Crystallization of urine mineral components may depend on the chemical nature of *Proteus* endotoxin polysaccharides

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Formation of infectious urinary calculi is the most common complication accompanying urinary tract infections by members of the genus *Proteus*. The major factor involved in stone formation is the urease produced by these bacteria, which causes local supersaturation and crystallization of magnesium and calcium phosphates as carbonate apatite [$\text{Ca}_{10}(\text{PO}_4)_6 \cdot \text{CO}_3$] and struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$), respectively. This effect may also be enhanced by bacterial polysaccharides. Macromolecules of such kind contain negatively charged residues that are able to bind Ca^{2+} and Mg^{2+} , leading to the accumulation of these ions around bacterial cells and acceleration of the crystallization process. The levels of Ca^{2+} and Mg^{2+} ions bound by whole *Proteus* cells were measured, as well as the chemical nature of isolated LPS polysaccharides, and the intensity of the *in vitro* crystallization process was compared in a synthetic urine. The results suggest that the sugar composition of *Proteus* LPS may either enhance or inhibit the crystallization of struvite and apatite, depending on its chemical structure and ability to bind cations. This points to the increased importance of endotoxin in urinary tract infections.

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INTRODUCTION

Proteus species are motile, Gram-negative bacteria within the *Enterobacteriaceae* that cause urinary tract infections, primarily in patients with long-term urinary catheters in place or structural abnormalities of the urinary tract (Warren, 1996). *Proteus* infections are known to be frequently persistent and difficult to treat and can lead to several complications such as acute or chronic pyelonephritis. Additionally, *Proteus* species are the most common bacilli associated with the formation of bacteria-induced bladder and kidney stones (about 70 % of all bacteria isolated from such urinary calculi) (Lerner *et al.*, 1989; Rodman, 1999; Coker *et al.*, 2000). Urease is the essential virulence factor of these bacteria involved in stone formation. Ammonia, produced by the enzymic hydrolysis of urea, elevates urine pH, causing supersaturation and crystallization of magnesium and calcium ions as struvite ($\text{MgNH}_4\text{PO}_4 \cdot 6\text{H}_2\text{O}$) and carbonate apatite [$\text{Ca}_{10}(\text{PO}_4)_6 \cdot \text{CO}_3$], respectively (Griffith *et al.*, 1976; Gleeson & Griffith, 1993; Kramer *et al.*, 2000).

It has been found that, in addition to urease activity, bacterial exopolysaccharides contribute to stone formation. Polysaccharide produced by bacteria may aggregate precipitated urine components to form a stone (McLean *et al.*, 1989). *Proteus* bacilli have capsular polysaccharide (CPS) and lipopolysaccharide (LPS, endotoxin) on their surfaces. CPS is the most external surface component of these bacteria, but detailed studies have shown that only a few strains can

synthesize a capsule antigen, and its structure is identical to the O-specific chain of their LPS (Beynon *et al.*, 1992; Perry *et al.*, 1994; Różalski *et al.*, 1997). LPS is the main component of the outer membrane and one of the major virulence factors of these bacteria. It consists of a polysaccharide part, containing an O-specific chain (O-antigen, O-PS), and a core region, as well as a lipophilic region, termed lipid A, which anchors the LPS to the bacterial outer membrane. It has been well documented that *Proteus* is an antigenically heterogeneous genus, principally because of structural differences in the O-specific polysaccharide chain of LPS. In most *Proteus* strains, O-specific polysaccharides have been found to be acidic due to the presence of uronic acids and various non-carbohydrate acidic components, including phosphate groups (Knirel *et al.*, 1993; Różalski *et al.*, 1997).

The role of acidic polysaccharides in the pathogenicity of *Proteus*, especially in urinary tract infections, is controversial. The negatively charged polysaccharides are important barriers against the bactericidal action of the complement system (Kaca *et al.*, 2000). They also play an important role in the migration of swarm cells by the reduction of surface friction (Gygi *et al.*, 1995). The acidic character of *Proteus* extracellular polysaccharides may play a crucial role in stone formation within the urinary tract. Clapham *et al.* (1990) had previously hypothesized that anionic groups found on bacterial polysaccharides influence struvite and carbonate apatite formation because they enable these macromolecules

to bind cations (Ca^{2+} , Mg^{2+}) via electrostatic interactions that accelerate supersaturation and crystallization of these ions. Dumanski *et al.* (1994) speculated that the structure and anionic character of *Proteus mirabilis* O6 CPS enhances struvite formation by weakly concentrating Mg^{2+} ions during struvite crystal formation. As mentioned before, O-specific polysaccharides also reveal a partially anionic character and, unlike CPS, they are located on the surface of each *Proteus* strain. Hence, the goal of this study was to establish the role of these polysaccharides using crystallization experiments *in vitro* in the presence of *Proteus* strains or their acidic polysaccharides.

METHODS

Bacterial strains and growth conditions. *P. mirabilis* and *Proteus vulgaris* strains came from the Czech National Collection of Type Cultures (Institute of Epidemiology and Microbiology, Prague, Czech Republic). *Proteus penneri* 25 was from the Department of General Microbiology, University of Łódź. Before the experiment, bacteria were maintained on a slant of tryptic soy agar overnight at 37 °C and then suspended in synthetic urine.

Synthetic urine. The synthetic urine was made using a modified version of the method previously described by Griffith *et al.* (1976) and was formulated to contain components always present in normal urine, each at a concentration equivalent to the mean concentration found in a 24 h period in the urine of normal human males. It contained the following components (g l⁻¹): $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.651; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.651; NaCl, 4.6; Na_2SO_4 , 2.3; KH_2PO_4 , 2.8; KCl, 1.6; NH_4Cl , 1.0; urea, 25.0; creatine, 1.1; and tryptic soy broth, 10.0. Immediately prior to experiments, the pH was adjusted to 5.8 and the urine was passed through a 0.45 µm pore-size filter.

Isolation of polysaccharide from LPS. Bacteria were cultivated aerobically in nutrient broth (BTL) supplemented with 1 % glucose. LPS was isolated from dried bacterial cells by phenol/water extraction and purified by treatment with DNase and RNase as described previously (Bartodziejska *et al.*, 1998). Polysaccharide was obtained after delipidation of LPS using aqueous 1 % acetic acid at 100 °C until lipid A was precipitated. The precipitate was removed by centrifugation (13 000 g, 20 min) and the supernatant was freeze-dried to give a polysaccharide fraction.

Urease-negative mutants. Bacterial cultures were washed to remove the medium and suspended in maleic buffer (44 mM maleic acid, 50 mM Tris base; pH 6.0). Chemical mutagenesis was performed using 2.5 mg MNNG (*N*-methyl-*N'*-nitro-*N*-nitrozoguanidine; Sigma) ml⁻¹ for a period of time that allowed 1 % survival of the culture. The survivors were plated on LB agar (1⁻¹: tryptone, 10 g; yeast extract, 5 g; NaCl, 10 g; agar, 15 g) and, later, single colonies were used to inoculate 96-well titration plates containing urease test broth (BBL, Becton Dickinson) covered with mineral oil. Cultures that were not able to hydrolyse urea (lack of pink colour) were used for further analysis.

Urease activity. The urease activity of whole bacterial cells was analysed using the phenol/hypochlorite/ammonia assay of Weatherburn (1967). Urease activity was expressed as µg NH₃ produced min⁻¹ (mg total bacterial protein)⁻¹. The amount of protein was measured by the method of Lowry *et al.* (1951).

Crystallization experiments. The experimental protocol used for *in vitro* production of crystals was described previously (Clapham *et al.*, 1990). Briefly, this involved placing a sample (1 × 10⁶ c.f.u. bacteria or

1 mg polysaccharide ml⁻¹) in a dialysis sac (12 000–14 000 molecular mass; Serva) in a culture flask containing 800 ml synthetic urine. Sterile synthetic urine was added continuously to the flask at the rate of 60 ml h⁻¹, i.e. urine production rate of human males. In experiments with polysaccharide and urease-negative mutants, the pH was elevated to 8.5 (to mimic urease activity) by the addition of 1 M NH₄OH to induce crystallization. Experiments were performed for 5 h at 37 °C.

Crystal analysis. Analysis consisted of direct examination by phase-contrast microscopy and determination of the chemical composition of crystals. Before analysis, crystals were washed twice by centrifugation in 0.05 M Tris/HCl, pH 8.6. Phosphate concentration was determined by the colorimetric method (Ames & Dubin, 1960) and atomic absorption spectroscopy (SpectrAA-300; Varian) was used to analyse calcium and magnesium concentrations.

Coulter multisizer analysis. Following the production of crystals in the presence of polysaccharide, samples were suspended in Isoton (an electrolyte solution; Beckman Coulter) and analysed for particle number and size using a Coulter counter (Beckman). The multisizer was calibrated using standard calibration particles measuring 6 µm in diameter (Molecular Probes). Crystals from 3 to 64 µm could be detected.

Metal binding. Whole bacterial cells or polysaccharides were suspended in synthetic urine and incubated at room temperature for 1 h (to allow binding of cations). Samples were then placed in dialysis tubing and unbound metals were removed through dialysis against water. Bound calcium and magnesium ions were analysed by atomic absorption spectroscopy.

Statistical analysis. Analysis was based on Student's *t*-test. Statistical differences between groups are indicated in the text.

RESULTS AND DISCUSSION

Metal binding by *Proteus* bacilli and their polysaccharides

The aim of this work was to show that negatively charged polysaccharide, being a part of the *Proteus* LPS, may bind the cations present in urine. Such binding leads to the accumulation of cations around bacterial cells and increases the crystallization rate and formation of urinary tract stones. Of 49 *Proteus* strains for which the chemical structure of the O-PS has been established, those containing acidic residues such as phosphates, uronic acid or their amides with amino acids were chosen (Fig. 1). In the first stage of this study, the effect of differences in the chemical structure of the polysaccharide component of LPS on the ability of strains to bind Ca^{2+} and Mg^{2+} cations from synthetic urine was analysed. As shown in Table 1, all bacterial cells tested and their polysaccharides had the ability to bind Ca^{2+} and Mg^{2+} from synthetic urine. The concentration of calcium ions bound by extracellular components of whole *Proteus* cells was about twofold higher than the concentration of magnesium ions bound in each sample. All polysaccharides tested bound Ca^{2+} and Mg^{2+} ions at comparable levels, except that from *P. vulgaris* O12, which bound significantly smaller amounts of these cations ($P < 0.05$). Comparison of the amounts of Ca^{2+} and Mg^{2+} bound in the experiment revealed no significant differences between bacteria and their polysac-

Fig. 1. Chemical structures of *Proteus* sp. O-PS. Gro-1-P, Glycerol phosphate. Data from Radziejewska-Lebrecht *et al.* (1995), Arbatsky *et al.* (1997) and Róžalski *et al.* (2002).

Table 1. Metal binding by bacteria and polysaccharide in synthetic urine

Values represent means $\pm$ SD of 8–10 experiments. Each polysaccharide represents the polysaccharide part of LPS, as outlined in Methods.

Sample	Cations bound ($\mu\text{g ml}^{-1}$)	
	Ca ²⁺	Mg ²⁺
Whole cells		
<i>P. vulgaris</i> O12	1.06 $\pm$ 0.90	0.68 $\pm$ 0.49
<i>P. vulgaris</i> O47	7.64 $\pm$ 2.25	2.02 $\pm$ 0.74
<i>P. mirabilis</i> O28	2.23 $\pm$ 1.01	1.63 $\pm$ 0.64
<i>P. mirabilis</i> O6	2.48 $\pm$ 0.54	1.50 $\pm$ 0.48
<i>P. vulgaris</i> O15	2.25 $\pm$ 0.78	1.50 $\pm$ 0.38
<i>P. penneri</i> 25	2.65 $\pm$ 0.10	1.66 $\pm$ 0.46
Polysaccharides		
<i>P. vulgaris</i> O12	1.40 $\pm$ 0.80	0.42 $\pm$ 0.20
<i>P. vulgaris</i> O47	2.65 $\pm$ 0.75	1.56 $\pm$ 0.61
<i>P. mirabilis</i> O28	2.37 $\pm$ 0.75	1.50 $\pm$ 0.34
<i>P. mirabilis</i> O6	2.25 $\pm$ 0.43	1.60 $\pm$ 0.52
<i>P. vulgaris</i> O15	2.36 $\pm$ 0.65	1.23 $\pm$ 0.42
<i>P. penneri</i> 25	2.65 $\pm$ 0.51	1.10 $\pm$ 0.28

charides, which confirms that polysaccharide, not other surface structures, is responsible for cation binding. The only exception was observed in the case of *P. vulgaris* O47 cells, which bound more Ca²⁺ ions than other bacteria or

their polysaccharides ($P < 0.001$). This is probably because of the presence of other surface structures with a higher affinity for calcium cations, for example CPS. According to present knowledge, the structure of *Proteus* CPS is identical to that of its O-PS counterpart, although the polysaccharide of the capsule is arranged more loosely on the cell surface. Such organization may allow more negatively charged residues to be available for cation binding. Moreover, cations may penetrate the loose capsule structures much more easily than in the case of O-PS (McLean *et al.*, 1990, 1996).

In vitro crystallization experiments in the presence of *Proteus* cells and their polysaccharides

Crystallization of urine mineral components was tested using *P. vulgaris* strains O47 and O12 and *P. mirabilis* O28, because their LPS O-specific parts revealed the biggest differences in chemical structure (Fig. 1). As described above, these strains also differed in the pattern of Ca²⁺ and Mg²⁺ binding. Moreover, as shown in Table 2, *P. vulgaris* O12 urease had the lowest activity, while *P. vulgaris* O47 revealed the highest urease activity ($P < 0.05$). In order to perform crystallization experiments under conditions that resembled the physiological situation in the urinary tract, a dialysis sac containing an inoculum of each strain [1×10^6 c.f.u. (ml synthetic urine)⁻¹] was placed in a flask with a continuous flow of synthetic urine. After 5 h incubation at 37 °C, an increase in pH up to 8.5–9.0 was observed in each dialysis sac and crystallization occurred. Microscopic examination revealed a large number of amorphous materials and crystals of characteristic morphology in all samples except the control without bacteria (Fig. 2). Additionally, it was observed that, in the sample containing *P. vulgaris* O12 cells, there were fewer crystals of defined morphology than amorphous precipitate, probably because of the low urease activity of this strain. Chemical analysis of these crystals indicated the presence of calcium, magnesium and phosphate ions, which might suggest that struvite and apatite had been formed (Table 2). There were some differences in the number of ions in crystals formed by particular strains, but they were not statistically significant ($P < 0.05$).

The low level of variation observed in the results described may be explained by the presence of several factors that influence the formation of urinary calculi and by the dominant role of urease, which may overcome the effect of factors such as polysaccharide. Moreover, the strains chosen for the experiments revealed differences in urease activity, which makes interpretation of the results even more difficult. Thus, we decided to obtain urease-negative counterparts of the tested *Proteus* strains and to use them for crystallization experiments. *Proteus* strains were mutagenized using MNNG as described in Methods. Urease-negative mutants were later analysed phenotypically in order to eliminate those in which other phenotypes had been affected. Only the colonies that passed the API test, displayed a similar swarming pattern and similar reactivity with homologous serum using Western

Fig. 2. Morphology of crystals formed in the presence of *Proteus* bacilli (a) and control without bacteria (b). Bar, 10 μm .

blot and ELISA and revealed an identical O-PS chemical structure (data not shown) were used for crystallization experiments.

As expected, in contrast to the wild-type strains, urease-negative mutants growing in the synthetic urine under the

same experimental conditions were not able to increase the pH inside the dialysis sac or to induce crystal production (data not shown). Therefore, the pH of the synthetic urine in the flask was elevated to 8.5 by titration with 1 M NH_4OH to stimulate crystallization. In this case, crystallization occurred in all tested samples including the control without bacteria. As seen in Fig. 3, the crystals produced in this experiment had more irregular shapes than the crystals that appeared as a result of urease activity. It was also observed that crystals formed in the presence of *P. vulgaris* O47 ure^- were significantly bigger than those formed in the presence of other tested strains. These crystals, as with those formed in the presence of urease, consisted of magnesium ammonium and calcium phosphate, since calcium, magnesium and phosphate ions were present in all samples tested. The intensity of crystallization expressed as a concentration of these ions per ml of sample was highest for *P. vulgaris* O12 ure^- and significantly lower ($P < 0.05$) for *P. vulgaris* O47 ure^- . It is worth mentioning that crystallization in the presence of *P. vulgaris* O12 ure^- was intense compared with the control, while only a few crystals were present in the samples containing *P. mirabilis* O28 ure^- and, especially, *P. vulgaris* O47 ure^- .

It was observed that there were some differences in crystal morphology and intensity of crystallization between the samples containing urease-negative strains, which might depend on the chemical character and structure of polysaccharide components of *Proteus* cell wall. To confirm this hypothesis, in our last experiment, crystal production was examined in the presence of polysaccharides isolated from previously tested *Proteus* strains. Consequently, the polysaccharide in the dialysis sac was incubated in a flask with urine, titrated by 1 M NH_4OH . The sample was then measured using the Coulter counter technique, which enabled differences in the number and size of crystals formed in the presence of the polysaccharides to be visualized. As shown in Fig. 4, the major population of particles detected was in the range of 3–15 μm in diameter. Crystals detected in samples containing *P. vulgaris* O12 polysaccharide and those present

Table 2. Urease activity and intensity of crystallization in the presence of urease-positive and -negative strains

Values represent means $\pm$ SD of 8–10 experiments. Urease activities are listed as $\mu\text{g NH}_3$ produced min^{-1} ($\text{mg total bacterial protein}^{-1}$).

Strain	Concentration in crystals ($\mu\text{g ml}^{-1}$)			Urease activity
	Ca^{2+}	Mg^{2+}	PO_4^{3-}	
<i>P. vulgaris</i> O47	252.45 $\pm$ 32.78	88.06 $\pm$ 20.61	389.62 $\pm$ 59.20	127.00 $\pm$ 8.60
<i>P. vulgaris</i> O12	264.10 $\pm$ 48.50	50.34 $\pm$ 17.20	446.86 $\pm$ 79.34	52.06 $\pm$ 8.95
<i>P. mirabilis</i> O28	276.83 $\pm$ 53.71	66.00 $\pm$ 15.80	429.60 $\pm$ 62.98	106.23 $\pm$ 4.16
<i>P. vulgaris</i> O47 ure^-	22.05 $\pm$ 2.81	3.48 $\pm$ 0.99	35.25 $\pm$ 5.02	2.45 $\pm$ 1.80
<i>P. vulgaris</i> O12 ure^-	29.08 $\pm$ 3.40	5.82 $\pm$ 1.21	69.03 $\pm$ 9.42	2.91 $\pm$ 2.22
<i>P. mirabilis</i> O28 ure^-	29.79 $\pm$ 5.04	4.92 $\pm$ 1.38	43.83 $\pm$ 9.54	1.63 $\pm$ 1.41
Control	36.10 $\pm$ 3.61	5.80 $\pm$ 0.17	51.8 $\pm$ 2.26	–

Fig. 3. Crystal growth in the presence of urease-negative mutants *P. vulgaris* O47 ure⁻ (a), *P. vulgaris* O12 ure⁻ (b), *P. mirabilis* O28 ure⁻ (c) and control (no bacteria added) (d). Bar, 10 μm.

Fig. 4. Coulter counter analysis of crystal parameters. Crystals were induced by polysaccharides from *P. vulgaris* O47 (a), *P. vulgaris* O12 (b) and *P. mirabilis* O28 (c) in the presence of NH₄OH. (d) Control (no polysaccharide in the presence of NH₄OH).

in the control (synthetic urine without polysaccharide) were significantly larger than crystals produced in the presence of polysaccharide from *P. mirabilis* O28 and *P. vulgaris* O47. The total number of crystals (means $\pm$ SD of five independent experiments) formed in the presence of particular polysaccharides was compared. Only *P. vulgaris* O12 polysaccharide was able to stimulate the formation of larger numbers of crystals ($24\,966 \pm 14\,471$ crystal particles ml⁻¹) in comparison with the control, in which $19\,045 \pm 7807$ crystals ml⁻¹ were detected. For the samples containing *P. mirabilis* O28 and *P. vulgaris* O47 polysaccharide (respec-

tively 5982 ± 3330 and 2013 ± 1139 crystals ml⁻¹), there were significantly fewer crystals than in the control sample ($P < 0.05$). These results correspond with those obtained for the urease-negative mutants (Table 2), showing the relationship between Ca²⁺ and Mg²⁺ cation-binding ability and the intensity of crystallization in the presence of bacterial polysaccharides.

Our results show that polysaccharide of *P. vulgaris* O12 bound magnesium and calcium ions weakly but increased the crystallization rate, whereas *P. mirabilis* O28 and *P.*

vulgaris O47 cells were able to bind large amounts of the cations but inhibited the process of crystallization *in vitro*. A similar relationship was observed by Dumanski *et al.* (1994), in a study in which the crystallization induced by bacterial capsules of different chemical character was analysed. Only CPS of *P. mirabilis* O6, which had the lowest affinity for magnesium ions, enhanced the crystallization process. The authors suggested that this polysaccharide bound magnesium in a weak manner, and the cations could therefore be released easily. This phenomenon causes local supersaturation of the solution and leads to increased rates of crystallization.

Our studies allowed us to determine the role of *Proteus* LPS polysaccharides in crystallization of urine mineral compounds. We conclude that the polysaccharide part of *Proteus* LPS may either enhance or inhibit the process of crystal formation, depending on the chemical composition of the molecule and its affinity for cations. One has to bear in mind that, in the host, bacterial endotoxin is present not only as a component of glycocalyx in biofilms, but also as free molecules originating from dead cells or released from urinary stones during their surgical removal, which causes serious health problems (Boelke *et al.*, 2001; McAleer *et al.*, 2002). Hence, it is possible that the same polysaccharide, through its cation affinity, may increase or inhibit the process of crystallization, depending on its location. Free endotoxin molecules with a high affinity for cations may act as crystallization inhibitors, since cations bound to such macromolecules would be washed out by the flow of urine. Conversely, in the biofilm, local accumulation of ions by anchored endotoxin would lead to stimulation of the crystallization process.

Urinary calculi formed as a result of bacterial infections constitute only 10–15 % of all stones appearing within the urinary tract, but they pose a serious health problem. In 50 % of cases, this is recurrent illness, which can lead to the loss of kidneys or even death if not properly treated (McLean *et al.*, 1988; Bihl & Meyers, 2001). The development of infectious urolithiasis may be caused by several factors and, despite long-term clinical and experimental investigation, some of the specific mechanisms responsible for urinary calculi formation remain a mystery. The results of our investigation are introductory in our understanding of the role of polysaccharides in the formation of urinary tract stones but, despite this, even at this stage, they show a new and interesting aspect of LPS sugar compound activity in this process.

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