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IN MEMORIAM

Prof. dr med. Janusz Jeljaszewicz (1930–2001)
at the 5th anniversary of his death

Professor Janusz Jeljaszewicz was born in Wilno, Poland, in 1930, as the son of a family of Polish Tatars with strong patriotic traditions. He has graduated from pharmaceutical studies in Poznań, 1954. He worked at the Medical Microbiology of the Medical School in Poznań, receiving his PhD degree in 1959. In 1963 he begun to work in the Microbiology Department of the National Institute of Hygiene in Warsaw. This became the centre of his working life, and it was here that he gained the title of Docent, and subsequently Professor. He was Chief of the Laboratory, Departmental Head, Deputy Scientific Director, and finally Director from 1996 to 2000.

During his years in Warsaw he went overseas to study and work, frequently. He was awarded a Fellowship at the National Institute for Medical Research in London (1959–1960). Later, he worked at the Centre for Disease Control, Atlanta (1965–1966), and studied at the University of Cologne (1971–1972). These stays resulted in numerous contacts and fruitful cooperation. A particularly long-term cooperation was established with the Centre at the University of Cologne, headed by Professor G. Pulverer, with whom he also had a lasting friendship.

The Professor’s scientific activities were prolific – he had almost 500 publications in important Polish and foreign journals. Amongst these were Nature, J. Bacteriology, J. Clin. Microbiology, Zentralblatt Bakteriologie, J. Infect. Dis., and many books. The list of his publications can be found in the Main Medical Library and in the National Institute of Hygiene. The books edited by him, on “Staphylococci and Staphylococcal Infections” and others, showed his great theoretical knowledge, but also summarised his scientific research with internationally-renowned groups.

His talents included also organising scientific circles of common interests, some with a substantial reputation. He took the initiative in supporting a number of grants in Poland and abroad from the Polish Committee for Scientific Research. As an example, studies on the biological properties of staphylococci, financed by the Center for Disease Control in the USA. These studies have been described in 170 publications.

There were several Polish centres with which the Professor actively cooperated. He also undertook educational activities – besides many lectures in his own country, he gave about 200 abroad, in the UK, Germany, France, Sweden, USA, and other countries. He took part in over 100 congresses, mostly international, and was active as the organiser, or as chairman of a section. He had the ability to share his knowledge with others, and enjoyed doing so; eg. he was a visiting professor at the Department of Medicine in the University of Cologne from 1971 to 1972.
The main subjects of his scientific research were staphylococci: toxins and enzymes, eg. investigation of the coagulation cascade and fibrinolytic systems during staphyloccocal infections. His name obviously features in the list of 138 Polish biomedical scientists most frequently quoted in the scientific bibliography (2003). He was quoted 1734 times out of 245 of his publications.

Professor Janusz Jeljaszewicz was a very social person, active in scientific circles. One of his most significant tasks was to chair the Coordinating Group for the Polish-American Scientific Commission. He was also an adviser and expert for the World Health Organisation in the field of bacterial infections. Finally, in Poland he was Vice-Chairman of the Central Commission for Scientific Degrees and Titles. Professor was a corresponding member of the Polish Academy of Sciences, and a member of the Polish Academy of Arts and Sciences.

His interest in the problems of health promotion made him an excellent populariser of science. The “Diagnosis” series, with his participation, of TV and Polish Radio 2 programmes, were very well-known. This aspect of his social activity was valuable to many scientists. The Commission for Polish-American Co-operation allowed Polish scientific workers to obtain US fellowships. Again, this created a valuable opportunity for scientific development for those persons in difficult times. In this way the Professor became a creator of personal careers. Thus, he contributed to the rising level of Polish science.

“He was a man full of charm, and many people liked him a lot”, wrote Professor Danuta Naruszewicz-Lesiuk, a colleague from the Epidemiological Review.

The Professor showed himself to the world as a high-class scientist. In this way, he was also showing Poland, as a place where such a man can be born, live, and be an equal partner in the effort to advance science and to improve the world.

Prof. dr hab. pharm. Wanda Parnowska
Professor Emeritus, former Head
of Department of Pharmaceutical Microbiology,
Institute of Drugs, Warsaw, Poland

Translated by Prof. dr med. Kazimierz Madalinski, National Institute of Hygiene, Warsaw; with language verification by Dr John Anderson.
Bactericidal Activity of Normal Bovine Serum (NBS) Directed against Some Enterobacteriaceae with Sialic Acid-containing Lipopolysaccharides (LPS) as a Component of Cell Wall

GABRIELA BUGLA-PŁOSKONSKA* and WŁODZIMIERZ DOROSZKIEWICZ

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Received 28 April 2006, accepted 15 June 2006

Abstract

The sensitivity of bacteria to the bactericidal activity of serum depends on the structure and organization of the bacterial outer membrane. Sialic acid has been found in the O-specific region of bacterial lipopolysaccharide (LPS) and it plays an essential role in protecting Gram-negative bacteria against the bactericidal activity of human and animal serum. The susceptibility of Gram-negative bacilli with sialic acid-containing LPS to the bactericidal action of normal bovine serum (NBS) was determined. The examined strains (Escherichia coli O104 (PCM 270), E. coli O24 (PCM 195), E. coli O56 (PCM 2372), Citrobacter braakii O37 (PCM 2346) and Salmonella enterica ssp. enterica serovar Toucra O48 (PCM 2359) showed variable sensitivity to the bactericidal effect of the serum. The role of the mechanisms of complement activation in the killing process was also determined.

Key words: Gram-negative bacteria, lipopolysaccharide, normal bovine serum (NBS), sialic acid

Introduction

The complement system plays an important role in protection higher organisms against bacterial infection. The complement system can be activated in three ways, known as: the classical, lectin, and alternative pathways. The classical pathway plays the most important role in the bactericidal action of serum. The alternative and the lectin pathways are considered to be less important. The latter two pathways can be activated with or without the participation of antibodies. Activation of the classical complement pathway is dependent on the recognition of antigen by IgG or IgM antibodies, whereas activation of the alternative and lectin pathways is not necessarily dependent on the presence of antibody and is therefore a first line of defense for the host organism (Mokracka-Latajka et al., 1996; Matsushita et al., 1998; Rautemaa and Meri, 1999).

The sensitivity of Gram-negative roods to the bactericidal action of serum depends on their cell wall structure. The phenomenon of serum resistance of bacteria has a multifactorial basis, and the structure of the outer membrane has an essential role in protecting Gram-negative bacteria against the action of serum. The O-specific side chains of lipopolysaccharides (LPS), capsules and outer membrane proteins (OMP) play a decisive role in this phenomenon (Lachowicz et al., 1999; Mielnik et al., 2001; Cisowska and Jankowski, 2004; Bugla et al., 2004).

Sialic acids (N-acetylneuraminic acid) are important constituents of glycoconjuggates in animal tissues. In eukaryotic cells, sialic acids stabilize glycoconjuggates, mediates cell-cell regulation, and regulates trans-membrane receptor function (Vimir et al., 2004). Sialic acid is not a common component within the bacterial cell, but may occur as a component of the capsule, e.g. K1 and K92 antigens in E. coli, type III capsular polysaccharides of streptococci of group B, capsules of meningococci of serogroup B, (Egan et al., 1977; Marques et al., 1992; Ram et al., 1999) or as a component of LPS. The sialic acid has been found in LPS of E. coli (serotypes O24; O56; O104) (Jann and Jann, 1977; Orskov et al., 1977; Gamian et al., 1992;
In the pathogenesis of Gram-negative bacilli sialic acid plays an essential role in protecting them against the bactericidal activity of serum by inhibition of the alternative pathway of complement activation. The presence of sialic acid on the bacterial surface and its direct interaction with factor H of complement can provide resistance against complement attack (Rautemaa and Meri, 1999). Sialic acid may also contribute to the pathogenicity of bacteria by taking part in epitopes that resemble host tissue components (molecular mimicry). Interesting phenomenon of the molecular mimicry was observed in the case of the LPS of C. braakii O37 which shared epitopes with equine and human erythrocytes. This serological mimicry may contribute to the pathogenicity of these bacteria (Gamian, 1996). Gamian et al. (1992b) reported that the other erythrocytes tested namely sheep, goat, pig, cat and bovine were not agglutinated by anti-O37 Citrobacter serum.

In our laboratory we analyzed the mechanisms of the activation of complement in NBS by Escherichia, Citrobacter and Salmonella strains containing LPS with sialic acid. Our previous results (Mielnik et al., 2001) indicated that the strains of Gram-negative rods possessing sialic acid in the O-specific antigen demonstrate different degrees of sensitivity to the bactericidal activity of normal cord serum which indicates that the presence of N-acetylneuraminic acid in LPS does not play a decisive role in determining of bacterial resistance to the bactericidal complement activity of normal cord serum.

The scope of the present investigation was to determine the effect of the presence of N-acetylneuraminic acid in the LPS structure on the sensitivity of Gram-negative bacilli to the bactericidal activity of NBS.

### Experimental

#### Materials and Methods

**Bacterial strains.** The study was carried out on Gram-negative strains which contain sialic acid in the O-specific side chain of LPS. The tested strains were: Escherichia coli O104 (PCM 270), E. coli O24 (PCM 195), E. coli O56 (PCM 2372), Citrobacter braakii O37 (PCM 2346) and Salmonella enterica ssp. enterica serovar Toucra O48 (PCM 2359). The strains were kindly provided by Polish Collection of Microorganisms (PCM) in Wrocław.

**Serum.** Normal bovine serum (NBS) was obtained from five healthy animals not treated with any antimicrobial drug. The serum samples were collected, pooled and kept frozen (−70°C) for a period no longer than three months. The suitable volume of serum was thawed immediately before use. Each portion was used only once.

**Bactericidal activity of NBS.** The bactericidal activity of NBS was determined as described previously (Doroszkiewicz, 1997). Briefly, the strains were grown overnight, and then bacterial cells in an early exponential growth phase were transferred to fresh YP and incubated at 37°C for 1 hour. After incubation, the bacterial cells were centrifuged (4000 rpm for 20 min) and suspended in saline. Then the bacteria were mixed with 12.5, 25, 50 or 75% NBS (the serum was diluted with 0.1 M NaCl). Bacteria with serum were incubated in a water bath at 37°C. After 0, 60 and 180 min, samples were collected, diluted, and cultured on nutrient agar plates for 18 h at 37°C. The number of colony forming units (CFU) at time 0 was taken as 100%. Strains, which had a survival above 100% after 180 min of incubation in NBS, were regarded as resistant.
Thermal inactivation of serum. The control was NBS decomplemented by heating the sample at 56°C for 30 min (NBS 56°C).

Treatment of sera. The alternative pathway of complement activation was blocked by incubation of NBS for 20 min at 50°C (NBS 50°C) (Edinger et al., 1977). The classical and lectin pathways of complement activation were inhibited using ethylene glycol-bis (β-aminomethyl ether) N,N,N',N'-tetraacetic acid (EGTA) (NBS MgEGTA). This effect of preparation was obtained by removing of cations Ca²⁺ from serum by EGTA supplemented with MgCl₂. The final concentration of EGTA and MgCl₂ in the serum was 10 mmol/l. The EGTA solution was prepared according to Fine et al. (1972).

Results

Four serum concentrations (12.5%, 25%, 50% and 75%) were used. The tested strains demonstrated varied resistance to the bactericidal activity of NBS. Two strains: E. coli O24, and E. coli O104, were resistant to the bactericidal effect of complement protein. E. coli O56, C. braakii O37, and S. Toucra O48 demonstrated higher sensitivity to the bactericidal activity of complement than the E. coli O24, and E. coli O104 strains, and NBS bactericidal activity was more effective against these strains. To determine the effect of incubation time on killing of the bacterial cell, we determined the survival of bacteria after one and three hours. It was shown that for E. coli O56, Citrobacter braakii O37, and S. Toucra O48 strains, one hour of incubation was sufficient to achieve a high bactericidal effect of the serum. The results concerning the sensitivity of the Gram-negative bacilli with sialic acid-containing LPS to NBS are given in Tables II–VI. As shown in these tables the bacteria proliferated very intensively in the inactivated NBS (NBS 56°C).

In next step we determined the role of the particular mechanisms of complement activation in the killing of Gram-negative bacilli with sialic acid-containing LPS. We examined the biological activity of serum in which the alternative pathway of complement activation was thermally inhibited (NBS 50°C) and serum in which

<table>
<thead>
<tr>
<th>Time of incubation (min)</th>
<th>Serum concentration (%)</th>
<th>CFU cf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.5</td>
<td>25</td>
</tr>
<tr>
<td>0</td>
<td>69 × 10⁵</td>
<td>29 × 10⁵</td>
</tr>
<tr>
<td>60</td>
<td>41 × 10⁵</td>
<td>60 × 10³</td>
</tr>
<tr>
<td>180</td>
<td>56 × 10⁴</td>
<td>36 × 10³</td>
</tr>
</tbody>
</table>

<sup>a</sup> CFU, colony forming units (relevant for Tables II–VI)  
<sup>b</sup> NT, not tested (relevant for Tables II–VI)  
<sup>c</sup> NBS, decomplemented by heating at 56°C for 30 min (relevant for Tables II–VI)
the classical and lectin pathways of complement activation were blocked (NBS MgEGTA). In this way the study of the bactericidal mechanisms of serum involved determination of the survival of bacteria in sera with certain bactericidal factors removed. In these experiments we used only 

\textit{E. coli} O56, \textit{S. Toucra} O48 and \textit{C. braakii} O37 which were sensitive to the bactericidal action of complete NBS, because the strains resistant to complete NBS would also be resistant to the bactericidal action of modified serum (Tables VII–IX).

The obtained data indicate the same mechanisms of activation of complement. An important role for the classical and lectin pathways’ mechanisms of complement activation was not observed. The dominant mechanism of activation of NBS was the independent activation of complement by the classical, lectin, and alternative pathways, in spite of the fact that all the tested strains have sialic acid in the O-specific antigen.

It was shown that the presence of sialic acid in LPS does not play a decisive role in the determination of bacterial resistance to the bactericidal activity of complement and that the presence of sialic acid in LPS is not sufficient to block of the amplification of the alternative pathway.

**Discussion**

The differences between rough strains (R form) and smooth strains (S form) in their sensitivity to bactericidal action of serum have been demonstrated many times, including genetic confirmation of LPS O-specific chains participation in protection of the bacterial cell from the bactericidal action of complement (Delabac, 1968; Taylor, 1995). It has been shown (Doroszkiewicz et al., 1994; Jankowski et al., 1996; Mielnik et al.,
2001; Bugla et al., 2004) that many smooth strains are sensitive to bactericidal action of complement. Our previous results also confirmed that the sensitivity of Gram-negative bacteria to normal cord serum (NCS) is variable (Mielnik et al., 2001).

In the present experiments we used normal bovine serum (NBS). NCS and NBS presented different bactericidal activity. The bactericidal activity of NCS against some Gram-negative bacteria is less efficient than that of NBS. This effect is caused by a deficiency of complement components and a physiological deficiency of IgM antibodies in NCS (Jankowski, 1994; Cisowska and Jankowski, 2004).

E. coli O56 has a small number of repeating units in the O-chains of LPS (Gamian et al., 1994) therefore these cells may be more sensitive to NBS than the S forms of E. coli O24, E. coli O104, S. Toucra O48 and C. braakii O37 (Gamian et al., 1992; Gamian et al., 1992a; Gamian et al., 1994; Gamian et al., 2000). S. Toucra O48 and C. braakii O37 (S form) were sensitive to the bactericidal action of NBS. The LPS of S. Toucra O48 and that of C. braakii O37 contain terminal non-reducing sialic acid (Neu5Ac), but the internal sialic acid in S. Toucra O48 LPS is 4-substituted, whereas that in C. braakii O37 is 7-substituted (Gamian and Kenne, 1993). In the tested strains of LPS endotoxins, except those of E. coli O104 the biological O-specific units are terminated at non-reducing end by sialic acid. In E. coli O104 antigen the biological O-specific unit has a terminal nonreducing galactose residue (Table I). Immunochemical analysis of the outer membrane showed that the differences in LPS structure could play an important role in determining the susceptibility of Gram-negative strains to the action of serum (Doroszkiewicz et al., 1994; Doroszkiewicz, 1997). The phenomenon of serum resistance of bacteria has a multifactorial basis and the mechanism of bactericidal sensitivity and resistance to the bactericidal action of complement is not fully understood. In conclusion, sialic acid seems to be immunodominant in most of the studied antigens, but the presence of this molecule in LPS is not sufficient for bacterial resistance to bactericidal serum activity.

The Neisseriae strains can sialylate their lipooligosaccharide (LOS) and mimic sialylated lactoneo-glycosphingolipids, which may serve as a camouflage the bacterium to the host (Moran et al., 1996). Serum-resistant strains of gonococci are known to be more sialylated (Rautemaa and Meri, 1999). Sialic acid, as a component of the cell of these bacteria, plays an essential role in protecting Gram-negative bacteria against the bactericidal activity of serum. Its protective effect is in the enhancement of the binding of factor H to the C3b component of the system which blocks the amplification loop, limiting the activation of the alternative pathway of complement activation. Factor H inhibits the alternative pathway’s C3-convertase by dissociating Bb from the C3bBb complex and promotes the inactivation of C3b by factor I (Rautemaa and Meri, 1999). However, Neu5Ac, as a component of capsule K1 of E. coli does not interact directly with factor H and the mechanisms of alternative pathway blocking are not inhibited (Meri and Pangburn, 1990).

We determined the role of the particular mechanisms of complement activation in the process of killing Gram-negative bacilli with LPS containing sialic acid. The alternative complement pathway killed all the tested strains without the participation of antibodies. The independent activation of the classical, lectin, and alternative pathways in the serum was observed. This suggests that the tested strains do not have the ability to block the alternative pathway. Devine and Roberts (1994) showed that the possibility of blocking the alternative pathway is perhaps related to the amount of sialic acid on the cell surface. The exact role of sialic acid in LPS is not know. Microbial, immunological, structural and immunochemical studies on these LPS are necessary to understand the role of the sialic acid in interactions of prokaryotes cells with the cells of human and animal hosts.

**Acknowledgements.** The authors thank Prof. A. Gamian (Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw, Poland) for the E. coli, Salmonella, and Citrobacter strains from the Polish Collection of Microorganisms.

**Literature**


Characterization of Coagulase-Negative Staphylococci Isolated from Cases of Ostitis and Osteomyelitis

IWONA WILK¹, ALICJA EKIEL¹, PIOTR KLUCIŃSKI¹, JOLANTA KRZYSZTOŃ-RUSSJAN² and GAYANE MARTIROSIAN¹,³*

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Abstract

Coagulase-negative staphylococci (CoNS) are often responsible for cases of chronic ostitis and osteomyelitis, especially in patients with orthopedic prosthesis/implants. The aim of this study was to characterize CoNS isolated from ambulatory patients with chronic ostitis/osteomyelitis and to compare them by PFGE (pulsed-field gel electrophoresis). Out of 263 bacterial strains isolated from wounds/sinuses of patients with chronic ostitis/osteomyelitis, 41 were identified as CoNS. Twenty methicillin-resistant strains were selected for this study. Our results confirm the superior performance of cefoxitin disk test to detect methicillin resistance in heterogenous population of CoNS. High level of antibiotic resistance was observed among the studied strains: majority of CoNS were resistant to tetracycline and erythromycin and also to clindamycin and ciprofloxacin. Importantly, in 15 out of 20 studied CoNS different phenotypes of macrolides, lincosamides and streptogramin – MLS resistance was suggested. Eight strains demonstrated resistance to both erythromycin and clindamycin, suggesting constitutive MLSB phenotype. Seven remaining strains presented resistance to erythromycin and susceptibility to clindamycin with negative D-test results, suggesting the presence of macrolides and streptogramines type A efflux pump. All studied strains were sensitive to vancomycin (MIC 0.75–2.0 µg/ml), teicoplanin (MIC 0.125–8.0 µg/ml), and quinupristin/dalfopristin (MIC 0.19–1.0 µg/ml). No clonal relatedness was observed in PFGE patterns.

Key words: coagulase-negative staphylococci, ostitis/osteomyelitis, antibiotic resistance

Introduction

Bacterial infections cause serious complications and constitute an important problem in orthopedic patients, especially in those after orthopedic surgery. The microorganisms most commonly causing deep wound infection in orthopedic patients are staphylococci (Wilk et al., 2004). Many publications describe cases of osteomyelitis caused by Staphylococcus aureus (Issartel et al., 2005). Coagulase-negative staphylococci (CoNS), however, are often responsible for the cases of chronic ostitis and osteomyelitis, especially in patients with orthopedic prosthesis and cause about 90% pin tract infections. Particular problems with correct obtaining samples for culturing exist for wounds that are in fact sinuses overlying a focus of chronic ostitis/osteomyelitis. For this reason, very often microbial results are interpreted incorrectly.

CoNS are important pathogens especially in cases of sternal osteomyelitis following median sternectomy (Rupp and Archer, 1994). The vertebral bodies are a typical site of haematogenous osteomyelitis. Cases of spondylodiscitis following CoNS bacteremia have been also reported (Bucher et al., 2000). Of 32 CoNS species validly published, only half are seen in specimens of human origin. More recently, species of CoNS were isolated, which significantly differed from all other Staphylococcus spp. based on phenotypic characteristics and 16 rRNA gene sequencing and a novel Staphylococcus pettenkoferi species was proposed (Trulzsch et al., 2002; von Eiff et al., 2002). Methicillin resistance in both community- and hospital-acquired strains

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of staphylococci has emerged as an important and growing resistance threat (Pottumarthy et al., 2005). The aim of this study was to characterize CoNS isolated from specimens taken from ambulatory cared patients with chronic ostitis/osteomyelitis and to compare of the appropriate species representatives by PFGE.

**Experimental**

**Materials and Methods**

This study included group of 263 patients with chronic bone infections (ostitis/osteomyelitis) from different regions of Upper Silesia, who attended the Department of Medical Microbiology, Medical University of Silesia in Katowice from 2000 to 2005 to identify etiological agents of chronic infection. These patients were not related epidemiologically with each other and were not hospitalized at the same time in the same hospital ward. Patients were considered to this study if isolation of the same type of CoNS was confirmed at least twice and no other microorganism was isolated simultaneously.

Sterile swabs were used to obtain material for culturing of aerobic/anaerobic microorganisms. Swabs obtained from deeper part of wounds/sinuses were cultured by using routine microbiological techniques (Baron et al., 1999). Isolated colonies were identified by colony morphology, Gram staining, catalase activity, determination of clumping factor (Slidex Staph-Kit, bioMerieux, Marcy l’Etoile, France) and coagulase activity by using rabbit plasma. Biochemical identification was performed by using ID-32 Staph panels (bioMerieux, Marcy l’Etoile, France). Each isolated strain was tested for methicillin sensitivity with oxacillin (1 µg) and cefoxitin (30 µg) disks, according to CLSI guidelines. All isolates were tested for the mecA gene by PCR (Murakami et al., 1991).

Antibiotic susceptibility testing was done by disk-diffusion methods for trimethoprim/sulfamethoxazole; chloramphenicol; tetracycline; erythromycin; clindamycin; gentamicin; ciprofloxacin; fusidic acid; moxifloxacin; quinupristin/dalfopristin; and linezolid (Oxoid, Basingstoke, UK), inhibition zone diameters were interpreted according to ranges recommended by CLSI.

Pulsed-field gel electrophoresis (PFGE) was performed using CHEF DRII apparatus (Bio-Rad Laboratories, Hercules, CA, USA), according to Chung and coworkers (Chung et al., 2000). PFGE patterns were compared with the use of Molecular Analyst software, version 1.12 (Bio-Rad, CA, USA).

**Results and Discussion**

Out of 263 bacterial isolates 41 were identified as strains of CoNS (Table I). Twenty strains were methicillin-resistant (MRCoNS) and were selected for this study. Among these MRCoNS: 6 strains of *Staphylococcus epidermidis*, 7 strains of *Staphylococcus haemolyticus*, 3 strains of *Staphylococcus simulans*, and single strains of *Staphylococcus sciuri*, *Staphylococcus cohnii*, *Staphylococcus hominis* and *Staphylococcus lentus* were identified. Clinical and microbiological data concerning these isolates are presented in Table II.

Comparison of methicillin susceptibility testing by oxacillin and cefoxitin disks demonstrated non-concordance (oxacillin-resistant/cefoxitin susceptible) in 5 cases (strains no 3, 13, 16, 19 and 20). However the presence of mecA gene was not demonstrated in these strains. These results confirm the superior performance of cefoxitin disk test to detect methicillin resistance in heterogenous population of CoNS. Similar observations in studied population of coagulase-positive and coagulase-negative staphylococci were published by other authors (Pottumarthy et al., 2005; Sharp et al., 2005). All studied strains were sensitive to vancomycin (MIC 0.75–2.0 µg/ml), teicoplanin (MIC 0.125–8.0 µg/ml), and quinupristin/dalfopristin.

**Table I**

<table>
<thead>
<tr>
<th>Strains of CoNS</th>
<th>Otitis/Osteomyelitis</th>
<th>Endoprosthesis</th>
<th>Sternal osteomyelitis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. epidermidis</em></td>
<td>7</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><em>S. haemolyticus</em></td>
<td>11</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td><em>S. simulans</em></td>
<td>4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. hominis</em></td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><em>S. warneri</em></td>
<td>1</td>
<td>2</td>
<td>–</td>
</tr>
<tr>
<td><em>S. sciuri</em></td>
<td>–</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td><em>S. cohnii</em></td>
<td>2</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. lentus</em></td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>S. chromogenes</em></td>
<td>1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total</td>
<td>29</td>
<td>9</td>
<td>3</td>
</tr>
</tbody>
</table>
Coagulase-negative staphylococci from ostitis and osteomyelitis cases

Table II
Characteristics of coagulase-negative staphylococci

| No | Identification | Health -Care Unit | Ox<sup>2</sup> | Fox<sup>2</sup> | mecA<sup>3</sup> | STX | C | T | E | CC | Ge | Cip | Fa | MXF | Syn | LZD | PFGE |
|----|----------------|------------------|--------------|--------------|----------------|-----|---|---|---|----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | S. haemolyticus | A                | R<sup>4</sup> | R<sup>22</sup> | +              | S   | S | R | S | R | S | S | S | S | S | S | A1  |
| 2  | S. haemolyticus | F                | R<sup>14</sup> | R<sup>21</sup> | +              | S   | R | R | R | R | S | S | S | S | S | S | B1  |
| 3  | S. haemolyticus | U                | R<sup>17</sup> | S<sup>19</sup> | –              | S   | R | R | R | S | S | S | S | S | S | C1  |
| 4  | S. haemolyticus | U                | R<sup>4</sup> | R<sup>21</sup> | +              | R   | S | R | R | S | S | S | S | S | S | S | D1  |
| 5  | S. haemolyticus | E                | R<sup>4</sup> | R<sup>20</sup> | +              | R   | S | S | R | S | R | S | S | S | S | S | E2  |
| 6  | S. haemolyticus | E                | R<sup>4</sup> | R<sup>14</sup> | +              | R   | S | S | R | R | R | S | S | S | S | S | F1  |
| 7  | S. haemolyticus | Z                | R<sup>4</sup> | R<sup>17</sup> | +              | R   | R | R | R | R | S | S | S | S | S | G1  |
| 8  | S. epidermidis  | R                | R<sup>4</sup> | R<sup>14</sup> | +              | S   | R | R | R | R | S | S | S | S | H1  |
| 9  | S. epidermidis  | E                | R<sup>4</sup> | R<sup>11</sup> | +              | R   | S | R | R | S | R | S | S | S | S | I1  |
| 10 | S. epidermidis  | T                | R<sup>4</sup> | R<sup>22</sup> | +              | R   | S | R | R | R | S | S | S | S | S | J1  |
| 11 | S. epidermidis  | B1               | R<sup>4</sup> | R<sup>11</sup> | +              | S   | R | S | R | R | S | S | S | S | S | K1  |
| 12 | S. epidermidis  | B1               | R<sup>4</sup> | R<sup>21</sup> | +              | S   | S | R | R | R | R | R | S | S | S | S | L1  |
| 13 | S. epidermidis  | E                | R<sup>17</sup> | S<sup>19</sup> | –              | S   | S | R | R | R | S | S | S | S | S | M1  |
| 14 | S. simulans     | F                | R<sup>12</sup> | R<sup>24</sup> | –              | S   | R | S | R | S | S | S | S | S | S | N1  |
| 15 | S. simulans     | E                | R<sup>4</sup> | R<sup>11</sup> | +              | R   | R | R | R | R | R | S | S | S | S | O1  |
| 16 | S. simulans     | S                | R<sup>17</sup> | S<sup>19</sup> | –              | S   | S | S | R | R | S | R | R | S | S | P1  |
| 17 | S. cohnii       | B                | R<sup>4</sup> | R<sup>14</sup> | +              | S   | S | R | R | S | R | S | S | S | S | R1  |
| 18 | S. sciuri       | E                | R<sup>13</sup> | R<sup>24</sup> | +              | S   | R | S | S | S | S | S | S | S | S | S | S | S | S | S | S | SI  |
| 19 | S. hominis      | B1               | R<sup>17</sup> | S<sup>19</sup> | –              | S   | S | R | S | S | S | S | S | S | S | S | S | S | S | T1  |
| 20 | S. lentus       | U                | R<sup>17</sup> | S<sup>19</sup> | –              | S   | S | S | S | S | S | S | S | S | S | S | U1  |

Total 10 R – resistant  S – susceptible

10 R – resistant 15/20 7/20 6/20 15/20 9/20 7/20 8/20 0/20 2/20 0/20 0/20 20

1 Health Care Units in region of Upper Silesia, from where patients were directed to the Department of Medical Microbiology Medical University of Silesia in Katowice

2 Ox (1 µg) – oxacillin and 2Fox (30 µg) – cefoxitin; index demonstrates the zone in mm of bacterial growth inhibition in disk diffusion test

3 mecA – gene was determined by Murakami et al. (1991).

4 PFGE – Pulsed-field gel electrophoresis performed according to Chung et al. (2000).

(MIC 0.19 – 1.0 µg/ml) in E-test method. Majority of CoNS (15/20) were resistant to tetracycline and erythromycin. About half of them were resistant to clindamycin (9/20) and ciprofloxacin (8/20). Seven out of 20 studied strains were resistant to gentamycin and trimethoprim/sulfamethoxazole. Only 6 strains demonstrated resistance to chloramphenicol and 2 strains – to moxifloxacin. No resistance was observed to fusidic acid, linezolid and quinupristin/dalfopristin. Eight strains demonstrated resistance to erythromycin and clindamycin, suggesting constitutive resistance to MLS<sub>B</sub> agents. Macrolides and lincosamides are commonly used antibiotics in treatment of staphylococcal infections, particularly skin and soft tissue infections, ostitis and osteomyelitis and also as alternatives in penicillin-allergic patients. Seven strains demonstrated resistance to erythromycin and susceptibility to clindamycin, however D-test results were negative (data not shown). These strains were considered to be negative for inducible resistance, but could have an active efflux pump. In these cases according to Polish recommendations for susceptibility testing to antimicrobial agents of selected bacterial species (Hryniecowa et al., 2005) 14–15-carbon containing macrolides and streptogramins B are not recommended for treatment. Although clindamycin is a good alternative for treatment of methicillin-resistant/susceptible staphylococcal infections, results of antibiotic susceptibility testing to erythromycin and clindamycin should be analyzed very carefully to avoid therapeutic failures. Attention should also be drawn to the uncommon lincosamides modification by 3-lincomycin, 4-clindamycin O-nucleotidyl-transferase encoded by linA/linA’ genes in staphylococci (Lina et al., 1999). Inducible MLS<sub>B</sub>
resistance is not recognized by standard susceptibility testing, including standard broth-based or agar dilution tests. When staphylococci appear to be resistant to erythromycin and susceptible to clindamycin by routine tests it is very important to perform the D-test to demonstrate the occurrence of inducible resistance to clindamycin. To detect lincosamide resistance, susceptibility to lincomycin and clindamycin should be checked, because in vitro high levels of lincomycin resistance and susceptibility to clindamycin simultaneously have been described (Leclercq et al., 1987). Our CoNS strains resistant to erythromycin and susceptible to clindamycin, demonstrated susceptibility to lincomycin in disk diffusion test (data not shown), confirming the presence of active efflux pump mechanism. Susceptibility to quinupristin/dalfopristin is caused by the dalfopristin component, which is a streptogramin A (quinupristin is a streptogramin B) (Azap et al., 2005). Among 20 studied strains no clonal relatedness was observed in PFGE patterns, although some strains were cultured from patients treated in the same health care unit, but not during the same period of time (no 3 and 4; no 5, 6, 9 and 13; no 11 and 12 in Table II). Importantly, in 15 out of 20 studied CoNS different phenotypes of MLSB resistance were demonstrated. This is alarming news, especially for treatment strategies of ostitis/osteomyelitis due to CoNS. Further studies on a larger number of strains are required for detailed characterization of CoNS – causative agents of chronic ostitis and osteomyelitis.

Literature


S. aureus spp.


S. aureus spp.


Bioactive Compounds from *Streptomyces nasri* and its Mutants with Special Reference to Proteopolysaccharides

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**Abstract**

The use of microbial exopolysaccharides (EPS) in the food, pharmaceutical, and chemical industries has steadily increased during the past decade. A bioactive EPS producing microorganism, *Streptomyces nasri* was isolated from Kuwait tropical soil and the proteopolysaccharide was tested for its antimicrobial activity. The isolate was subjected to ultraviolet (UV) radiation and acridine orange (AO) treatment to select for superior proteopolysaccharide producers. Among eight (five derived from UV exposure and three from AO exposure) morphological variants of *Streptomyces nasri*, two mutants showed increased EPS production, from 1.8 g/l to 2.3 g/l. The SDS-PAGE profiles of exopolysaccharides were determined. The molecular weight of the proteopolysaccharide ranged from 18 to 200 kDa. Mutants derived from UV exposure produced polysaccharides with higher molecular weight than those derived from acridine orange exposure. Acridine orange derived mutants produced lower molecular weight polysaccharides. Culture supernatants have been partially characterized and they show antimicrobial activity against a wide range of microorganisms. The structure of the exopolysaccharide was determined using NMR spectroscopy. The polysaccharide was also tested for cytotoxic activity against human brain tumor cell line using SRB assay.

Key words: antimicrobial activity, bioactive compound, microbial polysaccharides, *Streptomyces* sp. mutations

**Introduction**

More than 70 years have passed since Sir Arthur Fleming’s outstanding discovery of a *Penicillium* colony, which inhibited the growth of a *Staphylococcus* culture. Today, these two features, the production of bioactive metabolites by microorganisms and their antibiotic effect against pathogenic microbes are continuing to attract scientific and public interest. Though almost 20 000 microbial metabolites and approximately 10 000 plant products have been described so far (Grabley and Thiericke, 1999). Secondary metabolism appears as an inexhaustible source of new antimicrobials, antivirals, antitumour drugs, agricultural and pharmacological agents. Therefore, continuous efforts are being made in the research on microbial secondary metabolites, which have potential applications, such as proteopolysaccharides.

Recent reviews on microbial proteopolysaccharides confirm that there is potentially a huge market (Sutherland, 2001). Moreover, the importance of various proteopolysaccharides for pharmaceutical purposes such as baker’s yeast glycans, eg. Scleroglucan and Krestin has a long historical background and increased considerably during the last decades (Franz, 1989). This is not only the case for polysaccharides...
used as exceptions essential for specific drug formulations but also for a series of carbohydrates polymers which have proven to be most useful as physiological active drugs like branched glucans of *Dictyophora* sp. and *Symphitum fructans* (Franz, 1989). Many interesting areas have been opened, which include their role in cancer therapy (Schizophyllan) and prevention of bacterial and viral diseases (Lentinan). It has been known that human malignant growths sometimes undergo regression following an acute bacterial infection. Polysaccharides complexes from *Staphylococcus aureus*, *Acetobacter xylinum*, different species of *Streptomyces* and *Pseudomonas* and some other bacteria were reported to be active against solid tumors.

Many microorganisms synthesize exopolysaccharides (EPS) that either remain attached to the cell surface or are found in the extracellular medium in the form of amorphous slime. Microbial polysaccharides are water-soluble polymers and may be ionic or non-ionic. The repeating units of these exopolysaccharides are very regular, branched or unbranched, and are connected by glycosidic linkages. Some microbial polysaccharides are commercially accepted products and others are at various stages of development. Currently a small number of biopolymers are produced commercially on a large scale. However, these limited groups of products exhibit an extensive range of physical properties (Sutherland, 1990; 1998).

Actinomycetes are saprophytic bacteria that secrete important hydrolytic enzymes, antibiotics and medicinally important secondary metabolites. Between 1988 and 1992 more than hundred different new molecules from actinomycetes were discovered. Approximately 75% of these originated from *Streptomyces* genus (Sanglier et al., 1993; Sacramento et al., 2004). Therefore and because of their ability to secrete valuable proteins, *Streptomyces* have been considered as an alternative host organism for producing recombinant proteins (Dela Cruz et al., 1992). Induction of mutation and selection to improve the productivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for many antibiotics (Venkateswarlu et al., 2000). The mutant strains can be achieved by inducing genetic variation in the natural strain with increased productivity.

In this study an actinomycetes strain (*Streptomyces* sp.), previously isolated from Kuwait tropical desert (Hashem and Diab, 1973), was chosen due its marked stability and tested for its double capacity to produce antimicrobial agents active against some pathogenic with special focus on the cytotoxic activity against brain human tumor cell line. The possibility of producing mutants from the parent strain has been explored. Direct cytotoxic activity against brain cancer cell line has been investigated. An attempt to determine the molecular weight and the structure of the isolated proteopolysaccharides was undertaken.

**Experimental**

**Materials and Methods**

**Organism and culture media.** *Streptomyces nasri* was isolated from the desert of Kuwait by Hashem and Diab (1973). This strain was found to produce an antibiotic active against Gram-positive bacteria. A loop-full of spore stock was spread on ISP-agar plate and incubated for 6–8 days at 30°C. 500 µl spore suspension was then used as an inoculum for each 250-ml Erlenmeyer flask containing 50 ml of the particular medium (M1, M2, M3) and incubated on a rotary shaker (Infors AG, CH-4103 Bottmingen) at 30°C for 7 days at 200 rpm. This culture was used for various mutagenic experiments and production of proteopolysaccharides. Three different media have been used throughout this work, namely M1, M2 and M3. Medium No 1 (M1) contains (g/l): glucose 30, NaN0₃ 3, yeast extracts 5, NaCl 4, MgSO₄ 0.5, K₂HPO₄ 1, CaCO₃ 1, pH 7.0. Medium No 2 (M2) contains (g/l): glucose 10, yeast extracts 3, peptone 5, KH₂PO₄ 1, K₂HPO₄ 1, pH 7.0. Medium No 3 (M3-ISP) contains (g/l): malt extract 10, yeast extract 4, glucose 4, pH 7.2.

**Mutagenesis.** In *Streptomyces nasri* two classical methods have been successfully used to produce mutants. This includes the use of UV light and acridine orange (AO).

**Ultraviolet (UV) treatment.** Ultraviolet (UV) mutagenesis was carried out according to Zhang et al. (1993). Photoreactivation was avoided by keeping the isolated colonies overnight in the dark by wrapping the plates in aluminum foil (Held and Kutzner, 1991) and a survival rate of not more than 1% was considered.

**Acridine orange (AO) treatment.** Two ml heavy spore suspension (10⁶ spores/ml) of the parent type *Streptomyces nasri* were subjected to a final concentration of 10 µg/ml acridine orange for 90 min to cause 99% killing. Spores were washed several times using LB medium by repeated centrifugation at 4000 rpm for 10 min and decantation. Washed spore pellet was finally suspended in 2 ml LB medium, vortexed, spread onto LB-agar plates, incubated at 30°C. Macroscopically different colonies were selected.

**Cell growth.** Samples were taken at the end of cultivation (7 days) and various analyses were performed. Growth was measured as the dry weight per volume by centrifugation (5000 × g for 10 min) and then dried to a constant weight in an oven at 60°C overnight to obtain cell dry weight (CDW).

**Extraction and separation of proteopolysaccharides (EPS).** Mycelial balls were separated by centrifugation at 3000 × g for 10 min. The supernatant was concentrated to 1/10 its initial volume by a rotary evaporator (Heidolph WB2000, Germany). The concentrates were mixed with equal volume of chilled absolute ethanol to precipitate the proteopolysaccharides. To enhance precipitation, these samples were stored at 4°C for 24 h. The precipitates were recovered by centrifugation at 4000 × g for 15 min and then dried at 55°C overnight (van Geel-Schutten et al., 1998).
Proton NMR spectrometry. Proteopolysaccharide (EPS) sample (50 mg) was dissolved in 1 ml dimethyl sulphoxide by ultrasonication for 10 minutes. The solution was introduced into a precision ground tube (5 mm diameter, depth 2–3 cm) then subjected to measurement by an NMR-spectrophotometer (JEOL, Japan).

SDS-PAGE electrophoresis. Protein samples were analyzed by electrophoresis in a 12% (w/v) sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) as described by Laemmli (1970).

Measurement of cytotoxicity by SRB assay. Potential cytotoxicity of proteopolysaccharides was tested using the method of Skehan et al. (1990). Briefly, U-251 brain cancer cell line was plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the proteopolysaccharides to adhere cells. Different concentrations of EPS dissolved in DMSO (0, 1, 2.5, 5, 6.5, 8.5 and 10 mg/ml) were added to the cell monolayer. Triplicate wells were prepared for each individual dose. Monolayer cells were incubated with the compound(s) for 48 h at 37°C in 5% CO₂ atmosphere. After 48 h, cells were fixed, washed and stained with sulforhodamine B stain. Excess stain was washed with acetic acid and attached stain was recovered with Tris-EDTA buffer. Color intensity was measured in an ELISA reader. The relation between the number of viable cells and EPS concentration was plotted to get the survival curve of tumor cell line after the specified compound.

Antimicrobial activity evaluation (Bioassay). Mueller-Hinton agar (MHA) medium was used as an assay medium. The agar medium at 45°C was mixed with 0.1 ml bacterial suspension containing approximately 10⁵ cfu/ml. The mixture was poured into 9 cm Petri dish and allowed to solidify. Sterile paper discs (5 mm) were placed on the dried surface of the medium. Each disc received 20 µl of the culture filtrate. Petri dishes were incubated at 37°C for 18 hours. The inhibition zone, if any, was measured in mm diameter (Amade et al., 1994). The test organisms for bioassay were the Gram-positive bacteria Bacillus subtilis ATCC 31324, Micrococcus luteus ATCC 10240, Sarcina lutea ATCC 10773, Staphylococcus aureus ATCC 6538 and the Gram-negative bacteria Bordetella bronchiseptica ATCC 4617, Escherichia coli ATCC 8739, Klebsiella pneumoniae ATCC 15050, Proteus mirabilis ATCC 12453, Pseudomonas aeruginosa ATCC 9027 and Salmonella typhi ATCC 3112.

Results and Discussion

Effect of AO and UV treatment on Streptomyces nasri. Five UV and three AO mutants were obtained (Figure 1). They differ in macroscopic colony characteristics including rate of growth, color and density of the spores, reverse colony color and production of soluble pigments. In addition they were all stable on subsequent cultivation in both solidified and liquid media considering their antimicrobial activity and EPS production.

Comparison between Streptomyces nasri mutants and parent strain for the production of EPS in shake flasks. Parent strain of S. nasri was used as a standard organism for screening and comparing productivity of the isolated mutants. The mutants were grown on a solid ISP-medium. Fermentation experiments in suspension culture were conducted in 250 ml shake-flasks by inoculating the mutant spores in 50 ml of three different media: M-1, M-2 and M-3.

Fig. 1. Colony morphology of S. nasri parent type, UV and AO mutants grown on oat-meal agar medium for 10 days at 30°C.
Table I shows the biomass as a dry weight and EPS production as well as final pH, in shake-flask cultures of parent \textit{Streptomyces} strain and UV as well as AO mutants cultivated in three different media. All mutants were able to grow in the three tested culture media with distinguishable differences in EPS production. The response of the parent type of \textit{S. nasri} and its UV- as well as AO-mutants to changes in the medium composition considering CDW, EPS, and pH was noticeably different. M-1 could produce a relatively very high record for the CDW (1.49 g/l) considering all mutants. Also the average for any of \textit{S. nasri} UV- or AO-mutants, M-1 was the best (1.10 and 2.14 g/l), respectively. Other media (M-2 and M-3) were inferior to M-1 (only 0.99 and 0.33 g/l with \textit{S. nasri} UV- and AO-mutants, respectively). For the average EPS production by all mutants, M-1 medium was also superior (3.21 g/l) and M-3 inferior (0.93 g/l). To simplify comparison between the three medium for the validity of EPS production, the EPS/CDW ratio (as a fold increase factor, or an index, for the specific activity for unit mycelial dry weight) was calculated for the three media compared to that of the parent type. EPS-fold increase for all mutants (UV and AO) when grown in M-1 was 6.82 times the parent type, 3.21 times in M-2 and only 1.11 times in M-3. The level of increase was unequally divided between UV- and AO-mutants. For instance, the increase of EPS and specific activity were dominated by the \textit{S. nasri} UV-mutants with the superiority in M-1 (7.68 folds) and inferiority in M-3 (1.21 folds). \textit{S. nasri}-AO mutants were superior in the level of increase of CDW measures but not in EPS production.

According to the previous results, \textit{S. nasri}-UV 135 mutant was chosen to be used in the foregoing investigations. To confirm the obtained results and to get some details about the nature, constitution and molecular weight(s) of the EPS produced by this strain, SDS-PAGE technique was applied. The antimicrobial activity and the cytotoxicity were checked. In addition, the proton-NMR fingerprint of the EPS was also determined.

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**SDS-PAGE for the EPS produced by parent strain of \textit{S. nasri}, its UV- and AO-mutants.** Figure 2 shows the SDS gel electrophoretic protein profile of the EPS of the parent \textit{S. nasri}, its UV- and AO-mutants. EPS isolated from each strain are a mixture of low and high molecular weight molecules ranging from 18–200 kDa. Generally, the parent strain was found to produce EPSs molecules whose molecular weights ranged from 29–97 kDa. \textit{S. nasri} UV-mutants showed band range relatively wider than that of the parent type (29–200 kDa). In addition, UV-\textit{Streptomyces} mutants produce more protein fragments than the parent
Bioactive compounds from *Streptomyces nasri* strain. On the other hand, protein bands of *S. nasri*-AO mutants showed a wider range of molecular weight, from relatively low to high MTs (18–200 g/l).

There is a correlation between the molecular weight and the biological activity of proteopolysaccharides. The activity depends on the size of molecules and degree of branching and conformation (Ohno *et al.*, 2001). Adachi *et al.* (1990) reported that while very large molecular weight proteopolysaccharides activate a wide variety of immune functions, after reducing the size of the molecules by heating the range and strength of the activity is proportionally reduced. Small compounds with low molecular weight show only the ability to activate glucose consumption possibly lowering blood glucose and synthesis of lysosomal enzyme (an antibacterial protective function). Mizuno *et al.* (1996) also reported that high molecular weight proteopolysaccharides appear to be more effective than those of low molecular weight.

**Antimicrobial activity and its relation with EPS.** Culture filtrates of different *S. nasri* UV- and AO-mutants grown on different 3 media, after full extraction of the EPS, were tested for their antimicrobial activity against 4 Gram-positive and 10 Gram-negative bacteria. Neither the parent nor its mutants could exhibit any anti Gram-negative activity. As for anti Gram-positive activity (Table II), M-1 was the best for production of antimicrobial agent(s) followed by M-2 then M-3. M-1 allowed production of broader antimicrobial spectra against the 4 test bacteria with the parent strain, UV-135 and AO-170 *Streptomyces* mutants. *S. nasri* UV-159 exhibited narrow activity against *S. aureus* only, whereas *S. nasri* UV-160 against *M. luteus*. M-2 was suitable for broad anti Gram-positive bacteria with the parent and AO-170 *Streptomyces*. *S. nasri* UV-135 mutant exhibited activity against the 4 tested bacteria whereas UV-159 and AO-170 failed to produce any active compounds. M-3 caused none of the producers to exhibit any broad spectrum activity in addition to failure of production with both AO-170 and UV-159. Maximum inhibition zone was recorded for UV-135 when grown in M-1 against *Micrococcus luteus* (28 mm) followed by *S. aureus* (25 mm).

Considering the antibacterial activity of EPS presented by the parent *S. nasri* and its mutants in different culture conditions, the activity of the parent strain was restricted to Gram-positive test bacteria only, without any Gram-negative activity (Table III). No active agents were produced against *Klebsiella* and only *S. nasri* UV-160 mutant could exhibit activity against *E. coli* when grown in M-2. Also, *S. nasri* AO-170 mutant failed to develop any activity against any of the tested organisms when grown in either M-2 or M-3, while *S. nasri* UV-135 was active against all Gram-positive and Gram-negative organisms except *E. coli* and *K. pneumoniae* when grown in M-1. Moreover, *S. nasri* UV-135 mutant recorded the widest antimicrobial
Table II
Antimicrobial activity of the culture filtrates of the parent strain *S. nasri* and its mutants grown on different media (M-1, M-2, M-3) after removal of EPS

<table>
<thead>
<tr>
<th>Medium</th>
<th>Organism</th>
<th>Bacillus subtilis ATCC 10240</th>
<th>Sarcina lutea ATCC 10773</th>
<th>Staphylococcus aureus ATCC 6538</th>
</tr>
</thead>
<tbody>
<tr>
<td>M-1</td>
<td>Parent <em>S. nasri</em></td>
<td>13 18</td>
<td>15 14</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-AO 170</td>
<td>21 16</td>
<td>22 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 135</td>
<td>14 28</td>
<td>18 25</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 159</td>
<td>0 0</td>
<td>0 21</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 160</td>
<td>0 15</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td>M-2</td>
<td>Parent <em>S. nasri</em></td>
<td>14 17</td>
<td>20 18</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-AO 170</td>
<td>0 0</td>
<td>0 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 135</td>
<td>17 11</td>
<td>13 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 159</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 160</td>
<td>0 16</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td>M-3</td>
<td>Parent <em>S. nasri</em></td>
<td>9 0</td>
<td>10 15</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-AO 170</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 135</td>
<td>0 11</td>
<td>9 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 159</td>
<td>0 0</td>
<td>0 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>S. nasri</em>-UV 160</td>
<td>0 12</td>
<td>0 0</td>
<td></td>
</tr>
</tbody>
</table>

(0) = No inhibition zone. Each 5 mm disc received 20 µl culture filtrate after removal of EPS.

Table III
Antimicrobial activity of the EPS produced by the parent strain *S. nasri* and its mutants grown on different media (M-1, M-2, M-3)

<table>
<thead>
<tr>
<th>Medium</th>
<th>Mutant No</th>
<th>Inhibition zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gram-positive bacteria</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bacillus subtilis ATCC 10240</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 0</td>
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<td>0 0</td>
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<td>0 0</td>
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<tr>
<td></td>
<td></td>
<td>0 0</td>
</tr>
</tbody>
</table>

(0) = No inhibition zone. Each 5 mm disc received 20 µl of EPS.
Bioactive compounds from *Streptomyces nasri*

Spectrum (against 8 test bacteria) among other producers followed by AO-170 when grown in M-1. Presence of high concentration of glucose, and presence of NaNO₃, NaCl, MgSO₄ and CaCO₃ in M-1 were advantageous for production of both non-EPS and EPS anti-bacterially active products by *S. nasri* and its mutants.

**Proton NMR spectroscopic analysis of separated proteopolysaccharides.** Proteopolysaccharides (EPS) produced by *S. nasri*-UV 135 mutant were analyzed by NMR analysis (Figure 3). Although NMR peaks are poorly resolved because of the viscosity, the characteristic signals for glucan (a branched polysaccharide chain with either 1,4 or 1,3 and 1,6 linkage) anomeric protons could be detected.

**Cytotoxic activity.** The exposure of U-251 brain cancer cells to various concentrations of EPS produced by *S. nasri*-UV 135 mutant (0, 1, 2.5, 5, 6.5, 8.5 and 10 µg/ml) exhibited a general slow decrease except at 2.5 µg/ml EPS and continued its decrease to reach 87% of the start number of U-251 brain cancer cells at the highest concentration 10 µg/ml as shown in Fig. 4.

β-1,3-glucan is widely used as a dietary supplement, with well-established stimulating effects on the immune defense system (Vetvicka, 2001; Ross *et al.*, 1999). A large body of published data supports this use. Browder *et al.* (1990) described strongly decreased septic morbidity with β-1,3-glucan administration. A series of well documented multicenter blind studies demonstrated that β-1,3-glucan-treated patients had significantly lower infection rates (Babineau *et al.*, 1994a; Babineau *et al.*, 1994b). Positive effects were also found in patients after cardiopulmonary bypass (Hamano *et al.*, 1999) and inhibition of antiviral activity has been found in HIV-infected patients (Itoh *et al.*, 1990). Some β-1,3-glucans are routinely used in patients for tumor immunotherapy (Kimura *et al.*, 1994; Nakano *et al.*, 1996).

Within the field of pharmaceutically active polymers, the area of immune-stimulating polysaccharides is an attractive field of tumor therapy. The interest in polysaccharides as antitumor substances came from the dissatisfaction with current cancer chemotherapy. Countless numbers of chemical compounds have been

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**Fig. 3.** Proton NMR spectrum of proteopolysaccharides (EPS) produced by *S. nasri* UV-135 mutant grown on M-1 medium at 30°C and 200 rpm for 7 days.
tested as cytostatic agents with possibly a high specificity for the cancer cell. But unfortunately in many cases, this specificity is rather low, and therefore many chemotherapeutic antitumor substances have considerable side effects. Extensive studies have been carried out on nontoxic antitumor substances and most interestingly, some polysaccharides were shown to be most useful. Several papers have reported that microbial EPS cannot have direct effect on tumour cells in-vitro (Chihara et al., 1987; Chihara et al., 1989). It has been known for more than a century that human malignant growths sometimes undergo regression following an acute bacterial infection. The earliest bacterial-derived EPS reported to have antitumour activity was attributed to Serratia marcescens infection and became known as Shear’s polysaccharide (Ooi and Liu, 2000). This polysaccharide could cause extensive cytotoxic damage to Sarcoma 37 tumours, but as it had serious side effects, clinical trials have not been performed.

It has been the experience of clinicians, as well as of laboratory experimenters, however, that successful total kill of cancer cells is apt to include the host too. Hence the enhancement or potentiation of host resistance emerged as a possible means of inhibiting tumour growth without harming the host. Starting from this point of view, extensive studies have been made on noncytotoxic and host mediated antitumour proteopolysaccharides. These studies are still in progress in many laboratories and the role of proteopolysaccharides as immunopotentiators is being especially debated. The proteopolysaccharides so far examined although may be strongly active in suppressing transplanted tumours, do not seem eligible for clinical trials in human cancer therapy. Therefore, search for new polysaccharide more strongly antitumour active than those thus far discovered stands as a challenge for further studies on antitumour proteopolysaccharides.

Literature


Hydrophobicity and Biofilm Formation of Lipophilic Skin Corynebacteria

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Abstract
Lipophilic corynebacteria isolated as natural flora of human skin were examined. Among 119 assayed strains 94% presented a hydrophobic cell surface and 75.6 % were able to form biofilms. These attributes, as well as aggregation in liquid media, were statistically connected with each other and promote the developing of biofilms on solid surfaces. This was characteristic of all the lipophilic Corynebacterium species found on human skin that were examined in this study. C. jeikeium and CDC group G2 strains dominated in this population, and they could be responsible for investigated features in the whole lipophilic skin bacterial population. These two groups are the most common coryneform bacteria isolated from nosocomial infections and these attributes most likely promote them to cause opportunistic infections.

Key words: biofilm, Corynebacterium, hydrophobicity

Introduction
Lipophilic species of Corynebacterium present a stable position among bacteria forming resident flora of the skin. Although their occurrence is not numerous, their specific contribution in this habitat is quantitatively stable. In this group of skin residents the most often represented taxa are C. jeikeium, CDC groups G1 and G2 and C. afermentans subsp. lipophilum. Lipophilic corynebacteria stay in dynamic equilibrium with coagulase-negative staphylococci (Roth and James, 1989; Tancrède, 1992; Kaźmierczak et al., 2005).

Many reports show that these bacteria, apart from being a natural flora, can participate in severe opportunistic infections. Such infections cause treatment difficulties because many strains are multiresistant to antibiotics (Coyle and Lipsky, 1990; Williams et al., 1993; Bayston et al., 1994; Funke et al., 1997). Ability to develop acquired hospital infections is strongly connected with long term survival in a hospital environment, easy selection of resistant strains or medical device colonization due to biofilm formation. Our knowledge concerning coryneforms in these fields is incremental. According to the estimation by the Centers for Disease Control and Prevention about 65% of bacterial infections in humans are connected with the formation of bacterial biofilms (Cvitkovitch et al., 2003). Many physico-chemical features of surfaces of colonised devices and bacterial cells affect biofilm creation. The main ones are: specific cell wall surface adhesion, bacterial hydrophobicity and the ability to synthesise extracellular slime substances (ESS) (Sutherland, 2001).

The aim of this study was to analyse bacterial cells hydrophobicity and the ability of the lipophilic Corynebacterium strains to form biofilms on artificial surfaces.

Experimental

Material and Methods
The population of 119 strains of Corynebacterium isolated from human skin on the back and the forehead was investigated: C. afermentans subsp. lipophilum (10 strains), C. diphtheriae var. intermedius (3 strains), C. jeikeium (29 strains), C. kroppenstedti (3 strains), C. urealyticum (4 strains), CDC group F1 (5 strains), CDC group G1 (15 strains), CDC group G2 (34 strains), Corynebacterium spp. (16 strains). The number of strains used in experiments reflected particular species participation within Corynebacterium genus population on skin (Kaźmierczak et al., 2005). All strains were cultured and stored on TYT80 medium (Kaźmierczak and Szewczyk, 2004).

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Microbial adherence to hydrocarbons was determined according to Rosenberg et al. (1980) method (MATH test). Strains were grown on nutrient broth (POCH) supplemented with 0.1% Tween 80 at 37°C for 72 h (orbital shaker – 90 cycles/min). Cells were harvested, washed twice with demineralised water and resuspended in a 10 mM potassium phosphate buffer adjusted to pH 5 with HCl. Optical densities of bacterial suspensions were standardized at 600 nm to approximately $3 \times 10^8$ CFU/mL $(A_0)$. 150 µL of $p$-xylene was added to a glass tube with 3 mL of bacterial suspension which was then vortexed vigorously for 60 s. After 15 minutes, phases were separated and aqueous phase was carefully removed to measure the optical density of cells remaining in suspension $(A_t)$. Hydrophobicity was calculated as the percentage of cells partitioning in the hydrocarbon phase. The % of $p$-xylene partitioning was determined by the formula: $= (A_0 – A_t/A_0) \times 100$. Strains were tested in triplicate. When the mean adherence to $p$-xylene was $\leq 30\%$, strains were considered hydrophilic, those whose values were $> 30\%$ were considered hydrophobic, and among them, highly hydrophobic strains gave values $\geq 70\%$.

Quantification of biofilm development was performed according to modified Tsai method (Tsai et al., 1988). Strains were grown in TSB broth (Biocorp) supplemented with 0.03% Tween 80 (ICN Biomedicals) at 37°C for 3 hours on an orbital shaker and incubated stationary for 93 hours. Culture medium was decanted and the biofilm formed on glass surface was washed three times with demineralised water, dried at room temperature and fixed using Carnoy’s solution (absolute ethanol, chloroform, glacial acetic acid 6:3:1) for 15 s. Fixed biofilm was stained with 0.1% safranine for 1 h, washed three times with demineralised water, dried and hydrolysed with 0.2 M NaOH at 85°C for 1 h. The optical density was measured at 540 nm with a Microplate Reader 680 (Bio Rad). All strains were assayed in triplicate. Biofilm-producing strains were arbitrarily defined to have mean $A_{540} > 0.06$. The biofilm-producing strains were classified as average ($0.06 < A_{540} < 0.2$) and abundant biofilm-producers ($A_{540} > 0.2$).

**Results**

Many investigated strains presented aptitude for spontaneous auto-aggregation when growing in nutrient broth. 23.5% of strains formed compact clusters during growth, sometimes suspended in completely clear medium. We could classify these strains as rough. This observation led to an assumption about their strong hydrophobicity. In Table I the percentage of rough and smooth strains in particular species are shown.

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of strains</th>
<th>Rough strains (%)</th>
<th>Smooth strains (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. afermentans subsp. lipophilum</td>
<td>10</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>C. diphtheriae var. intermedius</td>
<td>3</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>C. jeikeium</td>
<td>29</td>
<td>27.6</td>
<td>72.4</td>
</tr>
<tr>
<td>C. kroppenstedtii</td>
<td>3</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>C. urealyticum</td>
<td>4</td>
<td>25.0</td>
<td>75.0</td>
</tr>
<tr>
<td>CDC group F1</td>
<td>5</td>
<td>40.0</td>
<td>60.0</td>
</tr>
<tr>
<td>CDC group G1</td>
<td>15</td>
<td>13.3</td>
<td>86.7</td>
</tr>
<tr>
<td>CDC group G2</td>
<td>34</td>
<td>23.5</td>
<td>76.5</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>16</td>
<td>12.5</td>
<td>87.5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of strains</th>
<th>Number of hydrophobic strains</th>
<th>Number of hydrophobic strains*</th>
<th>Hydrophobic (% of all 119 strains of tested population)</th>
<th>Hydrophobic (% of all 119 strains of tested population)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. afermentans subsp. lipophilum</td>
<td>10</td>
<td>0</td>
<td>10 (3)</td>
<td>0</td>
<td>8.4</td>
</tr>
<tr>
<td>C. diphtheriae var. intermedius</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>C. jeikeium</td>
<td>29</td>
<td>4</td>
<td>25 (3)</td>
<td>3.4</td>
<td>21.0</td>
</tr>
<tr>
<td>C. kroppenstedtii</td>
<td>4</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>2.5</td>
</tr>
<tr>
<td>C. urealyticum</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>3.4</td>
</tr>
<tr>
<td>CDC group F1</td>
<td>5</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>CDC group G1</td>
<td>15</td>
<td>1</td>
<td>14</td>
<td>0.8</td>
<td>11.8</td>
</tr>
<tr>
<td>CDC group G2</td>
<td>34</td>
<td>0</td>
<td>34 (7)</td>
<td>0</td>
<td>28.5</td>
</tr>
<tr>
<td>Corynebacterium spp.</td>
<td>16</td>
<td>2</td>
<td>14 (5)</td>
<td>1.7</td>
<td>11.8</td>
</tr>
<tr>
<td>All strains investigated</td>
<td>119</td>
<td>7</td>
<td>112 (18)</td>
<td>5.9</td>
<td>94.1</td>
</tr>
</tbody>
</table>

* in brackets – number of strains presenting strong adhesion
The MATH method based on the degree of adherence to the interface of hydrocarbon (p-xylene) and water (buffer pH 5) showed that more than 94% of strains were hydrophobic. Eighteen strains presented very strong adhesion to hydrocarbons and only seven were hydrophilic.

The number of hydrophobic strains present in particular species is shown in Table II. Strongly hydrophobic strains belonged to *C. afermentans* subsp. *lipophilum*, *C. jeikeium*, CDC group G2 and many strains of *Corynebacterium* which were not identified to species level. The data presented help to estimate the influence of particular species on the general hydrophobicity of all strains forming the population of corynebacteria inhabiting human skin.

In the biofilm formation experiments 90 out of the 119 strains (75.6%) developed biofilm (Table III). Ten of them produced biofilm abundantly. Strains able to form biofilm came from all species. The biofilm-forming ability of the whole population of corynebacteria living on the skin depends on the two most numerously represented taxa CDC group G2 (22.8% producers) and *C. jeikeium* (17.6 %). The correlation between cell surface hydrophobicity and their ability to form biofilm was analysed. Among all MATH tested hydrophobic strains (112), one hundred were also able to form biofilms. Almost all strongly hydrophobic strains formed biofilms (94.4%). Statistical analysis by chi$^2$ test revealed strong interdependence of these features in the investigated strains (p<0.05).

Biofilm formation was also a trait of strains of corynebacteria that aggregated during growth in liquid media (Figure 1). The correlation of these two features was also statistically significant (chi$^2$ test; p<0.05).

![Fig. 1. Biofilm formation and rough type growth in liquid media](image_url)
Discussion

The emergence of opportunistic nosocomial infections is commonly known. These infections are frequently caused by opportunistic pathogens originating from natural physiological endogenous flora. So, characterization of well-known and newly described opportunistic pathogens is an incessant challenge for researchers. Among coryneform bacteria living on human skin almost 85% are lipophilic. This population is a very constant component of the natural flora of the skin in all examined samples. *C. jeikeium* and CDC group G2 predominate (Kaźmierczak and Szewczyk, 2004; Kaźmierczak et al., 2005).

The pathogenicity of some lipophilic species has already been characterized (Funke et al., 1997). The attention of authors has concentrated on the most often isolated *C. jeikeium* causing septicemia and various infections in surgical patients undergoing treatment using different medical devices. According to Williams et al. (1993) CDC group G are the second most frequently occurring corynebacteria isolated from opportunistic infections. *C. urealyticum* was connected with urinary tract infections and *C. afermentans* subsp. *lipophilum* was isolated from blood specimens and abscesses. Most clinically isolated strains were multi-resistant to antibiotics (Funke et al., 1997).

The constant occurrence and domination of these species of corynebacteria on human skin and their frequent isolation from nosocomial infections must be closely related and must be also an effect of their special relationships with skin and artificial surfaces *i.e.* hydrophobicity and other features that promote biofilm formation. Hydrophobicity plays a major role in bacterial adhesion (Absolom, 1988; Bendinger, 1993) and facilitates spread of the organism by bacteria ingested by phagocytes. Hydrophobicity increases and biofilm formation becomes more intensive under conditions of developing infection in the presence of serum (Olson et al., 2002) and iron limitation (Baldssarri et al., 2001; Moreira et al., 2003). Knowledge about these features in corynebacteria mainly concerns the adhesion ability of pathogenic *Corynebacterium diptheriae*. This is attributed to their hydrophobicity connected with the structure of the cell wall, specific for the whole genus. This contains different types of long-chain corynomycolic acids; chain length ranges from 22 to 36 carbon atoms (Bendinger et al., 1993). There is no data concerning skin habitants, especially lipophilic species. Lipids responsible for a barrier function of the skin make an excellent base for adherence of hydrophobic bacteria. The lipid layer is also the key to colonization of the skin as these bacteria need lipids for growth. Almost all lipophilic strains examined in this research were hydrophobic. The ability to form biofilm was presented by almost 76% of strains. The most hydrophobic and active in biofilm formation were *C. jeikeium* and CDC group G2. As they are the most numerous taxa represented among corynebacteria naturally inhabiting skin, the probability of contamination of wounds, implants and different medical devices must be very high.

Hydrophobicity and biofilm formation seems to be crucial for both skin colonization and developing opportunistic hospital infections. *Staphylococcus epidermidis* is well known as the main etiologic factor of these kind of infections. This species is recognized, among other staphylococci important for human pathology, as the best biofilm producer and often develops biofilms on the surfaces of intravenous catheters and other medical devices. Mixed populations of corynebacteria and staphylococci may form a mutually supporting “organism” which can efficiently oppose immune response and antibiotic action (Bayston et al., 1994). The presence and role of *Corynebacterium* strains in this kind of infection is still seldom noticed, due, as Funke et al. (1997) stated, to the relatively small database of knowledge gathered concerning these difficult to cultivation microorganisms. Their role must be significant considering our data presented here.

Acknowledgments. This study was supported by grant 502-305/2006 from Medical University of Łódź.

Literature


Iron Supply of Enterococci by 2-oxoacids and Hydroxyacids

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Abstract

Only 9 (11.2%) out of 80 studied bacterial strains were able to utilize iron saturated 2-oxo acids and hydroxyacids and grow on o-phenantroline containing media. These strains belonged to Enterococcus faecalis and Enterococcus faecium species and were isolated from clinical material. Iron sources utilized by all of these strains were Fe(III) complexes with pyruvic, 2-oxobutyric, 4-methylthio-2-oxobutyric, 2-oxo-3-methylvaleric, 2-oxoisocaproic and 2-oxoadipic acids. None of the nine strains released 2-oxoacids to environment during growth in iron excess Fe + medium and iron deficient – Fe – (Chelex) medium. In Fe – (phenantroline) medium, when the growth was strongly inhibited, only pyruvic acid was released. Iron uptake from 59Fe(III)-pyruvate was depended on iron deficiency during growth: cells harvested from Fe – (phenantroline) medium have acquired the most amount of iron. 2,4-Dinitrofenol was a strong inhibitor of 59Fe(III) iron uptake. Release of pyruvic acid is not subject to negative derepression and does not require the presence of iron as its inductor. It appears in the environment as a response to growth inhibiting stress caused by the iron deficiency but contrary to siderophores are not specially synthesized for iron assimilation. Therefore, it is only primary metabolism products released by damaged, but metabolic active cells.

Key words: Enterococcus spp., 2-oxoacids, siderophores, iron acquisition

Introduction

The free iron concentration – $10^{-18}$ M in human body is not sufficient to support bacterial growth (Ratledge and Dover, 2000). Therefore they need specific mechanisms for the acquisition of iron from their host. At least four different mechanisms by which bacteria acquire iron from its carriers have been proposed: the proteolytic cleavage of carrier resulting in the disruption of the iron-binding sites, reduction of bound Fe (III) to Fe(II) and consequent release of Fe(II), through a direct surface interaction between carrier and bacterial receptor and secretion of low molecular mass iron chelating agents – siderophores (Williams and Griffiths, 1992).

Growth promotion and iron transport studies revealed that certain primary metabolites – 2-oxoacids and hydroxyacids may serve as bacterial siderophores. The first evidence came from studies of Proteus mirabilis (Evanylo et al., 1984) and later the other Proteus species, related genera as Providencia and Morganella (Drechsel et al., 1993) and from E. coli (Reissbrodt et al., 1993), Pasteurella hemolytica and P. multocida (Reissbrodt et al., 1994), staphylococci and micrococci (Heuck et al., 1995). Moreover, strains of P. hemolytica and P. multocida that were unable to synthesize classical siderophores produced large amounts of pyruvic acid (Reissbrodt et al., 1994) while some producing siderophore coagulase-negative staphylococci excreted 2-oxo acids in high concentration under iron-restricted conditions (Lisiecki et al., 1994; Heuck et al., 1995).

Enterococci have produced siderophore belonging to hydroxamate chelator class (Lisiecki and Mikucki, 1999). Growth promotion test has indicated that Fe(III)-dicitrate complex stimulated growth of enterococci (Lisiecki and Mikucki, 2004). This paper reports investigations on 2-oxoacids and hydroxyacids functioning as siderophores in Enterococcus genus.

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Experimental

Materials and Methods

Bacterial strains. Eighty strains of Enterococcus genus were used in the study. Forty nine of them originated from the departmental collection, and others from the National Institute of Public Health in Warsaw, Czech National Collection of Type Culture (CNCTC), Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) and American Type Culture Collection (ATCC). Enterococci from departmental collection were identified with API-STREP system (bioMerieux).

Growth media. Strains were grown in the medium composed of (per litre): 3 g Casamino Acids Vitamin Free (Difco); 3 g Yeast Extract (Difco); 3 g KH₂PO₄; 5 g NaCl; 1 g NH₄Cl; 0.09 g MgCl₂; 0.01 g CaCl₂; 12.1 g Tris (BDH) and 20 g glucose (Sigma). pH of medium was adjusted to 7.2. Concentration of iron was reduced either by using polyaminocarboxylate resin Chelex 100 (200–400 mesh, BioRad) to approximately 3.5 × 10⁻⁷ M or by adding o-phenantroline at concentration corresponding to MIC or 50% MIC of the investigated strain. Solid medium contained 2% Agar N° 1 (Oxoid).

Suspensions density and viable count determination. The optical density of suspension and cultures was measured in UV/VIS Cary 1 (Varian) spectrophotometer at the 580 nm and viable count was estimated by using serial dilutions in buffered 0.155 M NaCl, pH 7.2 and standard plate methods on 4% Trypticase Soy Agar (Difco).

Growth conditions. Strains were initially iron-starved in Fe⁺ (Chelex) medium for 18 hours at 37°C with constant shaking. The starved suspensions were used to inoculate (5% v/v) media with different iron content: Fe⁺ with 100 µM iron in the form of FeSO₄·7H₂O, Fe⁺ (Chelex) subject to Chelex resin and Fe⁻ (phenantroline) containing o-phenantroline at concentration corresponding to 50% MIC of tested strains. Media were incubated at 37°C for 24 hours under constant shaking. Cultures were centrifuged (9500 × g, 15 min, 4°C) then the supernatant was filtered through the membrane filter (0.22 µm, Millipore).

2-oxo acids and hydroxycids used. The following chemicals were used: pyruvic acid, phenylpyruvic acid, 4-hydroxyphenylpyruvic acid, 3-indolylpyruvic acid, oxaloacetic acid, mesoxalic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxoisovaleric acid, 5-aminovalulinic acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid, 2-oxoglutaric acid, phenylpyruvic acid, 3-indolylpyruvic acid, oxaloacetic acid, mesoxalic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxoisovaleric acid, 5-aminovalulinic acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid, 2-oxoglutaric acid, phenylpyruvic acid, 3-indolylpyruvic acid, oxaloacetic acid, mesoxalic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxoisovaleric acid, 5-aminovalulinic acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid, 2-oxoglutaric acid, phenylpyruvic acid, 3-indolylpyruvic acid, oxaloacetic acid, mesoxalic acid, 2-oxobutyric acid, 4-methylthio-2-oxobutyric acid, 2-oxoglutaric acid, 2-oxovaleric acid, 2-oxoisovaleric acid, 5-aminovalulinic acid, 2-oxo-3-methylvaleric acid, 2-oxoisocaproic acid, 2-hydroxyisocaproic acid at 160 nmol mL⁻¹.

Determination of MIC for o-phenantroline. MIC for o-phenantroline was determined by agar dilution method on Mueller-Hinton 2 medium (bioMerieux) using twofold serial dilution of o-phenantroline at a concentrations ranging from 10 µg mL⁻¹ to 130 µg µL⁻¹ in accordance with CLSI standards (Clinical Laboratory Standards Institute, 2005).

Growth promotion tests. 0.1 mL of standardized suspension of the iron-starved strain (A₅80nm = 0.1) was added to 20 mL of melted agar medium with o-phenantroline at concentration corresponding to MIC for tested strains and then poured on plastic Petri dishes (Ø 14 cm, Nunc). After agar setting and 24 h storage of plates at 4°C, tissue paper discs (Whatman N° 3, Ø 6 mm) were placed on their surface and saturated either with 5 µl of iron complex with 2-oxo acid and hydroxycids (25 µg iron chelator per disc) or iron complexes of siderophores (25 µg iron chelator per disc), control iron solution (25 µg iron per disc) was also used. Growth around the discs demonstrating the ability of the tested strain to utilize iron sources was estimated after 24 and 48 h of incubation at 37°C.

Determination of iron concentration. Iron concentration in media was determined as described by Gadia and Mehra method (1977) with ferrozine (Sigma) using UV/VIS Cary 1 (Varian) spectrophotometer at 526 nm.

Determination of 2-oxoacids by high-performance liquid chromatography (HPLC). The assay of 2-oxoacids in supernatants of cultures was performed with HPLC of their 2-quinoxalinole derivatives according to the Hayashi et al. method (1993) to achieve the ratio 1:30 of iron to ligand.

Control iron solutions and siderophores. The following control iron solutions were used: ferric sulfate, ferric ammonium sulfate and ferric ammonium citrate (Sigma). Complexes of siderophores: rhodotorulic acid (Sigma), 2,3-dihydroxybenzoic acid (Sigma) and desferrioxamine B (Ciba-Geigy) with iron were prepared according to Dreschel et al. method (1993) to achieve the ratio 1:1 of iron to ligand.

Preparation of cells lysate. Protoplast of enterococci was prepared according to the method of Zorzi et al. (1996) and were lyzed as described by Lindberg (1981). Total protein concentration in lysates was determined by the method of Lowry (1951).

Enzyme assay. Deamination of L-alanine by cell lysates was assayed spectrophotometrically at 340 nm with L-alanine (Sigma) and NAD (Sigma) as substrates according to Bergmeyer (1985) method.

Statistical analysis. Statistical analysis was performed with the Statistica PL computer programme (StatSoft). Statistical significance was defined as p<0.05.

Results

All strains utilized iron from its control sources in form of Fe (II) and Fe (III) salts and were able to grow in media containing o-phenantroline. The minimal inhibitory concentration of o-phenantroline (MIC) values for investigated strains were varying within a range of 30–120 µg mL⁻¹ (Table I). Therefore, these strains
Iron supply of enterococci

Absolutely required iron in form of ferric and ferrous salts for the growth. The best growth was obtained for the organic Fe(III) salt – ferric ammonium citrate. In 63.3% of studied strains the growth zone diameter was larger than 20 mm. The smallest growth stimulating effect was observed for inorganic Fe(II) – ferric ammonium sulfate because in more than 98% of strains the growth zone diameter was at the range from 10 to 15 mm.

Ferric complexes with 2-oxo acids and hydroxyacids were utilized by only 9 (11.2%) out of 80 investigated enterococcal strains which growth was stimulated in o-phenantroline medium (Table I). These strains belong to *E. faecalis* and *E. faecium* species and were isolated from clinical material. Only *E. faecium* strain utilized Fe(III) of all iron complexes.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>MIC o-phenantroline (µg mL⁻¹)</th>
<th>Ferric complexes of 2-oxo and hydroxyacids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em> BD 122</td>
<td>100 ++ (+) – – +++ (+) ++ (+) (+) ++ ++ ++ – ++</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> BD 123</td>
<td>70 + – – ++ ++ (+) ++ ++ (+) ++ ++ – ++ + +</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> BD 160</td>
<td>30 + – – – ++ – + – – (+) – + + + (+) ++</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 449</td>
<td>70 ++ – – + ++ (+) ++ + + – +++ + ++ + +</td>
<td></td>
</tr>
<tr>
<td><em>E. faecalis</em> 605</td>
<td>110 ++ – – – – – – (+) ++ – ++ – ++ – (+) – (+)</td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> BY 1</td>
<td>120 ++ – – – – + (+) ++ ++ (+) – – ++ + + – ++</td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> BY 6</td>
<td>70 ++ (+) ++ + + + ++ + ++ (+) ++ + + + +</td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> BY 13</td>
<td>115 ++ (+) – ++ – – + (+) + + + + (+) + + + +</td>
<td></td>
</tr>
<tr>
<td><em>E. faecium</em> BY 49</td>
<td>115 ++ (+) – – (+) + + – (–) – + – + – +</td>
<td></td>
</tr>
</tbody>
</table>

--, no growth; (+), poor growth; +, growth zone 10–15 mm; ++, growth zone 16–20 mm; ++++, growth zone 21–30 mm.

Oxaloacetic and 2-oxoglutaric acids, intermediate metabolites of tricarboxylic acids cycle, differently stimulated growth of enterococci. Iron Fe(III) from oxaloacetic acid complex was used by all, except one (*E. faecalis* BD 160, 605 and *E. faecium* BY 49). 2-Oxoglutaric acid weakly (indicated as + and (+)) promoted the growth of six strains except for *E. faecalis* BD 160, 605 and *E. faecium* BY 49. Iron Fe(III) complexes with branched 2-oxoacid: 2-oxo-3-methylvaleric and 2-oxoisocaproic acids stimulated the growth of all strains. 2-Oxo-3-methylvaleric acid had the strongest effect (indicated as +++ and +++) while 2-oxoisovaleric acid was a very weak growth stimulator of only three strains. Iron Fe(III) complexes with 2-oxoadipic and 5-aminolevulinic acids were utilized by all examined strains, however 2-oxoadipic acid had a stronger growth stimulating effect (indicated as +++) (Table I).
Investigated strains were also able to utilize iron from its complexes with hydroxamate siderophores – ferrioxamine B and rhodotorulic acid, and catecholate siderophore – 2,3-dihydroxybenzoic acid. The lowest stimulated growth was observed for ferrioxamine B (Table II).

None of the nine strains released 2-oxoacids to the environment during growth on Fe⁺ medium with the iron excess and in Fe⁻ (Chelex) medium with the iron deficiency. Only the culture in Fe⁻ (phenantroline) medium containing chelator at 50% of minimal inhibitory concentration (MIC), with the high iron deficiency released 2-oxoacids (pyruvic acid) into the environment (Table III, Fig. 1.). Strains of *E. faecalis* released its low amounts: from 0.05 (BD 123 and 449 strains) to 0.21 nmol 10⁶ cfu⁻¹ (strain 605), an average 0.1 nmol 10⁶ cfu⁻¹. *E. faecium* strains released almost 60-times more of this metabolite: from 0.66 (strain BY 49) to 14.61 (strain BY 49) nmol 10⁶ cfu⁻¹, an average 5.9 nmol 10⁶ cfu⁻¹. On the other hand data calculated per mL of culture supernatants showed that *E. faecalis* strains released more of pyruvic acid: from 4.36 (strain 449) to 27.78 (strain 605) nmol mL⁻¹ (an average 11.58 nmol mL⁻¹). *E. faecium* strains released less of this metabolite: from 2.67 (strain BY 1) to 5.26 (strain BY 6) nmol mL⁻¹, (an average 3.79 nmol mL⁻¹). The differences in the amount of released pyruvic acid to the Fe⁻ (phenantroline) medium by *E. faecalis* and *E. faecium* strains were statistically significant (p<0.05). The ability of L-alanine deamination to pyruvic acid could not be detected in none of the enterococcal strains. The growth of all strains in Fe⁻ (phenantroline) medium was inhibited, less in *E. faecalis* and much more in *E. faecium* strains. In comparison with cultures in Fe⁺ medium, for *E. faecalis* strains it ranged from 39.1% (strain BD 122) to 80.9% (strain 449) of the number of the cfu mL⁻¹ and for *E. faecium* strains from 7.7% (BY 1) to 33.7% (BY 49) of the cfu mL⁻¹.

In the studies on 2-oxoacids participation in iron Fe(III) assimilation by enterococci and elucidation of the process kinetic ⁵⁹Fe(III) – pyruvic complex and resting cells of three strains: *E. faecalis* 449, *E. faecium* BY 1 and BY 49 were used. The cells were harvested from cultures with different iron concentration: Fe⁺, iron excess and iron deficiency of various degrees Fe⁻ (Chelex) and Fe⁻ (phenantroline). The crucial differences between these strains were the amounts of pyruvic acid released per 10⁶ cfu⁻¹ during the growth in Fe⁻ (phenantroline) medium: *E. faecalis* 449 has released 4.63 nmol mL⁻¹ and 0.05 nmol 10⁶ cfu⁻¹ of pyruvic acid, *E. faecium* BY 1 — 2.67 nmol mL⁻¹ and 4.23 nmol 10⁶ cfu⁻¹ and *E. faecium* BY 49 – 2.69 nmol mL⁻¹ and 0.66 nmol 10⁶ cfu⁻¹.

All strains harvested from Fe⁺ medium assimilated isotope ⁵⁹Fe(III). *E. faecalis* 449 strain took more quickly greater amounts

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**Table II**

<table>
<thead>
<tr>
<th>Iron saturated siderophores</th>
<th>Bacterial strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. faecalis</em> BD 122</td>
</tr>
<tr>
<td>Ferrioxamine B</td>
<td>++</td>
</tr>
<tr>
<td>Rhodotorulic acid</td>
<td>+</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid</td>
<td>+</td>
</tr>
</tbody>
</table>

MIC o-phenantroline (µg mL⁻¹): 100, 70, 30, 70, 110, 120, 70, 115, 115

+, no growth; (+), poor growth; +, growth zone 10–15 mm; ++, growth zone 16–20 mm; ++++, growth zone 21–30 mm.

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![Fig. 1. HPLC profiles of 2-quinoxalinol derivatives of 2-oxoacids in culture supernatant of *E. faecalis* BD 123 strains (A) and standard pyruvic acid (B). PA, pyruvic acid; IS, internal standard (2-oxocaprylic acid)](image-url)
of iron than *E. faecium* BY 49 and BY 1: after 30 minutes cells contained 13.29, 3.2 and 2.18 nmol $^{59}$Fe(III) per mg of dry weight of bacteria, respectively (28.3%, 7.3% and 4.6% of initial isotope dose) (Fig. 2, 3, 4). Bacteria harvested from Fe$^-$ (Chelex) medium with lower level of iron deficiency, did not uptake $^{59}$Fe(III) more actively. *E. faecalis* 449 strain harvested from Fe$^+$ medium utilized 13.29 nmol $^{59}$Fe(III) (28.3 % of initial isotope dose), and from Fe$^-$ (Chelex) medium – 8.82 nmol $^{59}$Fe(III) (18.8 % of initial isotope dose). Small and not increasing with time amounts of isotope taken by *E. faecium* BY 1 and BY 49 growing in Fe$^-$ (Chelex) were 2–3 times lower than those assimilated by cells derived from the Fe$^+$ medium (Fig. 3, 4).

### Table III

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Fe$^+$ medium</th>
<th>Fe (Chelex 100) medium</th>
<th>Fe$^-$ (phenantroline) medium$^{**}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Growth* cfu mL$^{-1}$ 24 h</td>
<td>Pyruvic acid nmol 10$^6$ cfu$^{-1}$</td>
<td>Growth* cfu mL$^{-1}$ 24 h</td>
</tr>
<tr>
<td><em>E. faecalis</em> BD 122</td>
<td>$156 \times 10^6$</td>
<td>0</td>
<td>$87 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecalis</em> BD 123</td>
<td>$545 \times 10^6$</td>
<td>0</td>
<td>$540 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecalis</em> BD 160</td>
<td>$78 \times 10^6$</td>
<td>0</td>
<td>$51 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecalis</em> 449</td>
<td>$126 \times 10^6$</td>
<td>0</td>
<td>$126 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecalis</em> 605</td>
<td>$233 \times 10^6$</td>
<td>0</td>
<td>$173 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecium</em> BY 1</td>
<td>$8.2 \times 10^6$</td>
<td>0</td>
<td>$7.40 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecium</em> BY 6</td>
<td>$4.1 \times 10^6$</td>
<td>0</td>
<td>$4.43 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecium</em> BY 13</td>
<td>$3.68 \times 10^6$</td>
<td>0</td>
<td>$1.96 \times 10^6$</td>
</tr>
<tr>
<td><em>E. faecium</em> BY 49</td>
<td>$19.6 \times 10^6$</td>
<td>0</td>
<td>$18.40 \times 10^6$</td>
</tr>
</tbody>
</table>

* cfu – colony forming units; $^{**}$ contained 50% of MIC o-phenantroline

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Fig. 2. Iron uptake by *E. faecalis* 449 from $^{59}$Fe(III)-pyruvate

Fig. 3. Iron uptake by *E. faecium* BY 1 from $^{59}$Fe(III)-pyruvate

Fig. 4. Iron uptake by *E. faecium* BY 49 from $^{59}$Fe(III)-pyruvate

Fig. 5. Iron uptake by *E. faecalis* 449 from $^{59}$Fe(III)-pyruvate in the presence of 2,4-dinitrophenol (DNF).

Cells were harvested from Fe$^-$ (phenantroline) medium.
High iron deficiency in cultures in Fe\(^{3+}\) (phenanthroline) medium caused more active uptake of iron \(^{59}\)Fe(III) by all strains. After 30 minutes \(E. \ faecalis\) 449 strain harvested from Fe\(^{3+}\) medium, used 13.29 nmol \(^{59}\)Fe(III) (28.3 % of initial isotope dose) per mg of bacteria dry weight, while from Fe\(^{3+}\) (phenanthroline) medium – 21.62 nmol \(^{59}\)Fe(III) (46.1% of initial isotope dose) (Fig. 2). \(E. \ faecium\) BY 1 utilized 2.18 nmol (4.6 % of initial isotope dose) and 10.87 nmol (5.1 % of initial isotope dose) \(^{59}\)Fe(III) per mg of dry weight of bacteria (Fig. 3). \(E. \ faecium\) BY 49 strain harvested from Fe\(^{3+}\) medium utilized 3.2 nmol (7.3 % of initial isotope dose) and from Fe\(^{3+}\) (phenanthroline) medium 5.7 nmol (13.1 % of initial isotope dose) (Fig. 4).

The influence of 2,4-dinitrophenol on the process of iron assimilation was tested using \(E. \ faecalis\) 449 strain cultured in Fe\(^{3+}\) (phenanthroline) medium and actively uptaking iron from its \(^{59}\)Fe(III)-pyruvic acid complex. This compound was a strong inhibitor of iron \(^{59}\)Fe(III) uptake. After 30 minute, in the presence of 2,4-dinitrophenol the cells were taking up 1.08 nmol \(^{59}\)Fe(III) (2.36 % of initial isotope dose) while those in absence of this compound – 19.9 nmol \(^{59}\)Fe(III) (42.4 % of initial isotope dose) per mg of dry weight of bacteria (Fig. 5).

Discussion

Only 9 (11.2%) out of 80 studied bacterial strains utilized iron Fe(III) complexes with 2-oxo and hydroxyacids. These strains belong to two species – \(E. \ faecalis\) and \(E. \ faecium\) isolated from clinical material.

The comparison of the iron sources utilization in the form of salts and complexes by enterococci, revealed that the iron salts are the best assimilated sources, subsequently followed by siderophores and 2-oxo and hydroxyacid complexes. Among the last two iron 2-oxoacid complexes were more often used. Fe(III) complexes with pyruvic acid and 2-oxo-3-methylvaleric acid stimulated the growth most effectively.

The structure of 2-oxo or hydroxyacid, particularly its side chain, can influence the transport of iron Fe(III) complex into the cell and growth stimulation. So far only bacteria from the genera \textit{Staphylococcus}, \textit{Micrococcus} (Heuck et al., 1995), \textit{Proteus}, \textit{Providencia} and \textit{Morganella} (Drechsel et al., 1993) have been tested for this ability.

Aromatic side chain of phenylpyruvic and 2-hydroxyphenylpyruvic acids and heteroaromatic chain of indolylpyruvic acid decreased the growth stimulation of enterococci, as compared to pyruvic acid. For \textit{S. epidermidis} phenylpyruvic acid was, similarly to pyruvic acid, the effective iron Fe(III) carrier (Heuck et al., 1995). Genera \textit{Proteus}, \textit{Providencia} and \textit{Morganella} utilized iron Fe(III) from phenylpyruvic and indolylpyruvic acids complexes only which stimulated growth the most effectively. Lipophilicity of side chains of these chelators did not influence the assimilation of iron Fe(III) by enterococci.

Most iron Fe(III) complexes with 2-oxoacids with an elongated non-polar side chain actively stimulated growth of enterococci. Only 2-oxoisovaleric and 2-hydroxyisocaproic acids were very weak iron carriers but not for all strains. 2-Oxoisovaleric acid is valine precursor, 2-oxoisocaproic acid – leucine and 2-oxo-3-methylvaleric acid – isoleucine. The source of these 2-oxoacids in enterococci is the reversible transamination of mentioned aminoacids with 2-oxoglutaric acid and the own cytoplasmic membrane. They may also be taken up from the environment (Chesbro and Evans, 1962). \textit{S. epidermidis} uses only some 2-oxoacids from this group as iron carriers: 2-oxoisocaproic, 2-oxoisovaleric, 2-oxo-3-methylvaleric and 2-hydroxyisovaleric acid (Heuck et al., 1995). The ability of valine and leucine deamination and corresponding 2-oxoacids release in the genera of \textit{Proteus}, \textit{Providencia} and \textit{Morganella}, results in active utilization of these chelators as iron carriers (Drechsel et al., 1993).

The length of the carrier carbon chain in enterococci had no effect on the growth stimulation. Iron Fe(III) complexes with short 2-oxoacids with polar side chains – pyruvic (C\(_3\)) or oxaloacetic (C\(_4\)) acids equally actively stimulated growth as complexes with C\(_3\) or C\(_6\) oxoacids – 2-oxovaleric, 2-oxo-3-methylvaleric and 2-oxoisocaproic acids. Similarly, such complexes influenced the growth of \textit{S. epidermidis} (Heuck et al., 1995). Genera \textit{Proteus}, \textit{Providencia} and \textit{Morganella} either did not use these complexes as iron carriers or their growth stimulation was insignificant (Drechsel et al., 1993).

However, lipophilicity does not always facilitate assimilation of iron Fe(III) complexes. Only hydrophobic 2-oxoacids with an elongated non-polar side chain were excellent (with some exceptions) iron Fe(III) carriers for enterococci, \textit{S. epidermidis} (Heuck et al., 1995) and bacteria from the genera \textit{Proteus}, \textit{Providencia} and \textit{Morganella} (Drechsel et al., 1993). Although pyruvic acid, the most active Fe(III) carrier in enterococci and \textit{S. epidermidis}, did not stimulate the growth of bacteria from the genus \textit{Proteus}, \textit{Providencia} and \textit{Morganella}, its aromatic and heteroaromatic derivatives having practically no influence on enterococci, well stimulated growth of mentioned bacteria (Heuck et al., 1995; Drechsel et al., 1993).
The attempts to detect the ability of alanine deamination in enterococci, which leads to the formation of pyruvic acid, the most effective iron Fe(III) carrier, failed. Enterococci do not deaminate aminoacids, only arginine can be deaminated as an energetic substrate of these bacteria (Holt et al., 1986). Genera Escherichia and Salmonella are also unable to deaminate aminoacids. In consequence, 2-oxoacids are not utilized as iron Fe(III) carriers in its transport to the cell (Reissbrodt et al., 1997). The analogous phenomenon is thus likely to occur in enterococci as the uses 2-oxo and hydroxyacids in iron transport by only 9 of 80 studied strains cannot be a common mechanism of iron uptake. Instead of it hydroxamate siderophores synthesis was found in all eighty examined strains (Lisiecki and Mikucki, 1999).

The decrease of iron concentration in a Fe⁺ (Chelex) medium to approximately 10⁻⁶–10⁻⁷ M, commonly known as the inducing derepression of siderophores synthesis, did not cause the release of pyruvic acid or any 2-oxo acids into the medium by the enterococci. In the similar conditions Salmonella Typhimurium secreted not only pyruvic acid at concentration 5 µmol mL⁻¹ but also 2-oxoglutaric, 2-oxoisovaleric and 2-oxoisocaproic acids (Reissbrodt et al., 1997). The increasing iron stress in a Fe⁺ (phenantroline) medium significantly reduced the growth of E. faecalis and inhibited it strongly in E. faecium, made the pyruvic acid appear in the supernatant of cultures. Similarly, in S. Typhimurium the release of 2-oxoacids was increasing, for pyruvic acid to 5 µmol mL⁻¹ when limited iron availability inhibited the growth of bacteria (Reissbrodt et al., 1997). Coagulase-negative staphylococci released a lot of 2-oxoacids (S. cohnii 80 nmol mL⁻¹ of pyruvic acids), when their growth was inhibited by the presence of iron chelator (Heuck et al., 1995). In comparison to these data the enterococci released more less amounts of pyruvic acid from 2.67 nmol mL⁻¹ to 27.78 nmol mL⁻¹. The synthesis and secretion of siderophores occur only during the bacterial growth as a result of an inductive signal while 2-oxoacids are products of primary metabolism and are similar to citric acid which promotes growth of E. coli (Ratledge and Dover, 2000; Silver and Walderhaug, 1992; Waggeg and Braun, 1981) and enterococci (Lisiecki and Mikucki, 2004). The increasing iron unavailability strongly reduced the growth of enterococci and the amount of released pyruvic acid was rising. E. faecalis cultures released greater amounts of pyruvic per mL of culture because it contained more cells releasing this metabolite. On the other hand, per number of cfu, E. faecium strains, strongly inhibited during growth, released more amount of pyruvic acid proving the main role of growth inhibition in this phenomenon. These relationships do not support the idea that pyruvic acid release is a process induced by the low iron availability which triggers chelator synthesis derepression or more active primary metabolite release. Pyruvic acid appears after 24 h in Fe⁺ (phenantroline) medium during growth inhibition and cells cover could be less tight (E. faecalis) or/and metabolic activity was continued even with growth inhibition (E. faecium). Therefore, pyruvic acid, in enterococci iron uptake system could be a primary metabolite secreted by metabolically active cells whose growth was strongly inhibited.

The ability of iron uptake from ⁵⁹Fe(III)-pyruvic acid complex by resting cells of three strains of E. faecalis and E. faecium has supported the results of biotests showing that this complex actively stimulated growth of enterococci. Growth promotion then depended on the iron uptake. The activity of assimilation depended, as in S. Typhimurium (Reissbrodt et al., 1997), on the level of iron reserves in the cell. Enterococci cultivated in medium containing o-phenantroline, a strong cell penetrating chelator and probably with the lowest iron reserves, were the most active. The cells harvested from a Fe⁺ (Chelex) medium were not more active in iron uptaking than cells from a Fe⁺ medium, which can be linked with intact endogenous iron reserves. It is necessary to discuss the contradiction between pyruvic acid releasing and iron acquisition from ⁵⁹Fe(III)-pyruvate complex by E. faecalis 449 and E. faecium BY 1 strains. In this two experiments the different bacterial model was used. The resting cells harvested from medium containing 50% MIC of o-phenantroline were used in iron uptake experiment and pyruvic acid was released by the cells growing in presence of 50% MIC of o-phenantroline. It seems that at such experimental differences simple relationship between pyruvic acid releasing and iron acquisition could not be expected. The comparison strains E. faecium 49 and BY 1 has supported this supposition. Both of them have released the same amounts of pyruvic acid – 2.67 and 2.69 nmol mL⁻¹ and acquired different amount of iron from ⁵⁹Fe(III)-pyruvate complex, respectively – 10.87 nmol mL⁻¹ and 5.7 nmol mL⁻¹ dry weight of cells.

In conditions of limited iron availability, 2-oxo and hydroxyacids take part in iron delivery only in few enterococci. The pyruvic acid release is not subject to negative derepression and does not require the presence of iron as its inductor. It appears in the environment as a response to growth inhibiting stress caused by the iron deficiency but contrary to siderophores are not specially synthesized for iron assimilation. Therefore, it is only primary metabolism products released by damaged, but metabolic active cells.

Acknowledgments. This research was supported by the grant from Medical University at Łódź (No 502-13-224)
Literature


The Growth, Ferrous Iron Oxidation and Ultrastructure of *Acidithiobacillus ferrooxidans* in the Presence of Dibutyl Phthalate

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**Abstract**

The iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* is an example of strictly chemolitotrophic extremophile occurring in acidic environments. The prime niche of these microorganisms is an environment with low pH and high concentrations of iron, sulfide minerals or sulfur. Besides these environments, *A. ferrooxidans* is also isolated from heavy metal contaminated environments such as soil and sewage sludge and is known to be useful in bioremediation processes of these environments. In the current study, the influence of dibutyl phthalate on the growth, activity and ultrastructure of *A. ferrooxidans* ATCC19859 was shown. The presence of dibutyl phthalate in 9K medium did not influence *A. ferrooxidans* growth or ability to oxidize ferrous iron although changes in growth medium were accompanied by changes in the protein expression profiles of periplasmic fractions and remarkable changes in ultrastructure of the cell.

**Key words:** *A. ferrooxidans*, dibutyl phthalate (DBP), ferrous iron oxidation, ultrastructure of the cell

**Introduction**

The iron-oxidizing bacteria *Acidithiobacillus ferrooxidans* is an example of obligate chemolithotroph and an extremophile occurring in acidic environments. Extreme acid environments of pH 2–5 are commonly associated with coal mines, draining waters and mining effluents. In addition to a low pH, these environments are characterized by high concentrations of reduced sulfur and iron compounds (Kelly and Harrison, 1989). As an obligate extreme acidophile and chemolithotroph, *A. ferrooxidans* needs to be cultivated under special conditions. It requires inorganic energy sources and a comprehensive range of micronutrients. Additionally, it is highly sensitive to soluble organic materials, particularly organic acids, as well as anions. The 9K liquid mineral medium of Silvermann and Lungren (1959) is the best known medium for cultivation of *A. ferrooxidans* under laboratory conditions. For many years it was, however, impossible to obtain growth of different strains of *A. ferrooxidans* on solid media. One of the main reasons was the toxic effect of the hydrolysis products of gelling agents on these bacteria. The application of overlaid solid media made it possible to obtain colonies of all tested strains of this species (Johnson, 1996).

Besides strictly chemolithotrophic and acidic environments, *A. ferrooxidans* is also isolated from other heavy metal contaminated environments such as soil and sewage sludge. The occurrence of these bacteria in sewage sludge is still regarded as doubtful, although it has been reported by several research groups (Tyagi et al., 1993; Zagury et al., 1994, Gomez and Bosecker, 1999; Matlakowska and Skłodowska, 2003). Its presence there seems improbable, especially because of the high pH of sewage sludge, reaching 6–7, as well as the high concentration of organic matter. It should, however, be taken into account that acidic micro-niches can be created in this environment by microbial activity. Such microniches were also observed in the bioleaching of heavy metals from alkaline or slightly neutral ores (Ostrowski and Skłodowska, 1992). The existence of acidic niches in dehydrated digested sewage sludge seems possible because of the high...
concentration of metal sulfides as well as the presence of chemolithotrophic, neutrophilic, and moderate acidophilic sulfur-oxidizing bacteria in this environment (Tyagi et al., 1993). Initial acidification of the sludge can occur by the growth of indigenous, less acidophilic thiobacilli, followed by acidophilic thiobacilli, resulting in reduction of the pH to approximately 2.0. The existence of *A. ferrooxidans* in the presence of organic matter occurring in sewage sludge may also be explained by the fact that most organic compounds are insoluble in water what results that those organic particles are not in direct contact with inner structure of cell and may have influence on outer cell structures only.

The aim of the present study was to investigate the influence of dibutyl phthalate as a model compound of benzenedicarboxylic acid esters, one of the most abundant chemical components of sewage sludge and contaminated soils, on the growth of *A. ferrooxidans*, and on oxidation of ferrous iron by this bacteria. The morphology and ultrastructure of *A. ferrooxidans* cells as well as changes in the protein profile during growth in the presence of dibutyl phthalate were also investigated.

### Experimental

#### Materials and Methods

**Bacterial strain.** *A. ferrooxidans* ATCC 19859 strain obtained from the American Type Culture Collection was used.

**Chemicals.** Di-n-butyl phthalate (DBP) \([\text{C}_{16}\text{H}_{22}\text{O}_4/\text{C}_6\text{H}_4(\text{COOC}_4\text{H}_9)]\) of commercial grade was obtained from Sigma.

**Culture conditions.** Bacteria were cultivated in either 9K mineral medium or in mineral medium supplemented with DBP at a concentration of 25% (v/v). The cultures were maintained in 500 ml flasks on a rotary shaker (100 rpm) at room temperature (22°C) for 14 days. Two controls: sterile 9K and sterile 9K + DBP media, were incubated at the same condition.

**Microbial and chemical analyses.** The number of bacteria cells, pH, and total iron and ferrous iron concentrations in the cultures were analyzed. The cell count of a sample was determined using a microscope counting chamber and standard deviation was calculated with Microsoft Excel software. Total iron was determined by atomic absorption spectrometry (SOOLAR M6, TJA Solution, UK) following the acidification of samples with 6 M HCl. Standard deviation (SD) and relative standard deviation were calculated using Solar MAA software (TJA Solution, UK). Ferrous iron was assayed using the ortho-phenantroline colorimetric method (Hermanowicz et al., 1999). DU-65 Beckman UV-VIS Spectrophotometer with Soft-pac Module Quant II Linear for standard deviation calculation for colorimetric measurements was used.

**Preparation of samples for transmission electron microscopy (TEM).** Bacterial cells were fixed with 3% glutaraldehyde in sodium cacodylate buffer and then treated with osmium tetroxide for 4 hours. Increasing concentrations of ethanol was used for dehydration. Ultrathin sections of epon-embedded cells were cut and treated with uranium acetate and lead citrate.

**Protein isolation and analysis.** *A. ferrooxidans* proteins were separated into cytoplasmic, periplasmic, inner membrane (cytoplasmic) and outer membrane fractions using the procedures of Guiliani and Jerez (1999) and Neu and Heppel (1965). Protease inhibitor (50 µg PMSF ml⁻¹) was used. SDS-PAGE (10%) gel electrophoresis was carried out by the standard procedure of Schägger (1985) and von Jagow (1987). Proteins were visualized by silver staining and analyzed with Image Master 1D Elite (Nonlinear Dynamics) using molecular weight calibration kits (Amersham Pharmacia Biotech). Proteins containing hem groups were detected by o-dianisidine staining according to the method of Francis and Becker (1984).

**MS/MS analysis.** MS/MS identification of selected proteins that had been separated using SDS-PAGE was performed after trypsin digestion using a Micromas ESI-Q-TOF mass spectrometer. The results were analyzed with Mascot software (www.matrixscience.com). The MS analysis was performed at the Environmental Laboratory of Mass Spectrometry, Institute of Biophysics and Biochemistry (Polish Academy of Sciences, Poland).

### Results

**Comparison of growth and Fe (II) oxidation of *A. ferrooxidans* in different media.** In 9K + DBP medium two fractions were visible: water fraction being mineral salt solution and organic fraction of DBP. As is shown on Figure 1, the growth of *A. ferrooxidans* in 9K + DBP occurred only in water fraction. The presence of brown sediment (jarosite) in this fraction indicated the growth of iron-oxidizing bacteria. Bacterial growth was not observed in DBP fraction.

Comparison of the growth curves for *A. ferrooxidans* cultivated in either 9K medium or in 9K + DBP medium showed only slight differences (Fig. 2). The lag phase of both cultures of *A. ferrooxidans* lasted 1 day and the stationary phase was reached after 7 days. The number of bacteria after 14 days of cultivation was 1.23 × 10⁹ and 1.15 × 10⁹ respectively, starting from 4 × 10⁶ cells/ml.

No difference in the ability of iron oxidation was detected. Both cultures of *A. ferrooxidans* showed complete oxidation of ferrous iron after 5 days (Fig. 3). The Fe(II) oxidation observed in both sterile control (9K and 9K + DBP media) was exclusively a chemical process. pH elevation as a result of ferrous iron oxidation was observed in both cultures of *A. ferrooxidans* between days 2 and 4 (Fig. 4). Slow acidification...
Dibutyl phthalate influence on *A. ferrooxidans*

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Fig. 1. Cultures of *A. ferrooxidans* in 9K + DBP medium

subsequently happened. Figure 4 shows the concentration of total iron. Identical decreases in total iron concentrations were observed in both cultures due to precipitation of jarosite (Fig. 5).

**Morphology and ultrastructure of *A. ferrooxidans***. Cross- and longitudinal ultrathin sections of bacterial cells were examined using TEM. Morphological and ultrastructural differences between cells grown
in 9K and those grown in 9K + DBP media were clearly visible (Fig. 6). Cells that were grown in 9K medium showed the typical shape and ultrastructure characteristic for autotrophically grown bacteria. In contrast, cells grown in the presence of DBP were deformed and closely attached to each other. Additionally, the nucleoplasm of cells cultivated in 9K+DBP medium presented a characteristic consistency, which seemed to be very dense and tightly packed. Empty spaces in *A. ferrooxidans* cells were also observed.

**Protein profiles of bacteria grown in the absence and in the presence of dibutyl phthalate.** SDS-PAGE gel electrophoresis was performed to determine the patterns of protein produced by the studied *A. ferrooxidans* strain when grown in either 9K medium with ferrous iron or in the presence of dibutyl phthalate. Cells were fractionated and protein expression profiles of four cellular fractions were compared. The cytoplasmic as well as inner and outer membrane profiles did not show any significant quantitative differences. Proteins identified as: CsoS1C (10 kDa), CbbS (12 kDa) and CbbL (42 Da) were found in the inner membrane of *A. ferrooxidans* cells cultivated under both studied conditions (Fig. 7 B). Two cytochromes of molecular weights 22 and 51 kDa (detected using o-dianisidine staining, results not shown), as well as a subunit of cytochrome oxidase (25 kDa), were present in the inner membrane fraction of both cultures. The blue copper protein, rusticyanin required for the oxidation of iron, was identified in the periplasmic fraction of *A. ferrooxidans* cells grown in 9K medium as well as in 9K + DBP medium.
Significant differences were observed, however, in protein profiles of the periplasmic fraction. The presence of DBP in the medium resulted in a total lack of proteins with a molecular weight more than 20 kDa (Fig. 7 C).
In the presented study, the influence of dibutyl phthalate on the growth, ferrous iron oxidation and ultrastructure of A. ferrooxidans was studied. Phthalic acid esters (PAEs) are a large group of chemicals used as plasticizers in the production of plastics. They are widely distributed in sediments, natural water, wastewater and soil. PAEs are also detected at high concentrations in landfill leachate as well as in sewage sludge. As a result of low water solubility and a high octanol/water partition coefficient, PAEs tend to accumulate in soils and sediments. Phthalic acid esters have been found in sewage sludges at levels of 12–1250 mg kg⁻¹ dry weight and were not degraded during sewage sludge digestion as shown by Rogers, (1996).

The adaptation of A. ferrooxidans to growth in sewage sludge was the aim of our previous study (Matlakowska and Skłodowska, 2003; Matlakowska and Skłodowska, 2005). We investigated the effect of sewage sludge, which combines different chemical and physical factors acting simultaneously on the bacterial cell, e.g. the availability of energy substrates and oxygen, presence of organic compounds and heavy metals, pH, pulp density, sludge particle size, metal speciation. The adaptation of A. ferrooxidans to sewage sludge and its role in metal bioleaching was shown (Matlakowska and Skłodowska, 2003).

The results presented in this paper concentrate on the action of just one factor – dibutyl phthalate on A. ferrooxidans under stable conditions. Bacteria were cultured in the media containing optimal concentrations of ferrous iron and micronutrients, as well as optimal pH and temperature. The concentration of dibutyl phthalate used was 25% (v/v). This concentration was selected as a representative for the sum of all esters in dehydrated digested sewage sludge. It was shown that the presence of dibutyl phthalate in 9K medium did not affect A. ferrooxidans growth or ability to oxidize ferrous iron. In contrast, a prolonged lag phase lasting 4–5 days was observed when bacteria were grown in dehydrated digested sewage sludge containing sulphur and small amount of ferrous iron (Matlakowska and Skłodowska, 2003). Previously, a wide variety of chemicals have been shown to affect the growth and ferrous iron oxidation of A. ferrooxidans. Inhibition of growth and physiological activity was observed in the presence of carbohydrates, organic acids (Tuovinen et al., 1971; Fratini et al., 2000) as well as chemical compounds used for copper extraction (ACORGA, LIX 64, nonylphenol, izodecanol, Alamine) (Torma and Izkovitch, 1976; Bosecker, 1997; Mazuelos et al., 1999). Pronk et al., (1991) indicated that A. ferrooxidans ATCC 21834 could, however, be grown on formate when the substrate supply was growth limiting, for example in formate-limited chemostat cultures. A. ferrooxidans grown under such conditions retained the ability to oxidize ferrous iron at high rates and assimilated carbon using the Calvin cycle. Moreover, under anoxic conditions, A. ferrooxidans was able to reduce ferric iron with sulfur or hydrogen as electron donors (Pronk et al., 1992; Ohmura et al., 2002). Drobner et al., (1990) showed that A. ferrooxidans (ATCC 2327) and two other strains were able to grow using the oxidation of gaseous hydrogen as an energy source.

As described before, sewage sludge is known to have a destructive influence on the growth and cell structure of A. ferrooxidans (Matlakowska and Skłodowska, 2003). Cells grown in medium containing sewage sludge were slightly deformed and contained reserve material. Additionally, carboxysomes were not observed and these cells were embedded in slime and covered by mucosal biofilm. As mentioned above, cells grown in the presence of DBP showed structural changes in peripheral parts of the cell while the outer and inner membranes were unchanged. The mechanism by which DBP influences bacterial cells is unknown. It maybe related to some physical changes in the medium and thus is of an indirect nature. DBP is a well known solvent for a number of organic compounds and is a surfactant as well. Synthetic surfactants affect microbial surface structure, composition, properties and functions (Angelova and Schmauder, 1999). They cause changes in fatty acid compositions and the level of their saturation (Chevalier et al., 1988). Anionic surfactants enhance cell surface hydrophobicity (Marchesi et al., 1994) and increase membrane permeability (Laouar et al., 1996; Chan and Kuo, 1997). Decreased surface tension of 9K medium containing DBP was confirmed (unpublished results) and it may cause changes in the structure of the outer membrane, allowing periplasmic leakage and contact between the surfactant and periplasmic proteins. The result was visible on ultrathin sections (Fig. 6) and as the degradation of large proteins observed in the SDS-PAGE profile of the periplasmic fraction (Fig. 7C). Moreover, increased hydrophobicity as well as decreased relative surface charge of A. ferrooxidans cells were observed during growth in 9K medium with dibutyl phthalate (unpublished results).

The results of SDS-PAGE clearly show that the expression of proteins, which are important to the A. ferrooxidans electron pathway from ferrous iron to oxygen, is not repressed when the organism is grown in the presence of DBP. The changes in expression of proteins of electron pathway were detected in A. ferrooxidans cells cultivated in sewage sludge, and clearly indicate that under these conditions,
the energy demand of cells is lower and partially covered by assimilation of simple organic molecules, such as anaplerotic compounds.

The results of the present study clearly demonstrate that *A. ferrooxidans* can grow and oxidize ferrous iron in the presence of dibutyl phthalate. This compound is an example of chemicals, which does not inhibit its growth or iron oxidation. The ultrastructural changes observed in *A. ferrooxidans* cells are due to physical changes of the medium such as surface tension rather than to direct influence of DBP on bacterial cells. The most important conclusion from presented studies is that the presence of benzenedicarboxylic acid diesters is not major obstacle for industrial application of *A. ferrooxidans* in bioremediation of soils and sewage sludge.

**Acknowledgement.** We wish to thank Dr. E. Lewandowska from Laboratory of Electron Microscopy, Institute of Psychiatry and Neurology, Warsaw for performing TEM observations.

**Literature**


Drobner E., H. Huber and K.O. Stetter. 1990. *Marchesi* J.R., G.F. White, W. A. House and N.J. Russel. 1994. Bacterial cell hydrophobicity is modified during the growth or iron oxidation. The ultrastructural changes observed in *A. ferrooxidans* cells are due to physical changes of the medium such as surface tension rather than to direct influence of DBP on bacterial cells. The most important conclusion from presented studies is that the presence of benzenedicarboxylic acid diesters is not major obstacle for industrial application of *A. ferrooxidans* in bioremediation of soils and sewage sludge.

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Urea and Ureolytic Activity in Lakes of Different Trophic Status

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Abstract

Urea and uraease (U-ase) activity were determined in water samples taken from the surface layers of 17 lakes of different trophic status. Urea concentrations were inversely correlated with the trophic status of the studied lakes and varied from below the detection limit to 25 µmol l⁻¹. Maximal potential ureolytic activity (V_max) ranged from 0.2 to 7.0 µmol l⁻¹ h⁻¹. The highest urea concentrations and the lowest U-ase activities were recorded in the spring, whereas the lowest urea concentrations and the highest rates of urea hydrolysis were observed late in summer, during heavy phytoplankton blooms. Since in the majority of the Great Mazurian Lakes microplankton growth was limited by nitrogen supply, urea was an important N source for both auto- and heterotrophic planktonic microorganisms throughout the growth period. U-ase activity was mainly related to the seston. Only up to 25% of total activity could be attributed to free enzymes dissolved in lake water. In epilimnetic water samples the bulk of the ureolytic activity originated from seston-attached bacteria. However, a positive, statistically significant correlation between ureolytic activity and chlorophyll a (Chl_a) concentrations suggests that phytoplankton may also be responsible for at least a some of the observed ureolytic activity in the highly eutrophic Great Mazurian Lakes.

Key words: algae, bacteria, lakes, urea, ureolytic activity

Introduction

Urea may be present in significant concentrations in all types of aquatic environments (Remsen, 1971; McCarthy, 1972; Kristiansen, 1983; Mitamura and Saijo, 1986; Weeb and Haas, 1986; Park et al., 1997; Wilthshire and Lampert, 1999; Berman and Bronk, 2003). Numerous studies carried out in coastal and oceanic waters showed that urea plays a key role in the marine nitrogen cycle (Herbland, 1976; Savidge and Hutley, 1977). However, the function, fate, sources and turnover of urea in inland waters were only extensively studied and are still poorly known. Unlike oceanic habitats, in freshwater urea can be both of allochthonous and autochthonous origin. It is not only a major excretory product of man and terrestrial animals, but also a highly concentrated artificial N fertilizer and constituent of pesticides. Therefore, lakes receive substantial amounts of allochthonous urea as runoff from land and sewage from urban areas (Dugan, 1975). Some urea may also be introduced into natural waters due to atmospheric precipitation (Timperley et al., 1985).

In unpolluted freshwater ecosystems autochthonous urea predominates. The major supply routes of this compound are zooplankton excretion (Wiltshire and Lampert, 1999) and bacterial degradation of organic matter, especially purines, pyrimidines and arginine (Cho et al., 1996; Satoh, 1980; Pedersen et al., 1993; Vogels and Van der Drift, 1976; Therkildsen et al., 1996). Quantitative information on urea distribution in various types of marine environments is relatively rich (Price and Harrison, 1987). However, analogous data concerning freshwater environments are scant. As reported by Berman (1974), Satoh and Hanya (1976) and Mitamura and Saijo (1986) concentrations of urea in lakes and ponds reached 9.1 µmol l⁻¹ and most commonly ranged between 0.5 and 2.0 µmol l⁻¹.

Enzymatic hydrolysis of urea provides substantial amounts of NH₄⁺ and CO₃²⁻ which are the basic mineral N and C forms taken up and metabolized by planktonic microorganisms. Up to now, the majority of studies

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on the assimilation and degradation of urea and on the role of this compound in growth and metabolism of aquatic microorganisms were carried out in algal and bacterial cultures. Field ecological investigations were mainly focused on the utilization of urea as a nitrogen source for the growth of marine phytoplankton (McCarthy, 1972; Ignatiades, 1986) and lately, on the role of this compound as an important N source for toxic dinoflagellates responsible for red tides (Zehr and Ward, 2002). Consequently, our knowledge concerning urea transformations and the ecological significance of this compound for aquatic bacteria and the whole microplankton community in inland aquatic habitats is limited and requires detailed investigation (Berman and Bronk, 2003).

It is generally believed that in the photic zone of marine and freshwater environments urea is utilized primarily by phytoplankton rather than by bacteria (Remsen et al., 1972; McCarthy, 1972; Carpenter et al., 1972, Satoh and Hanya, 1976; Turley, 1986). For instance, Mitamura and co-workers (Mitamura et al., 2000) showed that the diel changes in urea-decomposing activity exhibited a similar pattern to that of the photosynthetic assimilation number, following the diel change in photosynthetic activity. Price and Harrison (1987) found that in the ocean much of urea could be taken up by phytoplankton directly, without its hydrolysis outside the cell. They also pointed out that NH$_4^+$ ions liberated from this compound by intracellular enzymes was not retained by algal cells. Those observations strongly suggested that phytoplankton would not only be competitive to bacteria in terms of urea N assimilation but also could participate in NH$_4^+$ regeneration processes. On the other hand, little is known about the participation of bacteria in urea metabolism in aquatic habitats. Whether bacteria are a source of urea or a sink for it is still an open question (Zehr and Ward, 2002). The wide distribution of urea-decomposing bacteria (Satoh and Hanya, 1976; Satoh 1980) and cyanobacteria (Flores and Herrero, 1994) in aquatic habitats, as well as documented by Park et al. (1997) active urea decomposition during the night (47.1–90 % of the daily urea decomposition activity) may prove indirectly that in freshwater environments also bacteria may substantially participate in urea decomposition processes.

Some authors (McCarthy et al., 1977; Probyn and Painting, 1985) considered that in marine and coastal environments urea nitrogen can be taken up by planktonic microorganisms even in the presence of nitrate and ammonium. However, as postulated by Kristiansen (1983) the uptake of urea by phytoplankton seems to be the most effective in the absence of reduced N sources. According to Kristiansen (1983) ammonium concentrations higher than 1 µmol l$^{-1}$ effectively inhibit urea assimilation. The highest absolute as well as relative urea uptake rates were noted by Kristiansen (1983) during the summer, when concentrations of inorganic N forms in the water were low. Therefore, one can presume that urea can be one of the most important factors affecting phytoplankton growth especially significant in highly eutrophic environments often nitrogen limited. In Chesapeake Bay urea constitutes a small percentage of total dissolved organic N pool. However, it has been shown to contribute from 60 to 80% of the nitrogen utilized throughout much of the year by the planktonic community (Glibert and Terlizzi, 1999).

The main aim of this study was to compare urea concentrations and ureolytic activity in lakes of different trophic status. Moreover we discuss the importance of this compound for N limited freshwater environments and the role of bacteria and phytoplankton in urea decomposition processes.

### Experimental

#### Materials and Methods

**Study area and sampling.** The studies were carried out in the spring and the summer of two vegetation periods. During three sampling sessions (April, August and September 2002) 51 water samples were taken under non-sterile conditions from surface layer (1 m depth) of 17 lakes of the Mazurian Lake District in northeastern Poland. From three of these lakes (Kuc, Mikolajskie and Tatoowski) surface water samples were collected in 2001 and 2002 more intensively i.e. 4 times a season. Additionally, in August 2002 samples from depth profiles of Lake Tatoowski and Lake Mikolajskie were analyzed. Lakes that were taken into consideration represented a wide range of trophic conditions – from mesotrophic to highly eutrophic (Table I).

**Determination of urea concentrations.** For determination of urea concentrations we modified McCarthy’s “enzymatic” technique (McCarthy, 1970) by applying the highly sensitive O-phthalaldehyde (OPA) method (Holmes et al., 1999) for NH$_4^+$ determination instead of the classical procedure of Solorzano (1969). Although according to Price and Harrison (1987) the “urease method” in comparison to chemical urea determination (Newell et al., 1967) underestimates the total urea concentrations, we decided to use it as a method that provides more precise information on readily available urea for planktonic microorganisms than the “chemical” method.

Filtered (0.2 µm Nuclepore) lake water sample was divided into six 5 ml portions and warmed up to 25°C. Three of them were supplemented with 0.25 ml (~10 U) of jack bean U-ase (Type IV, Sigma) solution whereas the next three served as controls. Commercially available U-ase was dissolved in deionized water to 40 U ml$^{-1}$ concentrations just before using. Subsamples with U-ase
and urease-free controls were incubated for 1.5 h at 25°C. After the incubation, 1 ml of each “sample” and “control” replicate was placed in plastic 8 ml vial and supplemented with 3 ml of combined OPA reagent (Holmes et al., 1999). To the controls 0.05 ml of U-ase working solution was additionally added. Combined OPA reagent was not only necessary for \( \text{HN}_4^+ \) assay but also terminated the enzymatic reaction. Addition of U-ase working solution to “controls” after their supplementation with OPA combined reagent equalized volumes of “sample” and “control” and corrected errors caused by contamination of commercially available urease by \( \text{HN}_4^+ \). The reaction with OPA reagent was carried out for 135 min at 20°C in darkness. Fluorescence of all subsamples was read in Shimadzu RF 1501 spectrofluorometer (330 nm ex., 455 nm em.). Ammonium concentrations were calculated from linear regression of the standard curve. Urea concentrations were obtained from the following equation:

\[
C_{\text{Urea}} = 1.05 \left( \frac{A - B}{2} \right)
\]

where: \( C_{\text{Urea}} \) – urea concentration; \( 1.05 \) – volume correction; \( A \) – concentration of \( \text{HN}_4^+ \) in subsamples treated with U-ase before incubation; \( B \) – concentration of \( \text{HN}_4^+ \) in subsamples supplemented with U-ase after incubation.

**Urease activity assay.** U-ase activity was determined as maximal velocity \( (V_{\text{max}}) \) of ammonium liberation in the course of hydrolysis of urea added to the tested water samples. To determine the kinetics of urea hydrolysis, a series of subsamples (10 ml) taken from each lake water sample was supplemented with 0.4 ml of working substrate solutions. Final concentrations of urea in assay were: 0.5, 1.0, 2.5, 5.0, 10.0, 20.0, 50.0 and 80.0 mmol l\(^{-1}\). Substrate stock solution (2.5 M) and working solutions (0.0125, 0.025, 0.0625, 0.125, 0.25, 0.5, 1.25 and 2.0 M), obtained by dilution of urea stock solution in deionized water, were freshly prepared before ureolytic activity determination. Concentrations of \( \text{HN}_4^+ \) were measured twice: at time 0 and after 1.0–3.0 h of incubation at 20°C by method of Holmes et al. (1999) described previously. Since we were interested only in an increase in fluorescence (ammonium concentration) during sample incubation, no additional controls and corrections were needed. For determination of U-ase activity in various seston size fractions differential filtration technique was used. Water samples were filtered using vacuum (up to 8000 Pa) through polycarbonate membrane filters 0.2, 1.0, 10.0 µm Nuclepore and 100.0 and 200.0 µm plankton net. To reduce possible overestimation of U-ase activity by \( \text{HN}_4^+ \) concentrations commonly observed in water of The Great Mazurian Lakes) could inhibit U-ase activity, samples taken from epilimnion (1 m depth) of L. Tałtowisko and L. Mikolajskie in August (ambient \( \text{HN}_4^+ \) concentrations 0.75 and 0.5 µmol l\(^{-1}\), respectively) were enriched with increasing amounts of \( \text{NH}_4\text{Cl} \). U-ase activity in \( \text{NH}_4\text{Cl} \) enriched samples was measured within 0.5 h after ammonium addition. Moreover we have also tested the influence of environmental conditions on U-ase activity. Epilimnetic (1.0 m depth)
and hypolimnetic (1.0 m above of the sediment) water samples (2.0 l) were filtered through Nuclepore membrane filters (0.2 µm). Seston from each sample was suspended in 20 ml of distilled water and divided into two 10 ml portions. One portion of “epilimnetic seston” resuspension was added to 0.45 l of epilimnetic water filtrate (control) whereas another one was added to the same volume of hypolimnetic water filtrate. Similarly, hypolimnetic water filtrate was supplemented with “hypolimnetic” (control) and epilimnetic seston resuspensions. Finally, in each combined sample U-ase activity was measured in respect to in situ temperature.

Other analyses. Temperature, pH, conductivity and oxygen concentrations were measured in situ, using a Water Analyzer H20 (Cole Palmer Instruments). Total numbers of bacterial cell (sum of seston-attached and free-living bacteria) and numbers of free-living bacteria were estimated in samples fixed with formaldehyde (2% v/v final conc.) by DAPI direct count method (Porter and Feig, 1980). To detach seston-attached bacteria for counting, non-filtered water samples were supplemented with sodium pyrophosphate (100 mmol l–1 final conc.), cooled and sonicated with a 5 mm tip (six 5 sec. pulses, ~ 400 W) in UDM-10 Sonicator Techpan, Poland. For counting of free-living microorganisms the same water samples were filtered through a 1.0 µm Nuclepore membrane filter before fixing. The numbers of attached bacteria were calculated as the difference between numbers of total and free living bacterial cells. Chl a was extracted from phytoplankton with 98% v/v ethanol and measured spectrophotometrically (Marker et al., 1980). Total phosphorus was assayed by the Koroleff (1983) method. L-leucine aminopeptidase activity (AMP) was measured according to Siuda and Chróst (2002). Concentrations of proteins and amino acids were determined fluorometrically with OPA (Roth, 1971).

Results

The results of three sampling sessions carried out in April, July and August 2002 in 17 Mazurian lakes of different trophic status and the data obtained during more intensive investigations conducted in three of these lakes during spring-autumn seasons of 2001 and 2002 are summarized in Table II and Table III. Generally, urea concentrations in surface waters of studied lakes varied from the detection limit (0.01 µmol l–1) in L. Mikołajskie in September 2002 to about 25.0 µmol l–1 in L. Kuc in April 2001 (Table III). Typically they averaged 0.5–0.8 µmol l–1 and only seldom exceeded 1.0 µmol l–1 (Table II and Table III). Amounts of urea in lake water were inversely related to their trophic status (Fig. 1 A). Commonly, elevated urea concentrations (12.0–25.0 µmol l–1) were noted in less eutrophic lakes in spring, lower – in more eutrophic environments throughout the rest of the growth period (Fig. 1 A, Table II and Table III). We found that urea was a quantitatively significant constituent of the labile dissolved organic N fraction (LDON),

<table>
<thead>
<tr>
<th>Lake</th>
<th>April 2002</th>
<th>June 2002</th>
<th>September 2002</th>
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<tr>
<td></td>
<td>Urea µM</td>
<td>Urease µmol urea l–1 h–1</td>
<td>Urea µM</td>
</tr>
<tr>
<td>Kuc</td>
<td>0.20 ± 0.04</td>
<td>0.57 ± 0.15</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>Manry</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>Dargin</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.68 ± 0.07</td>
</tr>
<tr>
<td>Przystań</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.50 ± 0.02</td>
</tr>
<tr>
<td>Kisajno</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>Labap</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.55 ± 0.06</td>
</tr>
<tr>
<td>Śniardwy</td>
<td>0.47 ± 0.07</td>
<td>n.d.</td>
<td>0.04 ± 0.03</td>
</tr>
<tr>
<td>Beldany</td>
<td>0.74 ± 0.05</td>
<td>n.d.</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td>Mikołajskie</td>
<td>0.17 ± 0.07</td>
<td>2.36 ± 0.11</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>Niegoćin</td>
<td>0.25 ± 0.04</td>
<td>n.d.</td>
<td>0.21 ± 0.07</td>
</tr>
<tr>
<td>Ryśkie</td>
<td>0.10 ± 0.06</td>
<td>n.d.</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>Boczné</td>
<td>0.23 ± 0.03</td>
<td>n.d.</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Szymoneckie</td>
<td>0.08 ± 0.02</td>
<td>n.d.</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Jagdno</td>
<td>0.15 ± 0.04</td>
<td>n.d.</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>Talty</td>
<td>0.00 ± 0.04</td>
<td>n.d.</td>
<td>0.18 ± 0.04</td>
</tr>
<tr>
<td>Szymon</td>
<td>0.07 ± 0.04</td>
<td>n.d.</td>
<td>0.26 ± 0.05</td>
</tr>
<tr>
<td>Tałtowisko</td>
<td>0.47 ± 0.02</td>
<td>1.81 ± 0.10</td>
<td>0.5 ± 0.04</td>
</tr>
</tbody>
</table>

n.d. – not determined, ± – standard deviation of triplicate determinations
that besides urea consists of other organic N compounds relatively easily available for planktonic microorganisms *i.e.* peptides, proteins and amino acids. During the spring-autumn period urea constitutes about 26–38% (in eutrophic) and up to 44% (in mesotrophic environments) of LDON fraction (Table III).

To test whether relatively high concentrations of urea noted in the early spring could be an effect of slow urea decomposition in this period of the year the relationship between temperature and U-ase activity was examined. We found that ureolytic activity (urea decomposition rate) increased exponentially with the temperature and at 5°C was about 2.25 times lower than at 20°C (Fig. 2).

Analysis of the depth profile of eutrophic L. Ta‡towisko in August exhibited the lowest amounts of urea (0.8–1.1 µM) in the upper epilimnion (Fig. 3A). In spite of some irregularities (distinct peaks in the central metalimnion and at 16 m depth) urea concentrations generally increased with depth and reached 6 µmols l⁻¹ above the bottom sediments. In contrast to L. Ta‡towisko, in waters of L. Mikolajskie in August urea content decreased with the depth (Fig. 3B). Higher urea concentrations were found in epilimnetic waters (0.40–0.75 µmol l⁻¹), while the lowest (<0.2 µmol l⁻¹) were observed at the bottom of the hypolimnetic zone. Similarly to L. Ta‡towisko, a distinct peak of urea (to 2.7 µmol l⁻¹) was observed in the middle of the metalimnetic zone.
Fig. 1. Urea concentrations (A) and urease activity (B) as a function of the trophic state index of the studied lakes. Trophic state index was calculated from total phosphorus according to Carlson (1977).

Fig. 2. The influence of temperature on activity of urease in lake water sample taken from surface layer of L. Mikołajskie in August 2002.
Fig. 3. Urea concentrations and urease activity in depth profiles of L. Taltowisko in August 2002 (A) and L. Mikolajskie in September 2002 (B).

For determination of urease activity in L. Mikolajskie 2 l samples taken from 1, 2, 4, 6 m (epilimnetic layer); 8, 10, 12, 14 m (metalimnetic layer) and from 18, 22, 25 m (hypolimnetic layer) were mixed and further analyzed as one integrated sample.
The maximal potential U-ase activity ($V_{\text{max}}$) was tightly correlated with the trophic status of the studied lakes (Fig 1B) and increased in the summer months from 0.18 µmol urea l$^{-1}$ h$^{-1}$ (Table II) to 7.25 µmol urea l$^{-1}$ h$^{-1}$ (Table III). The highest U-ase activity (4.5–7.2 µmol urea l$^{-1}$ h$^{-1}$) was observed in July and September in four of the most eutrophic lakes connected to each other and placed along the water flow (L. Jagodne, L. Szymoneckie, L. Szymon and L. Taltowisko) see Table II. In the rest of the studied lakes maximal potential activity of this enzyme was relatively stable and varied between 2.5 and 3.5 µmol urea l$^{-1}$ h$^{-1}$. Analysis of U-ase in depth profile of L. Taltowisko in August (Fig. 3A) showed that quantitatively important activity of this enzyme (8.1–10.1 µmol urea l$^{-1}$ h$^{-1}$) was only found in the epilimnion. In the other parts of the depth profile U-ase was almost undetectable (0.5–0.9 µmol urea l$^{-1}$ h$^{-1}$). Additional evidence suggesting minimal U-ase activity in the deeper parts of the eutrophic lake was also provided by analysis of integrated epi- meta- and hypolimnetic samples taken from L. Mikolajskie in August (Fig. 3B), when $V_{\text{max}}$ of U-ase in the meta- and hypolimnion was respectively about 3.5 and 21.5 times lower than in the epilimnion.

Fig. 4. Inhibition of urease activity by ammonium.
Samples were taken in August 2002 from epilimnion (1.0 m depth) of L. Mikolajskie (circles) and L. Taltowisko (squares).

Fig. 5. Modification of urease activity by environmental conditions.
Looking for factors that, like the low temperature, could cause depletion of U-ase activity in the hypolimnion, possible inhibition of this enzyme by high NH$_4^+$ concentrations or other inhibitors was also examined. As arises from Figure 4, ammonium concentrations up to 40.0 µmol l$^{-1}$ inhibited “epilimnetic U-ase” in only about 5%. However, in the hypolimnion U-ase could be evidently inhibited by unknown factors other than NH$_4^+$ (Fig. 5). “Epilimnetic enzyme” placed in hypolimnetic water exhibited only about 35% of its normal activity whereas “hypolimnetic U-ase” carried to epilimnetic water increased its activity by about 100%.

U-ase activity similarly as AMP activity was mainly associated with the seston. In surface waters of mesotrophic L. Kuc, eutrophic L. Mikołajskie and hypereutrophic L. Taltowisko only about 20 % of total U-ase activity was found in the liquid phase (fraction <0.2 µm) whereas 80 % was related to particulate material (Table IV). Simultaneously, the relative turnover time ($r_{Tt}$) of urea calculated for free urease activity was 3–11 times longer than calculated for the enzyme bound to the particles. The relative turnover time of urea calculated for the total ureolytic activity varied from 10 days to 2 months in highly eutrophic and from 3 to 4 months in less eutrophic environments.

A more detailed pattern of urease activity in various fractions of the seston demonstrated in Fig. 6A shows, that microorganisms (and perhaps free extracellular U-ase) attached to particulate material in fractions 10.0–100.0 µm and 1.0–10.0 µm were mainly responsible for urea hydrolysis in epilimnetic waters of L. Mikołajskie (31 % and 29 % of the total activity, respectively). Relatively low U-ase activities (up to 5% of the total activity) were found in fractions of larger particles (100.0–200.0 µm and >200.0 µm).

Fig. 6. Contribution of various seston size fractions to total urease and aminopeptidase activities in epilimnetic (A) and hypolimnetic (B) water of Lake Mikołajskie.
It was notable that in epilimnetic waters, excluding fraction <0.2 µm where L-leucine-aminopeptidase activity was almost undetectable, the distribution of U-ase and AMP activities among the tested seston size fractions was almost identical (R = 0.96, n = 5, P<0.01). In the hypolimnion, participation of U-ase activities in each fraction compared to overall activity of this enzyme was similar and did not exceed 23%. Moreover, in hypolimnetic waters, the correlation between U-ase and AMP activities in various fractions of the seston was not found.

Although our data suggest a strong relation of U-ase activity with the seston, we did not observe a direct relationship between U-ase activity and the number of seston-attached bacteria (Table V). In samples taken during June and September, no correlation between these parameters was observed. This was confirmed by analysis of all data collected during our investigations. On the other hand, we found an unexpected, strong correlation between U-ase activity and number of free-living bacteria (R = 0.81, n = 17, P<0.0001; R = 0.86, n = 12, P<0.0004 and R = 0.65, n = 36, P<0.0001 – for June, September and all collected results, respectively) and not so strong, but still statistically significant correlation U-ase activity with the total number of the bacteria (R = 0.54, n = 17, P<0.025; R = 0.68, n = 11, P<0.022; and R = 0.44, n = 36, P<0.008, respectively). The bacterial origin of U-ase can be confirmed additionally by comparing its activity with the activity of other, almost exclusively bacterial, enzyme – AMP. As can be seen in Figure 7, the activities of both of these enzymes were proportional to each other (U-ase activity =0.0047×AMP activity + 1.0215) and tightly correlated (R = 0.78, n = 27, P<0.0001) in the surface waters of all studied lakes.

### Table IV

Partition of total urease activity between particulate (>0.2 µm) and cell-free (<0.2 µm) fractions of integrated, epilimnetic water samples taken in August from the epilimnion of three lakes of different trophic status in August 2002

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Total</th>
<th>Attached</th>
<th>Free living</th>
</tr>
</thead>
<tbody>
<tr>
<td>V_{max} (µm urea l$^{-1}$ h$^{-1}$)</td>
<td>K_m (mM)</td>
<td>r_T (day$^{-1}$)</td>
<td>% of total activity</td>
</tr>
<tr>
<td>&gt;0.2 µm</td>
<td>&lt;0.2 µm</td>
<td>&gt;0.2 µm</td>
<td>&lt;0.2 µm</td>
</tr>
<tr>
<td>Kuc (mesotrophic)</td>
<td>1.27 ± 0.05</td>
<td>0.39 ± 0.04</td>
<td>3.06 ± 0.47</td>
</tr>
<tr>
<td>Miko‡ajskie (eutrophic)</td>
<td>2.34 ± 0.02</td>
<td>0.60 ± 0.02</td>
<td>1.54 ± 0.07</td>
</tr>
<tr>
<td>Ta‡towisko (hypertrophic)</td>
<td>7.19 ± 0.30</td>
<td>1.57 ± 0.03</td>
<td>1.60 ± 0.40</td>
</tr>
</tbody>
</table>

± standard errors

### Table V

Relationships between urease activity and bacterial numbers, aminopeptidase activity of (AMP-ase) and chlorophyll a (Chl$_a$) concentrations in surface waters of The Great Mazurian Lakes

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Total</th>
<th>Attached</th>
<th>Free living</th>
<th>AMP</th>
<th>Chl$_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>U-ase June</td>
<td>R = 0.54</td>
<td>R = 0.33</td>
<td>R = 0.81</td>
<td>R = 0.84</td>
<td>R = 0.93</td>
</tr>
<tr>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 17</td>
<td>n = 17</td>
</tr>
<tr>
<td>P &lt; 0.0250</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>September</td>
<td>R = 0.68</td>
<td>R = 0.51</td>
<td>R = 0.86</td>
<td>R = 0.62</td>
<td>R = 0.90</td>
</tr>
<tr>
<td>n = 12</td>
<td>n = 11</td>
<td>n = 12</td>
<td>n = 11</td>
<td>n = 12</td>
<td></td>
</tr>
<tr>
<td>P &lt; 0.0220</td>
<td>P &lt; 0.0004</td>
<td>P &lt; 0.0414</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>All results</td>
<td>R = 0.44</td>
<td>R = 0.24</td>
<td>R = 0.65</td>
<td>R = 0.78</td>
<td>R = 0.75</td>
</tr>
<tr>
<td>n = 36</td>
<td>n = 36</td>
<td>n = 36</td>
<td>n = 28</td>
<td>n = 37</td>
<td></td>
</tr>
<tr>
<td>P = 0.0080</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td></td>
</tr>
</tbody>
</table>

| Chl$_a$ June | R = 0.61 | R = 0.42 | R = 0.83 | R = 0.92 | n.s. |
| n = 17 | n = 17 | n = 17 | n = 16 | – |
| P = 0.0089 | P < 0.0001 | P < 0.0001 | P < 0.0001 | – |
| September | R = 0.67 | R = 0.57 | R = 0.67 | R = 0.62 | n.s. |
| n = 11 | n = 11 | n = 11 | n = 11 | n = 11 |
| P = 0.0249 | P = 0.0172 | P < 0.043 | – | – |
| All results | R = 0.57 | R = 0.02 | R = 0.43 | R = 0.78 | n.s. |
| n = 131 | n = 41 | n = 41 | n = 38 | – |
| P < 0.0001 | P = 0.0100 | P < 0.0001 | P < 0.0001 | – |

n.s. – correlation non-significant
Urea and ureolytic activity in lakes

Ureolytic activity was correlated not only with bacterial number but also with Chl a concentrations ($R = 0.93$, $n = 17$, $P < 0.0001$; $R = 0.90$, $n = 12$, $P < 0.0001$ and $R = 0.75$, $n = 37$, $P < 0.0001$ — calculated for June, September and from all collected results, respectively) (Table V). However, it should be pointed out that these correlations might only be apparent, because U-ase activity is a function of bacterial number, which depends directly on organic matter originating from algal production. To test this hypothesis, we examined relationships between Chl a and bacterial numbers (total, seston-attached and free-living) for results obtained in July, August and during the whole investigation period (Table V). Analysis of the results collected in Table V showed that, like ureolytic activity, also Chla concentrations were well correlated with total bacterial number ($R = 0.61$, $n = 7$, $P < 0.001$; $R = 0.67$, $n = 11$, $P < 0.001$; $R = 0.57$, $n = 131$, $P < 0.001$; June, September and all results, respectively) and with the number of free-living bacteria ($R = 0.83$, $n = 17$, $P < 0.001$; $R = 0.67$, $n = 11$, $P < 0.0172$; $R = 0.43$, $n = 42$, $P < 0.0100$ June, September and all results, respectively). Simultaneously, they were not correlated with the number of seston-attached bacteria.

### Discussion

Urea is one of the most significant components of the DON pool and easiest available N sources for aquatic microorganisms. Although this simplest organic N compound is ubiquitous in natural waters its content, biological transformations and importance to planktonic microorganisms and dissolved organic N pool in freshwater environments are still insufficiently investigated. Concentrations of urea in lake water noted during our investigations commonly changed from 0.2 to 7.0 μmol l$^{-1}$ h$^{-1}$. However, they were unusually as high as 11.37, 13.2 or even 25.0 μmol l$^{-1}$. In surface waters of the studied lakes concentrations of urea were inversely and closely correlated with their trophic status. Some indirect evidence suggested that in water of Mazurian Lakes urea was mostly of autochthonous origin. This is not fully consistent with the observations of other authors. For instance, the data obtained by Lomas et al. (2002) during long-term studies in waters of eutrophic Chesapeake Bay suggested that the external input of urea from the watershed was extremely significant in this estuarine system. Berman and Bronk (2003) also stated that human-derived pollution and runoff from agricultural areas are important allochthonous sources of urea in many lakes reservoirs and coastal marine waters. On the other hand, we did not observe higher urea concentrations in lakes Niegocin, Ryńskie and Mikołajskie receiving more sewage from surrounding urbanized areas than others. Indeed, relatively low and unpredictable urea concentrations could be there a consequence of high and dynamically changing rate of enzymatic decomposition of this compound by planktonic microorganisms. However, in these lakes systematically enriched with allochthonous urea U-ase activities did not differ distinctly from that noted in other lakes, less exposed to urea supply. Moreover in
waters of the studied lakes no statistically significant negative correlation between urea concentrations and U-ase activity was found. Such a correlation should be observed if urea concentrations would be depended directly on U-ase activity.

Increase in urea concentrations in the lower part of epilimnion and in metalimnion of L. Mikolajskie at the beginning of August (Fig. 3) followed not only massive phytoplankton blooms in surface waters but also the peak of the sailing season. This, by contrast, may suggest that, at least periodically, urea of allochthonous origin creates a substantial part of the total urea pool. The highest urea concentrations (up to 25 µmol l⁻¹) noted during the early spring in the surface waters of all studied lakes and in the deepest part of L. Taltowisko in July, if not overestimated, could be or an effect of urea accumulation in the environment resulted from low temperature of water (4.5–5.0°C) and/or from inhibition of U-ase activity at this time of the year and part of the lake depth profile. We found that the rate of urea decomposition in natural lake water sample increased exponentially with temperature and at 5°C was about 2.25 times lower than at 20°C (Fig. 2). Mitamura (1986) suggested that the optimal temperature for urea degradation in lake water was about 30°C. Q10 coefficient calculated for this reaction varied from 1.7 to 2.0.

Assimilation of urea by various groups of microorganisms in natural environments is still poorly understood. It is commonly believed that bacteria and cyanobacteria can utilize urea nitrogen as NH₄⁺, after enzymatic hydrolysis of urea by intracellular U-ase. The mechanism of urea assimilation by algae seems to be more complicated and is still not fully known. Some algal species (particularly green algae) and yeasts produce ATP-dependent urea amidolase instead of U-ase. The activity of this enzyme seems to be induced by urea and repressed by ammonium. Other planktonic algae (some diatoms and dinoflagellates), similarly to bacteria produce intracellular U-ase, or assimilate urea using metabolic pathways that are not yet known (Syrett, 1962; Morris, 1974; Park et al., 1993). The literature concerning utilization of urea by algae in pure cultures is relatively rich (Syrett, 1962). Moreover, field data (Remsen et al., 1972; Cho and Azam, 1995) also strongly suggest that urea is decomposed/assimilated primarily by phytoplankton rather than by bacteria in most freshwater and marine habitats.

Park et al. (1997) pointed out that in hypertrophic freshwater environments not only algae but also bacteria participate substantially in urea decomposition processes. Our results suggest that the role of bacterial U-ase was also significant in the surface waters of moderately eutrophic Mazurian Lakes. In meta- and hypolimnetic zones, bacterial U-ase produced in situ as well as exported from the epilimnion with bacteria attached to sinking seston was probably strongly inhibited/repressed by high HN₄⁺ concentrations or other not yet defined factors. Although we did not observe substantial U-ase inhibition by 40 µmol l⁻¹ of HN₄⁺ in laboratory conditions, but the concentrations of ammonium noted in the deepest layers of The Great Mazurian Lakes in late summer were occasionally as high as 140 µmol l⁻¹. Thus, one can expect that facultative and obligate anaerobic bacteria living in the profundal zone and in the bottom sediments may have produced urea rather than hydrolyzed it. Nucleic acid and protein decomposition supporting bacteria with urea precursors and inhibition or/reduction of U-ase activity at low temperature caused accumulation of urea in deep waters during the summer and its preservation in cold environment during the winter. Water mixing processes substantially increased urea concentrations in the photic zone of the studied lakes during the spring, when urea might serve, instead of NO₃⁻, as a “trigger” for early spring diatoms blooms.

It should be pointed out that the relatively low urea concentrations found in deep waters of L. Mikolajskie in August (Fig. 3B) contradict the hypothesis of urea conservation in the hypolimnion. However, they were probably only an exception to the rule. Later investigations showed that urea concentrations varied from 1 to 6 µM in profundal zone of the studied lakes and were generally much higher than in surface waters (Siuda, unpublished). The consistently elevated urea concentrations were also observed by Lomas et al. (2002) in the deep waters of Chesapeake Bay. According to these authors relative high concentrations of urea in deep waters could be an effect of decomposition of recently settled particulate organic matter by benthic microorganisms and/or could be due to decreases in authotrophic utilization of urea, relative to surface waters.

U-ase (EC.3.5.1.5) activity is a widely distributed property of aquatic bacteria including aerobes, facultative anaerobes and obligate anaerobes (Satoh and Hanya, 1976; Satoh, 1980). This mainly intracellular enzyme is specific for urea and hydroxyurea (Fishbein and Carbone, 1965) has a pH optimum between 6.7 and 8.0 and Kₘ of milimolar range (Palińska et al., 2000, Molyble and Hausinger, 1989). We found more than 75% of total U-ase activity in the particulate fraction, whereas the rest represented free dissolved enzyme activity. The relative turnover time (rT) of urea via U-ase varied from 10 days to 2 months in highly eutrophic and from 3 to 4 months in meso- and moderately eutrophic lakes. In water of the same lakes Tᵢ calculated for L-leucine-4-methyl-coumarinylamide (AMP substrate) commonly does not exceed 15 days and the half-life time of labile dissolved proteins reaches 8 days (Siuda et al., in preparation). Therefore,
one can speculate that urea may be a poorer N source for aquatic bacteria compared to peptides or proteins. Especially, as more complicated organic N compounds (like peptides or proteins) additionally support bacteria with organic C radicals.

Though the total U-ase activity was tightly correlated with the number of free-living bacteria (Table II), other collected evidence strongly suggests that it was produced mainly by bacteria attached to particles having dimensions from 1.0 to 100.0 µm. This fraction of the seston commonly exhibits also the highest overall microbial activity (Kiersztyń, unpublished, Long and Azam, 1996; Grossard and Simon, 1998). The crucial role of attached bacteria in urea decomposition processes was confirmed definitely by some other observations. First, by strong, statistically significant (P<0.0001) correlation between U-ase and AMP (Table V), an enzyme that is commonly produced by more efficiently attached bacteria (Siuda and Chróst, 2002). Second, by the fact, that U-ase activity in the studied lakes was proportional to their trophic state indexes, which were rather a function of quantity of the seston, than the number of free live bacteria in lake water, and finally by the partition of U-ase activity between various seston size fractions observed by Siuda in eutrophic Zegrzyński Reservoir (Siuda, unpublished). Contribution of free-living bacteria to the total U-ase activity was there smaller than 4%. Decisive role of attached bacteria in processes of urea hydrolysis was also postulated by Satoch and Hanya (1976). They found that in freshwater pond free-living bacteria did not decompose urea efficiently.

The data collected in Table V lead to more general conclusion that may define the function of attached and free-living bacteria in the environment. If we presume that, similarly as U-ase and aminopeptidase activity, also activities of other bacterial enzymes are primarily due to “colonists” – attached bacteria, we have to also accept that the number of free-living bacteria (“explorers”) in lake water must be dependent on “colonists” activity. Thanks to overproduction of variety of hydrolytic enzymes, attached bacteria support free-living heterotrophic microorganisms with readily utilisable monomeric substrates (Siuda and Chróst, 2002) and thus facilitate them to colonize other particles. Considering this, one could rather not expect the correlation of U-ase and AMP activities with the number of attached bacteria, especially that both of those enzymes might be also preserved in the matrix of biofilms, even after death of their producers. Inversely, the tight correlation between both of enzyme activities and the number of free-living bacteria observed during our investigations was obvious and well understood.

To explain the high correlation between ureolytic activity and Chl a concentrations (Table V) we propose four hypotheses: (1) as opposed to many individual algal species tested in pure cultures, natural phytoplankton assemblages (in the case of tested lakes dominated by cyanobacteria, diatoms and dinoflagellates), synthesized urease and utilized urea nitrogen after its hydrolysis to NH₄⁺ similarly as bacteria. Assimilation of urea could be especially favorable for algal species without anhydrase activity. During periods of intensive photosynthesis they could not take up inorganic C from insoluble carbonates but could, at least theoretically, obtain it from urea hydrolysed inside their cells; (2) urease was mainly produced by bacteria and correlation of its activity with Chl a was only apparent and misleading. It arose from the fact that Chl a concentrations, similarly as U-ase activity were well correlated with the total number of bacteria as well as with the number of free-living microorganisms; (3) considering that nitrogen limited primary production in majority of Great Mazurian Lakes and that urea composed the bulk of easy utilisable DON fraction, one could presume that NH₄⁺ liberated in excess from urea by bacterial U-ase not only covered bacterial N demand but also stimulated phytoplankton growth, (4) tight correlation of U-ase and AMP activities with Chl a concentrations was a consequence of peculiar algal-bacterial metabolic coupling (Francoeur and Wetzel, 2003). Bacteria attached to the living algal cells cooperated with algae supporting them, thanks to U-ase and AMP activities, with surplus NH₄⁺ and amino acids and utilizing easy available organic C compounds released extracellularly by algal cells. This mode of algal NH₄⁺ nutrition seems to be less energy-consuming than liberation of NH₄⁺ from urea via ATP-dependent urea amidolyase system.

Although none of the above hypotheses can be definitely proven on the basis of our experimental data, the last presumption seems to be the most promising and elegant. For better understanding the role of algae and bacteria in urea metabolism, importance of this compound in freshwater environments and mechanisms responsible for its production, assimilation and decomposition further extensive investigations are needed.

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**Literature**


Characterization of Selected Groups of Microorganisms Occurring in Soil Rhizosphere and Phyllosphere of Oats

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Abstract

Studies were carried out on the microflora of phyllosphere and soil rhizosphere of hulled (Chwat variety) and naked (Akt variety) oats. The material taken for study embraced samples of leaves and soil rhizosphere taken from cultivations differing in extent of nitrogen fertilization. The studies involved determination of total number of aerobic heterotrophic bacteria belonging to the genus Pseudomonas and microscopic hyphal fungi. Qualitative determinations focused on bacteria belonging to the genera Azotobacter and Azospirillum were also made.

Our results point to differences in number of microscopic hyphal fungi in the phyllosphere of both varieties of oats, depending on nitrogen fertilization dose. However, there were no significant differences in the number of bacteria of the different genera determined in the phyllosphere and rhizosphere. Strains of oligonitrophilic and diazotrophic bacteria were isolated from samples of the phyllosphere of oats and their N₂-fixing activity was determined by the acetylene reduction method using gas chromatography.

Key words: microflora, nitrogenase, oats, phyllosphere, rhizosphere

Introduction

The surfaces of the above-ground parts of plants are inhabited by various groups of microorganisms (Hirano and Upper, 2000). Only some of these occur in this environments as transients, being deposited on the surface of flowers or leaves with precipitation or carried there by wind or insects. Most of the phyllosphere microorganisms are able to grow and multiply in these conditions. This group is defined as epiphytic microorganisms. The most numerous among them are bacteria, for which the phrase phyllobacteria has been coined (Beattie and Lindow, 1999). The qualitative composition of epiphytic microorganisms depends on many environmental factors, especially at the beginning of spring, in the stage of leaf development. Of equal importance are the conditions that the plant itself ensures (Hirano and Upper 2000; Mercier and Lindow, 2000; Chmiel, 2004).

The role of epiphytic microorganisms has not been fully elucidated. It is known that this group includes both plant pathogens and microorganisms that provide a protective barrier against them. Several species of phyllobacteria have also been found to synthesize plant hormones and have been suggested to play a role in stimulating plant growth (Lindow et al., 1998; Beattie and Lindow, 1999).

The current study focuses on the effect of differentiated nitrogen fertilization on select groups of epiphytic microorganisms as well as those occurring in soil under oats cultivation.

Experimental

Materials and Methods

The studies embraced the microbiological analysis of leaf samples and rhizosphere soil taken from under oats cultivation. The material for the determinations was taken from field experiment set up at the Experimental Station of the Warsaw Agricultural University in Jaktorowo. In this experiment two types of oats were used: hulled Chwat and naked Akt. In each case, differentiated
mineral nitrogen fertilization was employed: 0, 30, 60, 90 and 120 kg N/ha. The material for the studies was collected in May 2004, two months after sowing the plants.

**Quantitative determinations.** Total number of heterotrophic bacteria was determined using the method and medium described by Bunt and Rovira (1955). The number of microscopic hyphal fungi was estimated using Martin’s medium (Martin, 1950) and the total number of bacteria using King B medium (King et al., 1954). Bacteria belonging to the genera Azotobacter and Azospirillum were enumerated by routine MPN method. In the case of Azotobacter cultures were set up in liquid nitrogen-free medium and the presence of the bacteria was determined based on macroscopic observations of individual cultures, followed by *in situ* observations in optical microscope for characteristic shape of the cells, as described by Döbereiner et al., (1976). To determine the MPN of bacteria for bacteria belonging to the genus Azospirillum a semi-liquid nitrogen-free medium, supplemented with malate (Ebelthigy et al., 2001), was used. Initial test for the presence of these bacteria was based on the presence in the culture of a delicate film or layer just under the surface of the medium, such growth being characteristic of microaerophiles, followed by *in situ* observation of preparations from the culture using phase-contrast microscopy for vibroid cells filled with numerous granules of β-hydroxybutyric acid, moving by spiral rotations.

In order to enumerate epiphytic microorganisms oats leaves were placed in 100 ml sterile water and shaken for about 20 minutes. The obtained suspension was diluted in series and plated on selective media. The obtained results were calculated as colony forming units (cfu) per 1 g air dried leaves. To determine the number of microorganisms in rhizosphere soil oats roots were first delicately shaken and then placed in 100 ml sterile water and shaken for about 20 minutes. Consecutive dilutions were plated out on selective media. To estimate the mass of rhizosphere soil the original suspensions were transferred to evaporation vessels and after evaporation of water the soil was dried at 105°C and then weighed. The obtained results were calculated per 1 g dry weight of rhizosphere soil.

**Qualitative determinations.**

Occurrence of bacteria belonging to the genera *Azospirillum* and *Azotobacter* in phyllosphere and rhizosphere soil of oats. Fragments of oats leaves and samples of soil rhizosphere were introduced in semi-liquid nitrogen-free medium with malate (NFb) as described by Döbereiner et al., (1976), and liquid azotobacter medium according to Girard and Rougieux (1967) supplemented with glucose. The routine procedures followed were basically as described above.

Characteristics of other groups of diazotrophic bacteria in the phyllosphere of oats. The enriched cultures obtained from phyllosphere of oats served as a source for the isolation of pure cultures of bacteria. Each of them was again checked for ability to grow in the absence of nitrogen compounds. Selected strains of oligotrophic bacteria were examined in detail for their systematic position. The determinations included biochemical traits as described in detail elsewhere (Becking, 1974; Winslow et al., 1974; Holmes et al., 1987). The obtained data were further corroborated with the use of the biochemical test kits API 20 E and NE and API LAB computer software for interpretation of the results and consequent identification of bacteria (bioMerieux).

Identification of nitrogenase activity of strains of diazotrophic bacteria. A culture enriched in bacteria from oats phyllosphere in nitrogen-free medium was used as a source of bacteria that were isolated to homogeneity and the obtained strains were tested for their ability to fix N₂. The nitrogenase activity of each of the bacterial strains was determined in cultures in semi-liquid medium: azotobacter medium with glucose; NFb medium supplemented with maleic acid; modified NFb medium, in which maleic acid was replaced by glucose, mannitol or sucrose.

The method used in the studies was acetylene reduction, expressed as Acetylene Reduction Activity (ARA) (Hardy et al., 1968). Measurements were made with UNICAM gas chromatograph – using flame ionization detector (FID) (temperature: doser 150°C, detector 200°C, column 60°C). The carrier gas was helium. Cultures of bacteria were set up in calibrated bottles containing 4 cm³ semi-liquid medium and incubated for 2 hours in an atmosphere of 10% acetylene at 28°C (volume of gas phase 7 cm³). The obtained results were calculated as number of nmoles C₂H₂ formed by a culture in one hour. All experiments were carried out in triplicate.

**Statistical elaborations.** The results of studies aimed at determining the number in soil and phyllosphere were subjected to statistical analysis using multifactor variance analysis. To compare the means from the studied experimental combinations, Tukey’s multiple comparison test, with significance level a = 0.05, was used (Blackman and Tukey, 1958). Uniform groups, that is means from experimental combinations between which there are no significant differences, are indicated on the individual figures using the same letters. On the contrary, means with different weights have been designated using different letters. Calculations were made using SAS 9.1 package (SAS Institute Inc., 2004).

**Results and Discussion**

Microorganisms colonizing the above-ground parts of plants usually occur in high numbers. A 1 cm² surface of a leaf may contain 10⁵ to 10⁷ bacterial cells (O’Brien and Lindow 1989; Hirano and Upper, 2000; Mercier and Lindow, 2000; Lindow and Leveau, 2002; Lindow and Brandl, 2003). These values can also be expressed as the number of bacteria per 1 gram fresh or dry weight of leaves (Brighigna et al., 2000). In such cases the number of bacteria per 1 gram fresh mass of leaves ranges from 10⁴ to 10⁶ colony forming units (cfu). Studies by Yang et al. (2001) indicate that generally microbial community structures are similar on different individuals of the same plant species, but unique on different plant species.

In our studies on the microflora of oats a relatively low number of phyllobacteria 3.8 x 10⁴ to 4.6 x 10⁵ cfu per 1 g dry weight was found (Figs. 1 and 2). The number of moulds was also low and ranged from 2.1 x 10⁶ to 6.3 x 10⁶ cfu (Fig. 3). It seems that this may be caused by conditions determined by the plant itself. According to many authors the occurrence of microorganisms on the above-ground parts of plants depends on a number of factors, of which a critical role may be played by the genus and even species of the host plant (Mercier and Lindow, 2000; Lindow and Brandl, 2003; Chmiel, 2004). This is related to the availabil-
ity of nutrients, above all saccharides (Wilson and Lindow, 1994a; 1994b; Wilson et al., 1995; Mercier and Lindow, 2000). Their low content in peas and cereals may strongly restrict the occurrence of epiphytic microorganisms on the surface of these plants.

It is known that the specificity of various plants as a habitat is also related to their secretions. Chmiel’s work (2004) suggests that the presence of certain compounds may directly affect the differentiated numerical force of moulds in the phyllosphere of certain plants. In studies of several years the author found a significantly lower number of microscopic hyphal fungi in the case of cole, oats and corn compared to white and red clover and bulb and root plants. In her opinion this phenomenon is caused not only by limited
nutrient resources in the phylloplane of these plants but also by organic compounds secreted by these plants. These include glycosides of pentacyclic triterpenoids, represented by avenacin A and alkylresorcinols with antifungal activity, which are synthesized by cereals.

In the view of many researchers the inhabiting of the phyllosphere of plants by microorganisms is strongly affected by environmental factors, such as humidity, temperature and chemical pollutants of the environment (e.g. Brighigna et al., 2000). This has been proven in the case of *Tilandsia caput medusae* and *Tillandsia schiedeana* in quantitative studies of phyllosphere depending on contamination of the environment with heavy metals. The sensitive group of organisms were epiphytic yeasts. Contamination of the environment with heavy metals resulted in their elimination from the phyllosphere of both species of the studied plants.

Surprisingly, there is practically no information in the literature regarding the effect of agrotechnical measures on the epiphytic microflora of cultivated plants. Consequently, this prompted our interest in determining the effect of mineral nitrogen fertilization on the number of microorganisms belonging to select groups inhabiting the phyllosphere of oats and the soil it is grown in. The studies embraced two varieties of oats – Chwat and Akt. Comparative analysis embraced the enumeration of moulds and heterotrophic bacteria, including bacteria belonging to the genus *Pseudomonas*. The results obtained in our studies suggest that the use of high N doses in mineral fertilization may stimulate the colonization of leaves by moulds and at the same time decrease the population size of heterotrophic bacteria. In the case of the Akt variety, an increase in N dose favoured the colonization of the surface of leaves by fungi (Fig. 1). In the case of the Chwat variety, such tendencies were observed in particular with fertilization of 90 and 120 kg N/ha. Statistical analysis did not reveal, however, any significant differences in the population sizes of moulds in the studied experimental combinations. However, it should be pointed out that the number of fungi in the phyllosphere of hulled oats was in general higher than in the case of naked oats. It therefore seems that not only the species but also the variety of a particular plants species can affect the qualitative composition of the population colonizing the leaves. The number of heterotrophic phyllobacteria in the studied oats’ combinations showed slight differentiation, but in some cases the differences were statistically significant (Fig. 2). For both oats varieties the lowest number of bacteria was found on leaves from cultivations fertilized with 90 kg N/ha, and in the case of the Akt variety, also at doses of both 90 and 120 kg N/ha. This indicates that the use of high N doses in mineral fertilization may to a certain degree inhibit the colonization of oats leaves by heterotrophic bacteria.

Determinations for bacteria of the genus *Pseudomonas* in the phyllosphere of both varieties of oats did not demonstrate any significant differences in population size in the studied experimental combinations (Fig. 3). In the case of the variety Chwat a statistically significantly lower number of bacteria were found at fertilization level 90 kg N/ha, and in the case of the variety Akt at doses of both 90 and 120 kg N/ha.

The population size of moulds in the rhizosphere of Akt and Chwat oats was similar in the studied experimental combinations (Fig. 4). This was confirmed by statistical analysis. A slight effect of fertiliza-
tion with mineral N was observed in the case of the population sizes of heterotrophic bacteria in the rhizosphere of the variety Akt (Fig. 5) and bacteria of the genus *Pseudomonas* (Fig. 6) in the case of both varieties. However, the obtained results do not allow any valid conclusions.

Quantitative determinations for N$_2$-fixing bacteria of the genera *Azotobacter* and *Azospirillum* were not successful. Plating out the suspension and its dilutions obtained after placing the leaves in sterile water gave negative results. The presence of these bacteria was determined only in the case of some samples when the media were directly inoculated with fragments of leaves. It seems that the number of bacteria of the genera *Azotobacter* and *Azospirillum* in the phyllosphere of oats is very low and it is possible that their presence could be determined only using highly sophisticated techniques, such as rRNA gene analysis, described by...
Yang et al. (2001). The specific location of these bacteria on the surface of the leaves as well as the possibility of their presence in internal leaf tissues can also be considered since some microorganisms are known to colonize not only the surface of a plant but also to penetrate into its interior (Reinhold and Hurek, 1988;

Table I
Occurrence of bacteria belonging to the genera Azospirillum and Azotobacter in phyllosphere and rhizosphere soil of hulled Chwat variety and naked Akt variety of oats

<table>
<thead>
<tr>
<th>Oats variety</th>
<th>N dose in mineral fertilization [kg N/ha]</th>
<th>Sample No</th>
<th>Phyllosphere</th>
<th>Rhizosphere</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Azospirillum sp.</td>
<td>Azotobacter sp.</td>
</tr>
<tr>
<td>Chwat</td>
<td>0</td>
<td>947</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>928</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>930</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>949</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>60</td>
<td>927</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>60</td>
<td>948</td>
<td>+</td>
<td>–</td>
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<tr>
<td></td>
<td>90</td>
<td>929</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>950</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>926</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>946</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Akt</td>
<td>0</td>
<td>936</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>968</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>939</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>969</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>940</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>966</td>
<td>–</td>
<td>–</td>
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<td>937</td>
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<td>+</td>
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<td>938</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>967</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Microorganisms in oats rhizosphere and phylosphere

Beattie and Lindow, 1999). In general bacteria of the genus *Azospirillum* are associated with the roots of plants and are the most frequent in the rhizosphere and rhizoplane of cereals and grasses (Kuliæska, 1983; Jaœkowska, 1995; 1994; Kirchhof et al., 1997). In combination with the plant, they form an association N₂-fixing system (Jaœkowska, 1989). Qualitative studies in the direction of N₂-fixing bacteria in the phyllosphere of oats revealed the presence of *Azotobacter* sp. only in the variety Akt (Tab. I). However, most of the studied of leaves of this variety (70 %) were colonized by bacteria belonging to the genus *Azospirillum*. In the case of the variety Chwat the percentage of positive identifications was 50 % of the samples.

The frequency of the occurrence of *Azotobacter* cells in the rhizosphere of oats was relatively low. For both varieties its presence was found in 5 out of 10 examined samples. On the other hand, bacteria of the genus *Azospirillum* were found in 7 samples of rhizosphere from hulled oats, whereas in the case of the variety Akt only two samples were positive for this species.

Enriched cultures set up from the phyllosphere of oats in nitrogen-free medium were used to isolated pure clones of bacteria. A comparison of the population of diazotrophic phyllobacteria on both varieties of oats indicated greater species differentiation in the case of naked oats (Tables I and II). In the case of leaves of this variety (70 %) were colonized by bacteria belonging to the genus *Azospirillum*. In the case of variety Chwat the percentage of positive identifications was 50 % of the samples.

The frequency of the occurrence of *Azotobacter* cells in the rhizosphere of oats was relatively low. For both varieties its presence was found in 5 out of 10 examined samples. On the other hand, bacteria of the genus *Azospirillum* were found in 7 samples of rhizosphere from hulled oats, whereas in the case of the variety Akt only two samples were positive for this species.

<table>
<thead>
<tr>
<th>Oats variety</th>
<th>Identification of bacterial strain</th>
<th>Concentration of ethylene in cultures of the studied strains (nmol C₂H₄/culture/h)</th>
<th>Substrate used in semi-liquid medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Malate</td>
<td>Glucose*</td>
</tr>
<tr>
<td>Chwat 927/1</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>8.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Chwat 927/2</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>0.0</td>
<td>5.8</td>
</tr>
<tr>
<td>Chwat 930/1</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>4.2</td>
<td>3.8</td>
</tr>
<tr>
<td>Chwat 950/c</td>
<td><em>Chryseomonas luteola</em></td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Akt 939</td>
<td><em>Azotobacter</em> sp.</td>
<td>20.5</td>
<td>nd</td>
</tr>
<tr>
<td>Akt 940/1</td>
<td>unidentified strain</td>
<td>8.01</td>
<td>3.1</td>
</tr>
<tr>
<td>Akt 940/2</td>
<td><em>Chryseomonas luteola</em></td>
<td>4.47</td>
<td>0</td>
</tr>
<tr>
<td>Akt 966</td>
<td><em>Chryseomonas luteola</em></td>
<td>6.2</td>
<td>0</td>
</tr>
<tr>
<td>Akt 967</td>
<td><em>Pseudomonas fluorescens</em></td>
<td>5.9</td>
<td>0</td>
</tr>
<tr>
<td>Akt 967/5</td>
<td><em>Chryseomonas luteola</em></td>
<td>15.7</td>
<td>13.1</td>
</tr>
<tr>
<td>Akt 967/12</td>
<td><em>Chryseomonas luteola</em></td>
<td>32.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Akt 968</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>6.7</td>
<td>2.2</td>
</tr>
<tr>
<td>Akt 970</td>
<td>unidentified strain</td>
<td>9.8</td>
<td>3.1</td>
</tr>
<tr>
<td>Akt 925</td>
<td><em>Agrobacterium radiobacter</em></td>
<td>13.2</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* – determination made for culture in semi-liquid NFb medium
** – determination made for culture in semi-liquid azotobacter medium
nd – not determined
lower ARA activity (3.78–19.36 nmoles C₂H₄/culture/h). The ability of C. luteola to fix atmospheric nitrogen has been described earlier by Hai-Lian et al. (1999). In this case the strain was an endophytic one, isolated from rice plant.

In Azotobacter cultures with glucose and mannitol the activity of nitrogenase was similar, being 31.2 and 30.8 nmoles C₂H₄/culture/h, respectively. The same strain in NFb with maleic acid reduced 20.5 nmoles C₂H₄/culture/h. It can be seen that the studied strains of diazotrophic bacteria showed varied nitrogenase activity, depending on the carbon source introduced into the medium. An unidentified strain designated (940/1), isolated from Akt oats leaves reduced acetylene in cultures with each of the substrates employed. It showed highest activity in azotobacter medium with glucose (10.0 nmoles C₂H₄/culture/h). Pseudomonas fluorescens, on the other hand, demonstrated activity only in NFb medium with malate.

Overall, our results point to differences in number of microscopic hyphal fungi in the phyllosphere of both varieties of oats, depending on nitrogen fertilization dose. However, we were not able to detect any significant differences in the number of bacteria of the different genera determined in the phyllosphere and rhizosphere.

Acknowledgements. The authors wish to thank prof. Zdzisław Wyszyński for kindly providing study material from an experiment conducted by the Department of Agronomy at the Field Station of the Warsaw Agricultural Academy in Jaktorowo and Dariusz Maękowski for assisting with the statistical elaboration of the results.

Literature


Rekosz-Burlaga H. and Garboliæska M. 3
A Simple, Direct Plating Method, Alternative to Dilution Plating, for Estimation of the Abundance of *Penicillium verrucosum* on Incubated Cereal Grain

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**Abstract**

Because dilution plating is more labor intensive than direct plating, we would like to propose the use of a direct plating technique for estimation of *Penicillium verrucosum* abundance in cereal grain in incubation studies, with use of very selective and indicative for the fungus agar DYSG medium. The proposed method is based on the measurement of the diameter of *P. verrucosum* colonies grown around cereal kernels placed on DYSG medium. In three different experiments wheat grain contained a great range of *P. verrucosum* CFU number (from <25 to 77×10^6 per 1 g). When *P. verrucosum* CFU number was at least as high as 10^2 per 1 g of the grain, 100% of the wheat kernels, placed on the surface of DYSG medium, were surrounded by colonies of *P. verrucosum*. The diameter (x, mm) of *P. verrucosum* colonies surrounding wheat kernels on DYSG medium was correlated with the fungal CFU number (y) on the wheat grain. The relationship is described by the exponential regression equation (y = 0.1258 e \(0.9309x\), \(R^2 = 0.96\)). The relationship became linear (y = 0.404 x – 0.901, \(R^2 = 0.96\)) after transformation of *P. verrucosum* CFU numbers to logarithms to base 10.

**Key words:** direct versus dilution plating method, *Penicillium verrucosum* abundance, wheat grain

**Introduction**

*Penicillium verrucosum* Dierckx is the major, if not the only, ochratoxigenic fungus in cereals in temperate climatic regions (Lund and Frisvad, 2003; Park et al., 2005).

Estimation of the abundance of *P. verrucosum* on cereal grain is in most cases necessary in ecological studies on ochratoxin (OTA) synthesis by the fungus. In incubation studies of grain inoculated with *P. verrucosum* (when the fungal propagule number is relatively high) dilution plating has been commonly used (Abramson et al., 1990; Lindblad et al., 2004; Ramakrishna et al., 1996). For evaluation of contamination of natural grain by the fungus (when the fungal propagule number is relatively low) direct plating is considered to be a more effective technique (Elmholt and Rasmussen, 2005; Lund and Frisvad, 2003; Park et al., 2005). Development of the very efficient selective and indicative medium DYSG for specific detection of *P. verrucosum* in cereals (Frisvad et al., 1992) offers excellent possibilities for estimating the abundance of the fungus in mixed populations of fungi, using both the dilution plating and the direct plating techniques (Elmholt et al., 1999; Lund and Frisvad, 2003).

Dilution plating is a more labor consuming method than the direct plating. So, we would like to propose the use of direct plating instead of dilution plating for estimation of *P. verrucosum* abundance in grain in incubation studies, with the use of agar DYSG medium.

**Experimental**

**Materials and Methods**

*Fungal strain* of *P. verrucosum* was isolated from grain of rye (LPH63 DE), harvested in 2002.

*The fungus growth medium.* The fungus was isolated and grown on DYSG medium (final pH 5.6) containing: glycerol (anhydrous) – 220 g; sucrose – 150 g; yeast extract (Difco) – 20 g; MgSO₄·7H₂O – 0.5 g; ZnSO₄·7H₂O – 0.01 g; CuSO₄·H₂O – 0.005 g; dichloran – 0.002 g; chloramphenicol – 0.05 g; agar – 20 g; dist. H₂O – 1000 ml (Lund and Frisvad, 2003).

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Nonsterile grain of spring wheat cultivar Histra, harvested in 2004, was used in the studies. On the basis of studies with methods described by Lund and Frisvad (2003), the grain was not contaminated with P. verrucosum. The grain used in our studies contained 12.1% water.

Inoculation of the grain with P. verrucosum. The 10 g samples of the grain were placed in beakers. The separate grain samples were inoculated with 1 ml of P. verrucosum spore suspensions in solution containing 0.1% peptone, 0.85% NaCl and 1 drop of Tween 80 (Frisvad, 1986) in Experiments I and II, and, in Experiment III, with 0.5 ml of suspension of the fungus spores in water. In Experiment I, 1 ml of P. verrucosum spore suspensions contained approximately 3.5×10^7, 3.5×10^6, 3.5×10^5, 3.5×10^4, 3.5×10^3, 3.5×10^2, 3.5×10^1 or 3.5×10^0 spores to obtain from 0.35 to 3.5×10^6 spores of the fungus per 1 g of the grain. In Experiment II, 1 ml of spore suspension contained approximately 1.7×10^6, 1.7×10^5 or 1.7×10^2 spores to obtain about 17×10^6, 17×10^3 and 17×10^2 per 1 g of the grain. In Experiment III, 0.5 ml amounts of spore suspension, contained approximately 1.5×10^6 spores were added to 10 g samples of the wheat grain containing 15% and 17% of water to obtain 150×10^3 spores per 1 g of the grain.

Incubation of the wheat grain before microbial analyses. Experiment I – the grain, wetted by the fungal inoculum, was equilibrated in a refrigerator at 1°C for 24 hours, with periodical shaking during this time. Experiment II – the wetted grain was incubated: (1) for 48 hours at 1°C; (2) for 24 hours at 1°C and then for 24 hours at 22.5°C; (3) for 48 hours at 22.5°C. Experiment III – the wetted grain was equilibrated in a refrigerator at 1°C for 24 hours, and after mixing, it was incubated for 2 weeks at 10, 15 or 20°C and at 20% or 22% initial water content.

Determination of fungal number on the inoculated grain. After the incubation, fungi were isolated from the grain by shaking with a solution containing 0.85% NaCl, 0.1% peptone and 0.1% Tween 80 (Frisvad, 1986). The numbers of colony forming units (CFU) of all fungi and P. verrucosum in the obtained suspensions were determined by dilution plating on DYSG medium. Inocula (0.1 ml per plate) were spread on the surface of the agar medium. The Petri dishes with DYSG medium were incubated in the dark at 22.5°C for 7 days. After this time P. verrucosum colonies had developed their characteristic terracotta-colored pigmentation on the DYSG reverse, caused by synthesis of an anthraquinone (Elmholt et al., 1999; Frisvad et al., 2005). The determinations were done in four replicates.

Determinations of diameter of P. verrucosum colonies around the wheat kernels placed on agar DYSG medium. Thirty six wheat kernels were placed on DYSG medium (9 kernels per 1 Petri dish). The diameters of the fungal colonies were measured in the crosswise direction of the kernels after 4, 5 and 6 days of incubation in the dark at 22.5°C (Experiment I) or after 5 days (Experiments II and III).

Competitive relation of P. verrucosum and other fungi was assessed on the basis of percentage of thirty six incubated wheat kernels, placed on agar DYSG medium, with growing colonies (beside the kernels) of P. verrucosum and other fungi, different from P. verrucosum (Experiment I only).

Statistical evaluations. The data were subjected to one-way analysis of variance and the means were separated with Student’s t-test. For statistical evaluation of significant differences between the percentages of wheat grains with colonies of P. verrucosum or with fungi different from P. verrucosum on DYSG medium, confidence intervals were calculated according to the equation (Oktaba, 1966):

$$ \frac{2Y + u_2^2 - K}{2(n - u_2^2)} < p < \frac{2Y + u_2^2 + K}{2(n - u_2^2)} $$

where: $p$ is a confidence interval, $K = u_2 \sqrt{\chi^2}$ and $x = u_2^2 + 4Y(1 - Y/n); Y$ is the number of kernels with growing colonies of fungi different from P. verrucosum; $n$ is the total number of kernels; $u_2$ is the Student’s $t$ value obtained from tables for an infinite number of freedom (1.96 for 95% confidence intervals). The confidence limits are presented as percentages ($p \times 100$).

For estimation of the relationships between the diameter of P. verrucosum colonies and the fungus CFU numbers or log of the CFU numbers, linear correlation analysis as well as linear and exponential regression analyses were applied. Together with correlation coefficients ($r$), probability ($P$) and the number of kernels ($n$) are presented. Determination coefficients ($R^2$) are presented with linear and exponential regression equations.

Results and Discussion

In these studies, a temperature of 22.5°C (the arithmetic mean of 20°C and 25°C) was chosen for incubation of DYSG Petri plates to determine both P. verrucosum CFU number and the diameter of the fungal colonies, because in many studies temperatures of 20°C or 25°C were used for incubation of Petri dishes with DYSG medium in both dilution plating and/or direct plating methods for enumeration of P. verrucosum CFU in grain or soils and for the number of cereal kernels colonized by the fungus (Elmholt, 2003; Elmholt and Rasmussen, 2005, Elmholt et al., 1999; Lund and Frisvad, 2003; Kristensen et al., 2005).

In Experiment I, where wheat grain was inoculated with a great range of number of P. verrucosum spores, the number of P. verrucosum CFU varied from less than 25 (under the detection limit) to more than 4×10^6 per gram of the grain (Table I). From 10^2 CFU number upward, 100% of the wheat kernels, placed on the surface of DYSG medium, were surrounded by colonies of P. verrucosum. However, P. verrucosum grown only beside 3–11% of these wheat kernels, inoculated with approximately 0.35–35 spores per 1g (about 23 kernels) of the grain (Table I). Simultaneously with a progressive decrease of P. verrucosum CFU number on the grain, a progressive increase in the percentage of the kernels (placed on DYSG medium) with growing fungi different from P. verrucosum was observed (Table I). In the case of P. verrucosum CFU number lower than 100 per 1 g of the grain, all wheat kernels, placed on DYSG medium, were surrounded by colonies of other fungi (Table I).
Diameter of *P. verrucosum* colonies surrounding wheat kernels on DYSG medium was correlated with the fungal CFU number on the wheat grain (Table I). The visible initial growth of *P. verrucosum* around wheat kernels placed on the surface of DYSG medium was observed earlier, when CFU number of the fungus was higher (results not shown), but later radial growth rates (only in experimental series with *P. verrucosum* CFU number higher than 100 per 1 g of the grain) were faster in the cases of series with lower CFU number, so the differences between the series gradually decreased with increasing time (Table I).

Experiment II was conducted to evaluate the significance of the physiological state of *P. verrucosum* on the diameter of the fungal colony around the wheat kernels placed on DYSG medium. In the series incubated at 1°C for 48 h *P. verrucosum* existed only in the form of spores; in the series incubated at 1°C for 24 h and at 22.5°C for 24 h the fungus predominantly existed in the form of germinated spores; and in series incubated at 22.5°C for 48 h it existed mostly in the form of short hyphae. Results of this experiment, presented in Table II, show that the physiological state of *P. verrucosum* was less important than CFU number of the fungus for its colony diameter around wheat kernels placed on the surface of DYSG medium. Nevertheless, the results of percentage of kernels with *P. verrucosum* colonies grown beside the kernels on DYSG medium show that the physiological state is important in competition with other microorganisms, because higher percentage of the kernels with growing *P. verrucosum* was observed in the case of the grain inoculated with the short fungal hyphae than with fungal spores (Table II).

### Table I

*P. verrucosum* abundance on wheat grain and growth rate of the fungus around wheat kernels placed on DYSG medium as well as the percentage of kernels with *P. verrucosum* and other fungi in Experiment I

<table>
<thead>
<tr>
<th><em>P. verrucosum</em> CFU numbers per 1 g of grain</th>
<th><em>P. verrucosum</em> colony diameter (mm)</th>
<th>Percentage of wheat kernels with <em>P. verrucosum</em> colony</th>
<th>Percentage of wheat kernels with colony of fungi different from <em>P. verrucosum</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Days of incubation of Petri dishes with wheat kernels</td>
<td>Days of incubation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>4165500 f ♣</td>
<td>13.06 c</td>
<td>17.31 c</td>
<td>21.44 f</td>
</tr>
<tr>
<td>368000 e</td>
<td>11.50 d</td>
<td>15.86 d</td>
<td>20.19 e</td>
</tr>
<tr>
<td>32372 d</td>
<td>9.53 c</td>
<td>13.97 c</td>
<td>18.33 d</td>
</tr>
<tr>
<td>3150 c</td>
<td>6.85 b</td>
<td>11.31 b</td>
<td>15.86 c</td>
</tr>
<tr>
<td>250 b</td>
<td>3.19 a</td>
<td>7.33 a</td>
<td>12.28 b</td>
</tr>
<tr>
<td>50 a n.m. ♯</td>
<td>5.00 a</td>
<td>7.00 b</td>
<td>−</td>
</tr>
<tr>
<td>&lt;25 n.m.</td>
<td>2.50 a</td>
<td>2.67 a</td>
<td>−</td>
</tr>
<tr>
<td>&lt;25 n.m.</td>
<td>0</td>
<td>1.00</td>
<td>−</td>
</tr>
</tbody>
</table>

♦ – not measurable; ♣ – the means in separate columns marked with different letters are statistically different at P < 0.01; ♦ – the differences between diameters after 6 days and diameters after 4 days; ♯ – the values in the parentheses are 95% confidence intervals. The percentages with confidence intervals overlapped each other did not differ statistically at P = 0.05;

### Table II

The effect of introduction of different amounts of *P. verrucosum* propagules to the wheat grain and physiological state of the fungus in the inoculum on its growth rate around the wheat kernels placed on DYSG medium in Experiment II

<table>
<thead>
<tr>
<th><em>P. verrucosum</em> spore numbers added to 1 g of wheat grain</th>
<th><em>P. verrucosum</em> colony diameters on DYSG medium (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Incubation conditions of <em>P. verrucosum</em> spores</td>
</tr>
<tr>
<td></td>
<td>2 days at 1°C</td>
</tr>
<tr>
<td>17×10⁶</td>
<td>18.50 c ⋆⋆</td>
</tr>
<tr>
<td>17×10⁷</td>
<td>12.28 b</td>
</tr>
<tr>
<td>17×10⁸</td>
<td>3.50 a (5.6%)*</td>
</tr>
</tbody>
</table>

⋆ – the values in parentheses are the percentage of wheat kernels with colonies of *P. verrucosum* (all kernels from the remaining experimental series are surrounded by colonies of *P. verrucosum*)

⋆⋆ – the means marked with different letters are statistically different at P < 0.01
Results of Experiment III, where the nonsterilized wheat grain, inoculated with *P. verrucosum*, was incubated for 2 weeks at different temperatures and different initial moisture contents, show, similarly to the previous experiments, a distinct relationship between *P. verrucosum* CFU number on the grain and the diameter of colonies of the fungus surrounding the wheat kernels placed on DYSG medium (Table III). However,

<table>
<thead>
<tr>
<th>Conditions of incubation: temperature/initial grain moisture</th>
<th><em>P. verrucosum</em> colony diameters on DYSG medium (mm)</th>
<th><em>P. verrucosum</em> CFU numbers on DYSG medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C/22%</td>
<td>20.54 c *</td>
<td>77.5 × 10^6 d</td>
</tr>
<tr>
<td>15°C/22%</td>
<td>20.13 bc</td>
<td>13.0 × 10^6 c</td>
</tr>
<tr>
<td>20°C/20%</td>
<td>19.47 b</td>
<td>78.1 × 10^6 c</td>
</tr>
<tr>
<td>15°C/20%</td>
<td>18.93 b</td>
<td>17.9 × 10^6 b</td>
</tr>
<tr>
<td>10°C/22%</td>
<td>16.26 b</td>
<td>18.8 × 10^6 a</td>
</tr>
<tr>
<td>10°C/20%</td>
<td>15.16 a</td>
<td>15.1 × 10^6 a</td>
</tr>
</tbody>
</table>

* – the means in separate columns marked with different letters are statistically different at P<0.01

Table IV

Linear correlation coefficients between diameter of *P. verrucosum* colonies surrounding the wheat kernels and CFU number (normal and presented as log_{10}) of the fungus on wheat grain

<table>
<thead>
<tr>
<th></th>
<th><em>P. verrucosum</em> colony diameters on DYSG medium</th>
<th><em>P. verrucosum</em> CFU numbers on DYSG medium</th>
<th>Log_{10} of CFU numbers of <em>P. verrucosum</em> on DYSG medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. I (n = 5)</td>
<td>1</td>
<td>0.653</td>
<td>0.996*</td>
</tr>
<tr>
<td>after 4 days</td>
<td>1</td>
<td>0.635</td>
<td>0.981*</td>
</tr>
<tr>
<td>after 5 days</td>
<td>1</td>
<td>0.633</td>
<td>0.982*</td>
</tr>
<tr>
<td>after 6 days</td>
<td>1</td>
<td>0.814</td>
<td>0.995*</td>
</tr>
<tr>
<td>Experiment II (n = 3)</td>
<td>1</td>
<td>0.590</td>
<td>0.955*</td>
</tr>
<tr>
<td>Experiment III (n = 6)</td>
<td>1</td>
<td>0.590</td>
<td>0.955*</td>
</tr>
</tbody>
</table>

* – The correlation coefficients are significant at P<0.01

Results of Experiment III, where the nonsterilized wheat grain, inoculated with *P. verrucosum*, was incubated for 2 weeks at different temperatures and different initial moisture contents, show, similarly to the previous experiments, a distinct relationship between *P. verrucosum* CFU number on the grain and the diameter of colonies of the fungus surrounding the wheat kernels on DYSG medium (Table III). However,
of results of Yong and Cousin (2001) are similarly related to A. parasiticus CFU number as the diameter of P. verrucosum colony to P. verrucosum CFU number in the present study. The present calculations on the basis of results of Yong and Cousin (2001) show that linear correlation coefficients between ELISA readings and A. parasiticus CFU numbers (r = 0.504 and 0.426 for naturally contaminated maize < n = 20 > and inoculated maize and peanuts < n = 37 >, respectively) are much higher and statistically significant, when the fungal CFU numbers are transformed to logarithmic values (r = 0.929 and 0.945).

The proposed, very simple method (which is a derivative of direct plating using DYSG medium, which enables to be distinguished P. verrucosum from other fungi) can be useful in the determination of the relative abundance of this fungus on cereal grain in incubation experiments.

Acknowledgements. We thank Prof. Anthony R. Dexter for correction of English grammar and syntax in our manuscript.

Literature


L-forms of \textit{Staphylococcus epidermidis} Induced by Penicillin

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\textbf{Abstract}

L-forms of \textit{S. epidermidis} were induced at 35°C with the use of an L-form medium with penicillin. The aim of this study was to evaluate the frequency of L-form induction and demonstrate whether the origin of the clinical strains affects the frequency of L-forms induction, as well as to study whether the time of action of the antibiotic has an influence on frequency of L-form induction.

\textbf{Key words:} L-forms, \textit{Staphylococcus epidermidis}

Cell wall deficient forms of \textit{Staphylococcus epidermidis} were isolated directly from clinical material of patients with jaw muscle infections (McGregor, 2000), urinary tract infections (Dominigue \textit{et al.}, 1993; Świerczewski and Reyes, 1970), secretory otitis media (Ataoglu \textit{et al.}, 1994). It is supposed, that L-forms of bacteria might be a cause of infections in patients with chronic idiopathic prostatitis (Dominigue and Hellstrom, 1998), and can be a cause of malignant tumors located on sun-exposed areas such as head, arms and legs (Cantwell, 2003). Some studies in the 1960s and 1970s showed that L-forms of \textit{Staphylococcus aureus} may be produced \textit{in vitro} by the action of penicillin (Banville 1964; Rosdahl and Vejlsgaard, 1970; Simon and Yin, 1970). However, there are no reports available concerning the studies of the evaluation of frequency of L-form induction of \textit{S. epidermidis}, the demonstration whether the origin of the clinical strains affects the frequency of L-form induction and examination if the time of action of the antibiotic has an effect on L-form induction.

36 strains of penicillin resistant \textit{S. epidermidis} were included in this study to determine \textit{in vitro} L-form induction. Strains were identified with the API Staph biochemical tests (bioMérieux). The \textit{S. epidermidis} strains, were isolated from blood (12), urine (12) and biomaterials (catheters, drains) (12) from patients of A. Jurasz University Hospital in Bydgoszcz. The \textit{S. epidermidis} strains were tested for induction to L-forms on Tryptic Soy Agar, TSA (BBL). Next, the bacteria were suspended in Tryptic Soy Broth, TSB (BBL) to a turbidity approximating 0.5° on the McFarland scale and were incubated for 2 hours. In order to induce L-forms, 0.05 ml of TSB suspension was added to 4.95 ml of Brain Heart Infusion, BHI (Difco) containing 100 U/ml penicillin G (Biochemie GmbH). The samples (0.1ml) were taken after 10 minutes of incubation and again after 24 hours. The samples were plated on BHI agar supplemented with 5% NaCl, 5% sucrose (Polskie Odczynniki Chemiczne, Gliwice), 0.5% yeast extract (Difco), 10% fresh horse serum and 100 U/ml penicillin G. All cultures were incubated at 35°C. The plates were checked for presence of L-form colonies for 8 days (Jakubczak \textit{et al.}, 2002; Owens, 1988). Homogenous growth of colonies which had irregular areas, “fried egg” shapes with centrally located core growing above the colony surface was taken as positive results.

In comparison with the parental strains colonies, the colonies of \textit{S. epidermidis} L-forms were larger than the vegetative cells they had been derived from. In this study only cultures of L-forms colonies with “fried egg” shapes, irregular areas, centrally located core growing above the surface of the colony were taken into consideration. After 10 minutes and 24 hours of incubation in BHI with penicillin 11.1% and 25.0% strains of \textit{S. epidermidis} transformed into L-forms, respectively. The same strains of L-forms grew after 10 minutes.
and 24 hours of incubation in BHI with penicillin. Table I shows the frequency of the induction of S. epidermidis L-forms from the strains of blood, urine and biomaterial.

The L-forms of bacteria have not been carefully studied yet, because it is difficult to characterize them by light microscopy. They do not grow on common media, but they can be cultured on hypertonic medium containing fresh horse serum and penicillin (Jakubczak et al., 2002; Owens, 1988). Dominique et al. (1993) isolated L-forms of Staphylococcus haemolyticus and Streptococcus agalactiae from a 22-year old woman with haematuria and routine culture-negative urine. They drew the conclusion that L-forms of bacteria were present in the genitourinary tract of the patient and caused idiopathic haematuria after antibiotic treatment. Ataoglu et al. (1994) cultured L-forms of coagulase negative staphylococci, for example S. epidermidis, from patients with secretory otitis media who had been treated by cefaclor or a combination of ampicillin and subbactam. However, there are no reports available about of the intentional in vitro induction of L-forms of S. epidermidis. It appears that the cause of phenomenon is the fact that S. epidermidis has been considered as pathogen in clinical cases in the 1980s and most of the studies of L-forms induction took place in 1960s and 1970s (Banville, 1964; Rosdahl and Vejlsgaard, 1970 and Simon and Yin, 1970). These studies showed that the strains of S. epidermidis which are penicillin resistant were capable to induce L-forms. Similar studies with the use of strains of S. aureus producing penicillinase were carried out by Rosdahl and Vejlsgaard (1970); Simon and Yin (1970). They concluded that it is possible that strains of S. aureus producing penicillinase induced L-forms. So it was supposed that the mechanism of bacterial resistance does not have an influence on the bacteria changing into L-forms. Jakubczak et al. (2002) and Owens (1988) proved that most of strains of S. aureus changed into L-forms after 10 minutes incubation in BHI with penicillin. Our studies suggest that the period of 24 hours is more optimal to induce L-forms of S. epidermidis strains. The origin of strains of S. epidermidis did not play a significant role in changing bacteria into L-forms. It was also proved by Jakubczak et al. (2002) in their studies of S. aureus. However, this problem requires further studies with the strains of S. epidermidis that cause clinically proved urinary tract infections, bacteriemia and shunt and catether-related infections.

### Literature


<table>
<thead>
<tr>
<th>Source of isolation</th>
<th>Number of strains producing L-forms isolated after 10 min of exposure to penicillin</th>
<th>Number of strains producing L-forms isolated after 24 h of exposure to penicillin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine (n = 12)</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Blood (n = 12)</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Biomaterials (n = 12)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>In general</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

n – number of strain
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