-HSLs receptor protein molecules as well as promoter regions linked to acyl-HSLs differ, which may affect biofilm formation and development. In the presented work, we have found that *Proteus* strains can produce homoserine lactone rings, similarly to other Gram-negative bacteria. Furthermore, by genetic analysis we have shown the presence of the gene responsible for the production of quorum-sensing molecules. The identification of the *luxS* gene coding S-ribosylhomocysteine lyase responsible for AI-2 synthesis proves that *P. mirabilis* uses a quorum-sensing communication system. Although *luxS* mutation does not disturb swarming motility (Schneider et al. 2002), it is possible that quorum sensing can interfere in the biofilm formation process as in the work of Viana et al. (2009). However, we have not identified types of acyl-HSLs produced by one *Proteus* strain tested. The presence and effects of acyl-HSLs are yet to be clarified. It is well-known that in enteric bacteria two quorum-sensing molecule systems are present (Kendall and Sperandio 2007). A system involving the AI-1 molecule base on acyl-HSL derivatives and engages *luxIR* genes. In our genetic studies, the presence of these genes in *P. mirabilis* O18 failed. However, we have shown that tested strain *P. mirabilis* O18 is sensitive to the examined BHL. That may suggest the presence of surface receptor proteins bindings BHL and changing the features of biofilm in stationary and continuous cultures. In the presented work, we identify gene *luxS* coding AI-2 molecules synthase. The existence of this system in *P. mirabilis* O18 using molecules derivative of furanones was described by others (Schauder et al. 2001). In conclusion, the *P. mirabilis* O18 strain reacted and changed the population features based on the acyl-HSL produced by other Gram-negative bacteria as well as AI-2 molecules. The correlation and dependence of AI-1 and AI-2 systems remains to be discovered.
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