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CORRIGENDUM

New Methods of Pathogenic Bacteria Elimination

KRYSTYNA I. WOLSKA, ANNA KRACZKIEWICZ-DOWJAT, ANNA M. GRUDNIAK, ANNA SAJKOWSKA and TATIANA WIKTOROWICZ


The legend to Fig. 1, p. 41, had erroneous designations and should be as shown below:

Fig. 1. Approach to killing of recipient cell.
Three methods of plasmid-based killing of bacteria are shown: A) killing by run-away replication, B) killing by activation of bacteriocin (lack of its neutralization), C) killing by derepression of gene encoding bacteriocin. ◊ – repressor of oriV, □ – repressor of bacteriocin transcription, ▶ – bacteriocin, ❃ – antidote.

Moreover, the authors sincerely apologize to Professor Marcin Filutowicz, University of Wisconsin, Madison WI, USA, for using Fig. 1, which was entirely his idea, without his explicit permission.
IN MEMORIAM

Professor Władysław J.H. Kunicki-Goldfinger (1916–1995)
(on the tenth anniversary of his passing away)

August 2005 saw the sad tenth anniversary of the death of prof. Władysław J.H. Kunicki-Goldfinger, an outstanding researcher, teacher, educator and Master of many Polish microbiologists. At the Professor’s grave, during the burial ceremonies in Lublin, the then Rector of Warsaw University, prof. Włodzimierz Siwiński, said “The old saying that no one is irreplaceable is not true. Professor Kunicki-Goldfinger with all certainty was such a man”. Round anniversaries usually prompt recollections, so this is the right occasion to recall the Professor’s biography, his scientific, social and organizational activity.

Professor W. Kunicki-Goldfinger was born in Kraków. His father, Zygfryd Goldfinger was a lawyer and his mother, Waleria Kunicka – a secondary school teacher. The Professor’s youth was spent in Lublin. In 1934 he commenced studies at the Faculty of Philosophy of the Jagiellonian University, majoring in the Natural Sciences in 1939. Because of his left-wing orientation he was not able to secure the position of assistant professor at his home faculty but after obtaining financial support began work at the Department of Agricultural Microbiology of the Jagiellonian University, where he met prof. Otton Bujwid, a world renown microbiologist. Shortly after the outbreak of the Second World War, he left for Lwow, from where he was deported to a work camp near Archangielsk. After an amnesty for Poles, he joined general W. Anders’s army, with which he did the whole combat trail.

The Professor returned to Poland in 1947 and started work at the Maria Curie-Skłodowska University in Lublin, where he defended his doctoral (1948) and habilitation (1950) theses, after which he set out on his own scientific trail, first establishing a Microbiology Workshop in the Division of Plant Physiology, and later the Department of General Microbiology. In 1955 Professor moved to Wrocław where he took over as the director of the Institute of Botany, Wrocław University and also established the Department of Bacterial Genetics in the Ludwik Hirsfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences. The Professor’s scientific itinerary ended in Warsaw, where he came in 1961 and immediately began work at the Department of Microbiology, and later Institute of Microbiology, Faculty of Biology and Earth Sciences at the Warsaw University. He was head of both for many years.

For several years the Professor was vice-director of the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences in Warsaw. In 1970 he accepted the position of “visiting professor” at the University of California at Davies. In 1965 he was elected corresponding member of the Polish Academy
of Sciences, and in 1980 – regular member. He also received honorary doctoral degrees from the Maria Curie-Skłodowska University in Lublin and from Wrocław University, and was laureate of State Award.

Professor Władysław Kunicki-Goldfinger (with his group) published many scientific articles in different fields of microbiology, including veterinary microbiology, environmental microbiology and bacterial genetics. Of these papers, close to 30 were published in leading scientific journals, under the joint title “Mechanism of Bacterial Conjugation and Recombination”. In his last years, the Professor’s interest focused on the area between biology (especially evolutionism) and philosophy. He published many treatises in philosophical and anthropological titles, had seminars at philosophical societies as well as at the Academy of Catholic Theology. The Professor was the author of many books, including academic textbooks. He had the gift of the written word, which is not that frequent among naturalists. All the Professor’s books, including those of a popular-science nature, were enormously successful and had many reprints. There were as many as 7 editions of his unconventional textbook “Życie bakterii” (The Life of Bacteria).

Words simply cannot adequately describe the didactic talent of the Professor. He was always surrounded by undergraduate and post-graduate students, assistants. Many of his students are now well known researchers and some of them hold high positions, not only in Poland. These “disciples” stem from three centres: in Lublin, Wrocław and Warsaw – all created and headed by the Professor. This achievement can be rivaled by very few scientists indeed.

The Professor’s biography cannot omit at least some of his organizational and social activities. The Professor was honorary member of Polish Society of Microbiologists, founding member of the Polish Genetical Society and the journal Acta Microbiologica Polonica, consultant for the PWN (Polish Scientific Publishers). He collaborated with the Committee for the Defense of Workers, the stipend fund for aggrieved researchers, participated in the activity of the Society for Supporting and Disseminating Knowledge and the “Flying University”.

In 1981 he was interned for his activities in the opposition. Later, in 1989, he took part in the historic “Round Table Discussions” in the section for science and education. To sum up this short biography, it should be emphasized once again that Professor Władysław Kunicki-Goldfinger was, without a doubt, a magnificent researcher, even though he himself used to say that “I am a so-called scientist”. He was also a charismatic teacher and educator who strongly influenced the attitudes of his students and followers. He was a Master and authority for many of us, the only one we have encountered in the course of our scientific activity. He was also an outstanding individuality. His pupils always had an important place in His heart and met with kindliness and help, and this aid sometimes was of key importance for more than one of us. However, the most precious thing in His life was his family – his wife, dr. Władysława Kunicka-Goldfinger and his children. The Professor’s wife, a senior lecturer at the Institute of Microbiology at Warsaw University for many years, “our beloved Mrs. Władka” died in March 2005. It should be mentioned that She was very happy hearing about a Conference (held in October 2005 in Warsaw) devoted to the memory of the Professor and took lively interest in its progress.

10 years ago, in valediction addresses, obituaries and recollections published in scientific journals, there were many voices ensuring that the memory of the Professor will persist a long time. These assurances were not hollow. A decade has passed since his death and memory about Him is still very much alive and will probably remain so for decades to come.

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Tuberculosis Bacilli Still Posing a Threat. Polymorphism of Genes Regulating Anti-Mycobacterial Properties of Macrophages

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

Abstract

One third of the earth’s population is infected with Mycobacterium tuberculosis, but only 5–10% of the infected individuals will develop active disease over their lifetime. To identify the genes responsible for the variation in the human susceptibility/resistance to tuberculosis (TB) we determined the polymorphisms of three genes crucial for the function of macrophages, in TB patients and healthy controls with no past history of TB. We found no association between the polymorphisms of the NRAMP-INT4, MBL (codons 52, 54, 57) and CD14-159 genes and TB in a Caucasian Polish population. However, we have suggested a possible involvement of CD14 and MBL molecules in the host-mycobacteria interactions on the basis of the significant increase in the serum CD14 and MBL in TB patients compared to healthy controls.

Key words: tuberculosis, CD14, MBL, NRAMP-1, polymorphism

Introduction

Tuberculosis (TB) carries serious health and economic implications in today’s society. The global burden of tuberculosis remains enormous, mainly because of poor control in Southeast Asia, sub-Saharan Africa and eastern Europe, and because of high rates of Mycobacterium tuberculosis and Human Immunodeficiency Virus (HIV) coinfection in some African countries (Dye et al., 1999). It has been estimated that M. tuberculosis infects about one-third of the world population and causes 8 million active cases of TB per year (WHO 2004). About 2 million people die of TB every year. The highest mortality of TB is notified in some African countries with high HIV rates. A new increasing problem is the appearance of multidrug-resistant strains of M. tuberculosis and this represents a failure in case management. The bacilli Calmette – Guérin (BCG), a live attenuated strain of M. bovis, is administered to approximately 100 million people every year. This vaccine is effective in preventing TB in children, however, it failed to reduce the incidence of the disease in the adult population. New drugs, more effective vaccine and better understanding of M. tuberculosis – host interactions are needed to counter TB.

Epidemiological data show that most of M. tuberculosis infected population neither develops disease nor becomes infectious, and clinical disease occurs in less than 10% of infected subjects. It suggests that genetic
differences in the host determine the immunological response, disease severity, and ultimate outcome of infection with mycobacteria. As intracellular pathogens, *M. tuberculosis* bacilli have the ability to survive within the host macrophages. Thus, we may expect that in the majority of *M. tuberculosis* infected subjects, the interaction between *M. tuberculosis* and phagocytic cells results in a dynamic balance between the host defence system and the virulence factors of mycobacteria allowing the persistence of *M. tuberculosis* in the absence of disease. Unfortunately, in about 10% of *M. tuberculosis* infected subjects, the interaction between macrophages and *M. tuberculosis* bacilli may result in tissue damage characterised by tissue necrosis with the formation of cavities and dissemination of the disease. To identify the genes responsible for differences in the human susceptibility to TB we investigated the polymorphism of three genes, *NRAMP-1*, *MBL*, *CD14*, which encode the proteins crucial for the functions of macrophages, in TB patients and healthy subjects who had no past history of tuberculosis. The *NRAMP-1* gene (Natural Resistance Associated Macrophage Protein-1) is a human homologue of mouse *Nramp-1* gene which confers increased resistance to infection with *Salmonella typhimurium*, *Leishmania donovani* and *M. bovis* BCG (North et al., 1996). The *MBL2* gene encodes mannose-binding lectin (MBL) a calcium-dependent serum lectin that acts as an opsonin to promote phagocytosis and activates complement via the classical pathway. The co-dominant single-base substitutions in codons 52, 54 and 57 result in reduced serum MBL concentrations (Turner, 1996). The *CD14* gene encodes a glycosylphosphatidylinositol-linked cell surface molecule CD14 which mediates mycobacteria induced activation of macrophages via Toll-like receptors (TLR), particularly TLR2 (LeBouder et al., 2003). The concentration of soluble CD14 (sCD14) and MBL in the sera from TB patients and controls was also determined.

**Experimental**

### Materials and Methods

**Subject characteristics.** Our study group consisted of 250 Caucasian Polish subjects, 126 patients with pulmonary tuberculosis (age 17–90 years, mean 51 ± 16) and 124 healthy volunteers (age 18–85 years, mean 50 ± 14) who had no past history of TB. In the patient group (TB patients), tuberculosis was confirmed by culture of *M. tuberculosis* from sputum. The study was approved by the Human Ethics Committee. All participants signed the written informed-consent documents.

**NRAMP-1, CD14 and MBL gene polymorphism.** DNA was isolated from EDTA-anticoagulated blood using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. The *NRAMP-1*/–159T/C polymorphism were determined by PCR method. The reaction was performed in 10 µl volume that contained 50–100 ng of DNA, 0.25 mM of paired primers, 1 X PCR buffer with 1.5 mM MgCl₂, 0.2 mM dNTP, 0.5 U Taq DNA polymerase (Promega, Madison, USA). The paired primers used for CD14/–159 polymorphism were, for C allele: 5'-CTC CAG AAT CCT TCC TGT TAC GAC-3' and 5'-TTG GTG CCA ACA GAT CAG GTT CAC-3' and for T allele: 5'-TTG GTG CCA ACA GAT CAG GTT CAC-3' and 5'-TGT AGG ATG TTT CAG GGA GGG GTA-3'. The PCR conditions were: 95°C for 5 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension of 72°C for 5 min. The primers used for *NRAMP-1*/–159T/C polymorphism were general primer 5'-CTC GCC TCC TCA CAG CTT CT-3' and G specific primer 5'-GGT TCT CCC TGT CCA GGC-3' or C specific primer 5'-GGT TCT CCC TGT CCA GGG-3'. The amplification consisted of a 2-min denaturation step at 94°C, 10 cycles of 10 s at 94°C and 60 s at 65°C, and 20 cycles of 10 s at 94°C and 50 s at 61° and 30 s at 72°C. PCR-amplified DNA were visualized by electrophoresis in 2% agarose gel stained with ethidium bromide (0.5 µg/ml) and visualized by photography under UV transillumination. In PCR for CD14/159 polymorphism, the assay yields a 381-bp band for the T allele and a 227-bp band for the C allele. Genotyping for MBL-2 variants was performed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR). To detect the mutations at codons 54(GGC→GAC, allele B) and 57 (GGA→GAA, allele C), a 685-bp fragment was amplified by PCR using the primers: 5'-AGT CGA CCC AGA TTG TAG GAC AGA G-3' and 5'-AGT TGT TGT TCT CCT GTT CAG-3'. The PCR product was digested with BanI and MboII restriction enzymes (Promega, Madison, USA). Detection of the mutation at codon 52 (GGC→GAC) was performed by MluI and HhaI restriction enzyme digestions of the 125-bp PCR-product amplified with the following primers: 5'-CAT CAA CGG CTT CCC AGGCAA AGA G-3' and 5'-AGT ATC CAG GCA GGT TCT CAC GGA AGG-3'. PCR was performed in a reaction volume of 10 µl, containing 50–100 ng of DNA, 0.25 mM of specific primers, 1 X PCR buffer with 1.5 mM MgCl₂, 0.2 mM dNTP, and 0.5 U Taq DNA polymerase (Promega, Madison, USA). All PCRs were initiated by a 5-min denaturation step at 95°C and completed by a 5-min extension step at 72°C. Amplification consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. PCR products were digested with the restriction enzymes BanI, MboII, MluI and HhaI at 37°C for 2 h, separated by electrophoresis in 2% agarose gel and stained with ethidium bromide.

**Serum levels of sCD14 and MBL.** The concentration of sCD14 in the sera was measured using an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine™ sCD14, R&D, MN, USA). MBL levels in the sera were determined by using a Human MBL ELISA test Kit (HyCult Biotechnology, Uden, The Netherlands). The optical density (OD) of each sample was determined using a multifunctional counter Victor 2 (Wallac Oy, Turku, Finland) set at 450 nm.

**Statistical analysis.** All analyses were performed using Statistica 5.0 (Statsoft). Comparisons between the various genotypes in TB patients and controls were made using the chi-square and Fisher’s exact test. Comparison of mean sCD14 values between the patients and controls was made by Mann-Whitney U-test. A *P* value <0.05 was considered significant.
Results

Allele and genotype frequencies of the patients with culture-proven tuberculosis (TB patients) and healthy individuals who had no past history of TB (controls) were analysed for the single nucleotide polymorphism (SNP) in the intron 4 of NRAMP-1 gene and promoter region of CD14/-159 gene. Three co-dominant single-base substitutions in codons 52, 54, and 57 in MBL gene were also investigated in TB patients and healthy controls. The NRAMP-1/INT4 polymorphism was determined for 126 TB patients and 114 healthy individuals. The distribution of alleles G and C as well as genotypes GG, GC and CC, was almost identical in both TB cases and control group (Table I). The NRAMP-1 GG genotype was the most frequent in the subjects who underwent the study (range 60–61%). The distribution of GC heterozygotes was almost the same in TB patients (36%) and controls (35%). Equally low frequency (4%) of CC genotype was noticed for the control group and TB cases.

The CD14/-159 polymorphism was determined for 126 TB patients and 122 healthy individuals. No association was found between the CD14/-159 gene polymorphism and the presence of TB (Table II). About half the subjects were CD14 CT heterozygous in both TB patients and controls (range 47–49%). Thirty eight percent of TB cases versus thirty one percent of healthy individuals were CC homozygous. This difference was not statistically significant; P>0.05. Also, no significant difference was found between the TB patients and control group in the frequencies of the carriers of the less frequent CD14 TT genotype (15 and 20% respectively).

A total of 108 TB cases and 92 controls were genotyped for the MBL polymorphism (Table III). The frequency of the wild type MBL allele was not different among TB cases and controls (range 61–63%). Also, no difference was found in the distribution of the B, C and D alleles of the MBL gene in TB patients and healthy individuals. One TB patient possessed two mutant alleles, B and D, for MBL gene.

The levels of sCD14 in the sera from 71 TB patients and 56 healthy individuals were tested by ELISA. Fig. 1 shows that there was no association between the serum sCD14 levels and CD14/-159 promoter polymorphism, either in TB patients or in controls. However, patients with TB exhibited significantly higher levels of sCD14 compared to healthy individuals. This increase was observed for all three CD14 genotypes (in CC 1611 ± 334 ng/ml versus 3450 ± 1646 ng/ml, P = 0.000001; in CT 1556 ± 441 ng/ml versus

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<th>Allele and genotype frequencies of NRAMP-1/INT4 gene polymorphism in TB patients and healthy controls</th>
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<th>Allele and genotype frequencies of CD14/C(-159)T gene promoter polymorphism in TB patients and healthy controls</th>
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<th>Allele and genotype frequencies of MBL (52, 54, 57 codons) gene polymorphism in TB patients and healthy controls</th>
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<td><strong>mb1-2 alleles</strong></td>
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The concentration of MBL in the sera from 108 TB patients and 92 healthy individuals was determined by ELISA. In agreement with the previous report, the co-dominant single-base substitutions in codons 52, 54 and 57 of MBL gene resulted in reduced serum MBL concentrations (Fig. 2). The average concentration of serum MBL determined for the healthy individuals with the wild type MBL gene was significantly higher (755 – 805 ng/ml) than serum MBL levels found for the healthy donors with variant alleles for this gene (157 – 140 ng/ml, \( P = 0.0002 \)). The pulmonary tuberculosis was associated with a significant elevation of the serum MBL concentration. The increase in serum MBL levels was noticed in TB patients with the wild type MBL gene (755 – 805 versus 1423 – 1138 ng/m, \( P = 0.0009 \)) and those with the B (159 – 136 versus 355 – 345 ng/ml, \( P = 0.05 \)) and D (153 – 161 versus 730 – 652 ng/ml, \( P = 0.029 \)) variant alleles for this gene.

### Discussion

One third of the earth’s population is infected with *M. tuberculosis*, but only 5–10% of the infected individuals will develop active disease over their lifetime. There is an increasing interest in the understanding of the role of genetic factors controlling susceptibility to TB in humans because this will allow us to develop effective strategies to combat this disease. A variety of studies have demonstrated that a large number of host genes are probably important in susceptibility to tuberculosis (Bellamy *et al.*, 1998a, b). The main route of infection for *M. tuberculosis* is the respiratory tract, where the bacilli are inhaled in airborne droplets. After entering lung, the first cell type encountered by the bacteria is the alveolar macrophage, which has the bactericidal ability to destroy most potential invaders. However, pathogenic mycobacteria prevent maturation of the phagosomes in which they reside inside macrophages and this is thought to be a major strategy
allowing them to survive and multiply within macrophages. The macrophages have multiple functions in *M. tuberculosis* infection including the antigen processing and presentation, production of cytokines that regulate the maturation and function of lymphocyte subsets, formation of granuloma that may retain mycobacteria inside cells and prevent their dissemination or eventually may result in tissue damage characterised by necrosis. The importance of the macrophages in the mycobacteria-host interaction prompted us to investigate the polymorphism in the *NRAMP-1*, *CD14* and *MBL* genes, which are involved in the expression of the macrophage functions. In mice, the *NRAMP-1* gene encodes a transmembrane protein that translocates to the phagocytic vacuole of macrophages following phagocytosis of bacteria and affects the intracellular survival of bacilli (Frehel et al., 2002). In this study the allele and genotype frequency of *NRAMP-1* gene polymorphism was almost the same in TB patients and controls. In contrast, in case-control study in Gambia, four polymorphisms in the *NRAMP-1* gene were found associated with TB (Bellamy et al., 1998a). In Denmark, Søborg et al., 2002, found that variant alleles in *NRAMP-1* gene were associated with increased mycobacterial replication rather than susceptibility to TB. Also Pacheco et al. (2003) demonstrated no association between the *CD14*-159 polymorphism and TB among a population of Colombia. A study in Malawi showed that a heterozygosity for a newly investigated CAAA insertion/deletion polymorphism in the *NRAMP-1* gene was associated with a protection against TB (Fitness et al., 2004). However, association of other variants of *NRAMP-1* gene with TB that was reported for other populations was not replicated in Malawi. This suggests that the genes and variants relevant to the susceptibility to TB may vary significantly between populations. Also, their distribution may be affected by the high rates of HIV infections in some African countries. MBL was included into our study as a key component of the innate immunity. It recognises peptidoglycan of Gram-positive bacteria via its C-type lectin domains and modulates the cytokine and chemokine releasing (Nadesalingam et al., 2005). MBL insufficiency due to polymorphisms in the MBL2 gene causes an opsonisation defect and predisposes to recurrent infections in children and adults (Turner et al., 1996). The frequency of the *MBL* gene mutations (37%) observed by us among healthy Polish subjects was slightly higher than those reported for a Gambian population (31%) (Bellamy et al., 1998b). We have also shown no association between the *MBL* gene polymorphism and a risk of TB in Caucasian Polish population. In contrast, the study among Gambians in West Africa, and African-Americans in Texas, showed a significantly lower frequency of the C and B alleles of *MBL* gene, respectively, among TB cases compared to healthy blood donors (Bellamy et al., 1998b; El Sahly et al., 2004). This suggested that MBL polymorphism was protective against TB in West Africans and African-Americans. However, in agreement with our data no protective effect of *MBL* gene polymorphism was observed among a white and Hispanic population in Texas, Africans in Malawi (Fitness et al., 2004) and a community in India (Selvaraj et al., 1999). Thus, the ethnicity may determine the association of *MBL* polymorphism with the resistance/susceptibility to TB.

The data presented in this paper confirmed the previous reports on a significant elevation of serum MBL levels in TB patients compared to controls (Garred et al., 1997; Bonar et al., 2004). It is known that MBL binds mycobacteria strongly (Nadesalingam et al., 2005) and it has thus been suggested that MBL may facilitate the uptake of mycobacteria by phagocytes. Because macrophages are the living environment for mycobacteria, high MBL serum levels could be a relative disadvantage for the host in relation to these bacteria. However, this suggestion contradicts the data of this study presenting the lack of association between a risk of TB and the MBL gene polymorphisms that result in serum MBL deficiency. Moreover, a previous study reported by us (Paziak-Domańska et al., 2002) has shown that the variation in serum MBL level does not affect the ingestion of *M. bovis* BCG by macrophages. On the other hand, our results do not exclude a possible influence of the increased levels of serum MBL on the host immune response to *M. tuberculosis*. Thus, MBL together with peptidoglycan of Gram-positive bacteria increases the production of chemokines, IL-8 and RANTES, but reduces that of TNF-α (Nadesalingam et al., 2005).

To understand the genetic background of the variation in human susceptibility to pathogenic mycobacteria we also determined the *CD14*-159 polymorphism for TB patients and healthy individuals. The macrophage *CD14* plays a pivotal role in innate immunity. It functions as a multifunctional receptor for bacterial cell wall components and enhances Toll-like receptors – mediated signaling (LeBouder et al., 2003). Previously, we found an association between the *CD14*-154 promoter polymorphism and development of delayed type hypersensitivity to tuberculin, in healthy volunteers vaccinated with *M. bovis* BCG vaccine. Thus it was relevant to investigate a possible relation between the *CD14*-159 polymorphism and TB. However, no difference was found in the distribution of the allele and genotype *CD14*-159 gene polymorphisms between TB patients and healthy individuals with no past history of TB. The lack of the association between the *CD14*-159 polymorphism and a risk of TB was also observed in a population of Caucasian and Mestizo ethnic groups in Colombia (Pacheco et al., 2004). However, the significant increase in sCD14 has suggested
a role of the CD14 molecule in the host-mycobacteria interactions. The same increase in sCD14 concentration was observed for TB patients carrying three different CD14/-159 genotypes (CC, CT, TT). Thus there was no association between sCD14 levels and CD14/-159 polymorphism. This finding is consistent with a previous report (Pacheco et al., 2004) but in contrast with the others showing an elevation of sCD14 in the individuals with the CD14/-159 TT genotype (Baldini et al., 1999; Eng et al., 2004). Several reasons might account for these discrepant findings including the presence of stratification of studied groups varying by the age, ethnic origin and health status. The elevated levels of serum sCD14 were observed in several infectious and non-infectious diseases. Although the function of sCD14 in human diseases has not yet been clarified, it is believed that elevated sCD14 in infectious and non-infectious diseases results from the state of activation of monocytes and macrophages. It is known that activation of monocytes results in increased shedding of sCD14. The elevated concentration of sCD14 may also reflects an increased need of plasma clearance of inflammatory stimuli such as mycobacterial lipoolarabinomannan.

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Literature


Mycobacterium bovis BCG Mycobacteria – New Application

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger
on the tenth anniversary of his passing away

Abstract

The polarized response of T helper-2 (Th2) lymphocytes to an allergen is considered to be the main cause of the pathogenesis of asthma. In this study, we asked a question whether M. bovis BCG mycobacteria which are known for the preferential stimulation of T helper-1 (Th1) immunity, diminish the effector functions of Th2 cells from allergic patients upon stimulation with a common house dust mite Der p-1 allergen. Our results allow a positive answer to this question. We demonstrate that BCG modulates the dendritic cell-dependent allergen presentation process and switches naive T lymphocytes towards an anti-allergic Th1 profile.

Key words: BCG, Der p-1 allergen, Th1/Th2 cytokines

Introduction

The live attenuated M. bovis BCG bacilli developed by Calmette and Guérin at the Institute Pasteur in Lille, France, has been a widely used vaccine against tuberculosis since 1921 (Griffin et al., 1999, Hoft et al., 1999, Williams et al., 2000). BCG vaccination of newborns and infants significantly reduces the risk of tuberculosis, by over 50%, on average. A protection has been observed across many populations. However an extraordinary variability in the protective efficacy of BCG vaccines was showed in clinical trials carried out in different parts of the world (Huebner, 1996). More than three billion people throughout the world have been vaccinated with BCG and this vaccine is considered to be one of the safest vaccines available. It appears to be safe even when given to perinatally HIV-1 – infected babies (Lallemand-Le Coeur et al., 1991). However, BCG vaccination did not eliminate the tuberculosis problem and a new more effective vaccine against tuberculosis is required. One newly invented vaccine against tuberculosis, the urease C-deficient recombinant BCG equipped with the membrane-perforating listeriolysin of Listeria monocytogenes, has been recently licensed to a pre-clinical trial (Kaufmann, 2005).

The study conducted by Marchant et al., 1999 indicated for the first time that human newborns can develop an acquired T-helper 1 (Th1) cellular response upon immunization with BCG. This response is characterized by a polarized production of type 1 cytokines including interleukin 12 (IL-12), interferon γ (IFN-γ) and IL-2. In this study, we addressed the possible interference of BCG with lymphocyte differentiation into T-helper 2 (Th2) cells upon stimulation with a common allergen from the house dust mite...
Dermatophagoides pteronyssinus (Der p-1). The polarized Th2 response to an allergen is considered to be the main cause of the pathogenesis of asthma. Th2 lymphocytes recognize the allergen peptides via T cell receptors and release interleukins which account for the joint involvement of B cells producing anti-allergen IgE (IL-4, IL-13), mast cells (IL-4, IL-10) and eosinophils (IL-5) in allergic inflammation (Romagnani, 2000). In order to investigate whether BCG interferes with the generation of Th2 response to Der p-1 allergen, we stimulated naïve T lymphocytes from the peripheral blood of the patients with allergy to Der p-1 and healthy donors with autologous dendritic cells (DC) presenting Der p-1 allergen in the presence or absence of BCG. Dendritic cells are the first immunocompetent cells to encounter inhaled allergens and they possess a unique capacity to initiate response in naïve T lymphocytes (Lambrecht and Hammad, 2003). The results show that in vitro BCG modulates the dendritic cell-dependent allergen presentation process and switches naïve T lymphocytes towards an anti-allergic Th1 profile. This allows speculating that BCG bacilli might be considered as a potential candidate for immunotherapeutic strategies in allergy.

Experimental

Materials and Methods

Patients. Blood was collected from allergic patients sensitive to Dermatophagoides pteronyssinus and from healthy donors. Allergic patients had a history of asthma and presented the usual features of house dust mite sensitization: specific IgE antibodies and positive skin prick tests toward D. pteronyssinus. The level of total IgE was higher than 100 IU/ml. Healthy donors did not display any of these characteristics; total IgE level was below 100 IU/ml and absence of specific IgE to D. pteronyssinus in serum.

Bacteria. M. bovis BCG, a skin lyophilized vaccine against tuberculosis; 1.6×10⁹ live bacilli/ml was diluted to 1×10⁸ and used for pulsation of DC.

Cell preparation. After depletion of platelet-rich plasma, blood was diluted with RPMI 1640 (1:1; Life technologies, Paisley, Scotland) and layered over a Ficoll density gradient (Pharmacia, Uppsala, Sweden). After centrifugation (400 g for 30 minutes) human PBMCs (Peripheral Blood Mononuclear Cells) were isolated, washed and resuspended in PBS (Phosphate Buffered Saline), pH 7.2, supplemented with 0.5% BSA (Bovine Serum Albumin; Sigma, Saint-Quentin Fallavier, France) and 2 mM EDTA. PBMCs were incubated on ice for 30 minutes with anti-CD14 antibodies coupled to magnetic micro-beads (Miltenyi Biotec, Germany), washed and applied onto a column placed in the magnetic field of a MACS separator (Miltenyi Biotec). After elimination of negative cells, the column was removed from the separator and the CD14⁺ cells were collected and resuspended in RPMI 1640 containing 10% heat-inactivated FCS (fetal calf serum; Life Technologies), 2 mM L-glutamine, and antibiotics (Ticarpen 1%; Smith Kline Beecham, Belgium) before plating (3×10⁶ cell / 3 ml per well) into 6-well flat-bottomed culture plates. CD14⁺ cells were differentiated into monocyte-derived DC for 6 days in medium supplemented with GM-CSF (20 ng/ml; Peprotech, United Kingdom) and interleukin 4 (IL-4) (200 IU/ml; R&D Systems, United Kingdom). Naive CD45RA⁺ CD4⁺ T lymphocytes were isolated from the eluted CD14⁺ cell fraction by using a CD4⁺ T cell isolation kit (containing CD8, CD11b, CD16, CD19, CD36, CD56 and CD45RO MACS microbeads). T cells were frozen in FCS containing 10% DMSO until used (Pochard, 2005).

Pulsation of DC. Monocyte derived DC (1×10⁵ cells/ml per well) were incubated for 24 hours with Der p-1 (1 µg/ml), BCG (1:1), both stimuli concomitantly or LPS (1 µg/ml; Sigma-Aldrich). T cell-DC cocultures. After washing, 1x 10⁵ pulsed DC were incubated with 1×10⁶ naïve T cells for 5 days. The levels of IL-4, IL-5 and IFN-γ in supernatants of the cultures were determined by specific ELISA (Diaclone). The sensitivity of the assays was 0.5, 5 and 5 pg/ml, respectively.

Statistical analysis. Nonparametric statistical analysis of the cytokine production by naïve T cells was performed using the Mann-Whitney U test. The Fisher exact test was used to determine the prevalence differences. Values of p≤0.05 were considered statistically significant.

Results

Immature monocyte-derived DC from allergic patients and healthy donors, pulsed or not with Der p-1, Der p-1/BCG, BCG, or LPS, were co-cultured, for 5 days, with the autologous naïve T cells at the ratio of 1:10. The concentrations of IL-4, IL-5 and IFN-γ were measured in the culture supernatants by ELISA. Data in Table I show that naïve T cells from all allergic patients produced the Th2 type cytokines, IL-4 and IL-5, when they were stimulated with autologous DC pulsed with Der p-1 antigen, BCG or both stimuli concomitantly. The prevalence of the release of IL-4 and IL-5 by identically stimulated naïve T cells from healthy donors was statistically decreased. LPS – pulsed DC used as a control stimulated the IL-4 and IL-5 production by the T lymphocytes from 3 out of 9 allergic patients and no healthy blood donor. The mean concentration of IL-4 produced by T lymphocytes from healthy donors stimulated with the allergen- or BCG-pulsed DC was very low (range 1.6–2.1 pg/ml) and just above the detection test sensitivity (data not shown). The concentration of IL-4 in the co-cultures of naïve T cells and allergen- or BCG-pulsed DC from allergic patients was easily measurable (Fig. 1). It is worth noting that the mean production of IL-4 by
New application of *M. bovis* BCG

T cells from allergic patients, stimulated simultaneously with allergen and BCG was decreased as compared with the mean IL-4 production driven by DC pulsed with Der p-1 alone. However this difference was not statistically significant.

As it was expected, naive T cells from allergic patients secreted significantly more IL-5 in response to autologous DC pulsed with Der p-1 allergen compared with the cells from healthy donors (*p* = 0.03) (Fig. 2). In contrast, BCG-treated DC stimulated the IL-5 production slightly more intensively in the autologous T cells from healthy donors than from allergic patients (*p* = 0.03). It is worth mentioning that naive T lymphocytes from both allergic and healthy subjects when stimulated with autologous DC pulsed with Der p-1

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**Table I**

Prevalence of the production of IL-4 and IL-5 by naive T cells from healthy donors and allergic patients, in response to DC pulsed with Der p-1, Der p-1 + BCG, BCG, or LPS.

<table>
<thead>
<tr>
<th>Blood donors</th>
<th>Der p-1</th>
<th>Der p-1 + BCG</th>
<th>BCG</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>3/6</td>
<td>3/6</td>
<td>4/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Allergic</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>3/9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blood donors</th>
<th>Der p-1</th>
<th>Der p-1 + BCG</th>
<th>BCG</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>3/6</td>
<td>3/6</td>
<td>4/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Allergic</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>3/9</td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± SEM. Mean intensity value for LPS represents the results for those individuals whose cells produced IL-4 in response to this stimulus.

---

**Fig. 1.** The concentration of IL-4 in the co-cultures of naive T cells from allergic patients, stimulated with autologous DC pulsed with BCG, Der p-1, Der p-1 + BCG or LPS.

**Fig. 2.** The concentration of IL-5 in the co-cultures of naive T cells from allergic patients, stimulated with autologous DC pulsed with BCG, Der p-1, Der p-1 + BCG or LPS.

Results are expressed as the mean ± SEM. Mean intensity values for LPS represent the results for those individuals whose cells produced IL-4 in response to this stimulus.
allergen and BCG concomitantly produced less IL-5 than when they were pulsed with Der p-1 allergen alone. However, this differences did not approached statistical significance.

Data on the production of Th1 type cytokine, IFN-\(\gamma\), are presented in Table II. This cytokine was detected in the cultures of naive T lymphocytes from all allergic and healthy subjects, stimulated with autologous DC pulsed with each stimulus. However, the mean concentration of IFN-\(\gamma\) was significantly lower in the cultures of naive T cells from allergic patients, stimulated with autologous DC pulsed with Der p-1 allergen, BCG or both than in the identically stimulated cultures of T cells from healthy donors (Fig. 3). However, no difference was noticed between the T cells from allergic and healthy subjects, responding by IFN-\(\gamma\) to DC educated with LPS. Moreover, the addition of Der p-1 to BCG-pulsed DC from allergic patients did not reduce their capacity to stimulate IFN-\(\gamma\) production by autologous naive T cells.

<table>
<thead>
<tr>
<th>Blood donors</th>
<th>Der p-1</th>
<th>Der p-1 + BCG</th>
<th>BCG</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
<td>6/6</td>
</tr>
<tr>
<td>Allergic</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
</tr>
</tbody>
</table>

**Table II**
Prevalence of the production of IFN-\(\gamma\) by naive T cells from healthy donors and allergic patients, in response to DC pulsed with Der p-1, Der p-1 + BCG, BCG, or LPS

Allergy is characterized as the strong immune response to allergens present in the environment. The term of atopy describes the genetic predisposition of organism to produce the increased amount of IgE antibodies against allergens. Atopic allergy is a chronic and systemic inflammatory process that currently affects a high proportion of population. According to the World Health Organization (WHO), 25–30% of the world population suffers from allergy (Geha, 2003). This disease affects up to 20% of the population in developed countries. It is currently estimated that next 15–20% habitants of developed countries produce the allergen-specific IgE antibodies so they represent the group of increased risk to allergy. Atopic diseases are the result of Th2-dominated responses to single or several allergens. The results of this study demonstrated that, at least in vitro, autologous BCG-educated dendritic cells modulated the response of naive T cells from the patients with allergy to the main dust allergen, Der p-1. Th2-dominated response of T cells from allergic patients, characterized by the enhanced production of IL-4 and IL-5 and diminished secretion of IFN-\(\gamma\), was polarized towards Th1 profile. This modulatory effect of BCG-pulsed DC resulted in the increased IFN-\(\gamma\) release and diminished secretion of IL-4 and IL-5. It is possible to speculate that in vivo, in the organism,
the decreased secretion of IL-4 and IL-5 will be associated with diminished synthesis of IgE including anti-allergen antibodies of this class which are directly responsible for allergic disorders.

The monocyte derived dendritic cells were used in this study because of their unique capacity to initiate differentiation of naïve T cells and their extraordinary potency in the regulation and maintenance of immune responses to antigens and allergens. The contact zone between DC and T cells is highly organized and the signaling between these two types of cells is called “immunological synapse” (Grakoui et al., 1999; Jacobelli et al., 2004). DC regulate immune response of Th1 and Th2 cells through cell-cell interactions and through the release of soluble mediators. In the presence of various antigens/allergens, DC possibly through the release of Th1- or Th2-attracting chemokines selectively attract these cells into inflamed tissue. In this study we demonstrated that BCG mycobacteria directed DC-dependent response of naive T cells from allergic patients toward a beneficial Th1 profile. Thus, our results suggest that BCG might switch the established Th2 response in allergic patients toward a beneficial Th1 profile. In conclusion, Bacille Calmette – Guérin mycobacteria, which are already used in the treatment of bladder cancer (Herr, 2005), might represent a new therapeutic strategy for the treatment of allergic diseases.

Acknowledgements. This work was supported by Marie-Curie Fellowship 5th Framework Programme and by MNiI grant No 2 PO5A 156 28. We thank healthy donors and allergic patients for their participation in this study. We also thank Professor G.A. Stewart (University of Western Australia) for providing purified native Der p-1 allergen.

Literature


Application of DNA Markers to Estimate Genetic Diversity of *Mycobacterium tuberculosis* Strains

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

**Abstract**

The obligatory human pathogen, *Mycobacterium tuberculosis*, is the most important etiological factor of tuberculosis. Unfortunately, there is little information about genetic diversity of this pathogen. The main aim of this research was the estimation of genetic diversity of *M. tuberculosis* on the basis of various categories of DNA markers. The genome of 32 strains were scanned by DNA markers such RAPD, IS6110 and catalase-peroxidase katG gene. All 162 identified loci were polymorphic. The genetic diversity coefficient (\(H_T\)) of *M. tuberculosis* was 0.32 for RAPD and 0.27 for IS6110. There were 14 alleles in katG gene. All strains were characterised by the individual molecular pattern. Genetic similarity varied from 0.13 to 0.94 (RAPD markers) and from 0 to 1 for (IS6110). *M. tuberculosis* strains did not represent a clonal structure, single source of transmission and epidemiological relationships as well. The applied DNA markers proved to be highly efficient for analysis of genetic structure of *M. tuberculosis*.

**Key words:** *M. tuberculosis*, genetic diversity (\(H\)), RAPD, IS6110, katG

**Introduction**

Tubercle bacillus, *Mycobacterium tuberculosis* is gram positive, acid fast and slow growing bacteria, regarded as main etiological factor of tuberculosis over the world (WHO, 2005). Research in biology and genetics of *M. tuberculosis*, resulted in very good description of ecology, genome construction and its sequencing in 1998 (Cole, 1999). However, in the last decade research focused on polymorphism of tubercle bacillus appeared. Papers concerned mutations in drug resistance genes in *M. tuberculosis* (Jou et al., 2005), selected sequences related to virulence (Gao et al., 2005), insertion sequences (Kulaga et al., 2004) and tandem repeats, as well (Fabre et al., 2004). The data of polymorphism in *M. tuberculosis* strains concern selected genes or sequence of medical and epidemiological meaning (Kulaga et al., 2004). It is not possible to generalize these data on the whole genome, because they are not representative (Tazi et al., 2004). Existing paradox is caused simultaneously by a very good recognition of the *M. tuberculosis* genome and the lack of data related to the level of this bacterium’s genetic variability. The use of all high-tech methods of molecular biology for structure and function research of chosen sequences in tubercle bacillus (Kaduma et al., 2003) should be correlated with its biological proprieties, especially with the type of reproduction and pathogenicity (Korzekwa, 2004). Therefore, we should get to know the level of *M. tuberculosis* genetic diversity to qualify genetic similarity among its strains and to estimate the efficiency of different classes of DNA markers in such investigations.

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Experimental

Materials and Methods

Thirty two strains (including 7 drug resistant) were isolated from sputum of patients hospitalised in Warmia and Mazury Centre of Tuberculosis and Pulmonary Diseases in Olsztyn. All isolates were cultured on Löwenstein-Jensen medium at 37°C and identified as *Mycobacterium tuberculosis* by Tuberculosis and Lung Diseases Institute in Warsaw. Finally, from each patient three samples were collected and joined as one bulk. Colonies from L-J medium were harvested then boiled for 10 minutes at 100°C. The DNA was extracted by CTAB (cetyl-trimethyl-ammonium bromide) method described previously by Chen and Ronald (1999) with modifications (Polok, unpublished data). Finally short 10 nucleotides (nt) and long 18 nt scanning primers were included (Table I). For each of 8 primers, the reproducibility of the patterns was tested three times for each stock. All bands obtained on RAPD gels were numbered and its presence was estimated. IS6110-PCR reaction was performed according to Ross and Dwyer (1993) with modifications (Polok, unpublished data). Two primers were included for right and left amplification of IS6110 region, divergently. For each of the 2 primers used (Table I) the reproducibility of the patterns was tested and all present bands obtained on gels were scored as locus. PCR for *katG* was performed according to Heym *et al.* (1995) with modifications (Polok, unpublished data). Total 24 primers (Table I) were included for amplification of *katG* that was divided into 12 fragments. The whole complete analysis was performed for 7 susceptible and 7 resistant strains to estimate the number of alleles and their frequencies in *katG* locus. Mutation sites were found with first fragment of *katG* analysis based on all 32 strains with first and second primers only. For each primer that was used the reproducibility of the patterns was tested and all present bands obtained on gels were scored as “amplification” or “mutation” site.

Taking into consideration that *M. tuberculosis* is haploid the obtained molecular phenotypes corresponded to genotypes. In every locus allele “1” or “0” were observed. Allele frequencies were calculated by POPGENE 1.32 software (Yeh *et al.*, 2000). All commonly used population genetics parameters of genetic diversity were calculated as: expected heterozygosity

\[
H = 1 – \sum_{i=1}^{m} p_i^2
\]

where \(p\) is frequency of \(i\) allele in locus in population with a mean value, through loci in population (\(H_S\)) and through loci in species (\(H_T\)) according to Nei and Kumar (2000). Additionally expected heterozygosity through loci for each starter was calculated to estimate primers efficiency. Coefficient of genetics similarity (I) was calculated on the basis of shareable bands between strains (\(I = 2 \times X_{ij} / X_1 + X_2\); where: \(X_i\) and \(X_j\) are number of bands from two different strains, \(X_{ij}\) are shared bands). Population was grouped by UPGMA algorithm (Nei and Kumar, 2000). Dendrograms were prepared by POPGENE 1.32.

### Table I

Sequences of the primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAPD primers</td>
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</tr>
<tr>
<td>ISJ3</td>
<td>5’TGCAGGTCAG 3’</td>
</tr>
<tr>
<td>OPD-01</td>
<td>5’ACCACGGGAGG 3’</td>
</tr>
<tr>
<td>OPD-02</td>
<td>5’GGACCCAACC 3’</td>
</tr>
<tr>
<td>OPD-03</td>
<td>5’GTCCGCCGCA 3’</td>
</tr>
<tr>
<td>OPD-05</td>
<td>5’TGACGCGACA 3’</td>
</tr>
<tr>
<td>OPD-08</td>
<td>5’GTGTGCCCCA 3’</td>
</tr>
<tr>
<td>ISJ-2</td>
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</tr>
<tr>
<td>ISJ-4</td>
<td>5’GTCGCCGGACAGGTAATG 3’</td>
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<tr>
<td>IS6110 primers</td>
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</tr>
<tr>
<td>IS-L</td>
<td>5’ACCCCATCCTTTCAAGAAC 3’</td>
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<tr>
<td>IS-R</td>
<td>5’GGCTGAGGTCTCAGATCAG 3’</td>
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<td>katG primers</td>
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<td>katG4-R</td>
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<tr>
<td>katG5-L</td>
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</tr>
<tr>
<td>katG5-R</td>
<td>5’GGTTCACTAGATCGACCC 3’</td>
</tr>
<tr>
<td>katG6-L</td>
<td>5’GCAAGAGGCTGACGGAG 3’</td>
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<td>katG8-L</td>
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Results

RAPD primers that scanned genomes of all strains showed 32 different molecular phenotypes. Short and long primers identified an average 32 and 30 phenotypes, respectively. Considered separately, short primers obtained 19 up to 30 different phenotypes while long – 17 and 29. All RAPD loci considered were polymorphic (P = 100%). Short RAPD primers disclosed 111 loci and long primers 32 loci. Both types of primers showed all polymorphic loci. On a locus, an average number of allele (A) for both types of primers was 2.0. In most of 143 studied loci “0” allele appeared with higher frequency than “1”. Mean total genetic diversity (HT) of M. tuberculosis strains obtained by RAPD marker was 0.34. Values of HT for individual loci ranged from 0.06 to 0.50. The parameter of average genetic diversity on locus in susceptible population (Hs) based on RAPD marker was 0.28 for short primers and 0.34 for long ones. Within RAPD primers more efficient were short primers, like the most efficient OPD-02 and 03, which revealed the highest number of loci. Genetic diversity for a given primer (Hp) was the highest in the case of OPD-01 (0.37). The best discrimination revealed OPD-02 which alone distinguished 30 from 32 strains. Summary RAPD analysis of 32 strains revealed similarity between them on level 0.88. Values of this parameter ranged from 0.13 to 0.88. The strains were divided into three main clusters and pseudo-clusters for RAPD markers (Fig. 1). Short and long RAPD primers showed a wide range of I values between 0.13–0.87 and 0.07–0.98, respectively. Both types of primers showed the same type of clustering with differences in particular branches.

Conterminal regions of insertion sequence revealed 31 different phenotypes. IS6110 marker showed 18 polymorphic loci (P = 100%). In all loci “0” alleles appeared with frequency of 0.80 and “1” with 0.20. Genetic diversity value (Hs) for IS6110 primers was 0.30 and Hp ranged from 0.06 up to 0.50. Mean H value for susceptible population (Hs) was 0.27 and H value ranged from 0.0 to 0.49. Efficiency of IS6110 primers revealed by Hp value (0.29) was lower even in comparison to long RAPD primers. Genetic similarity among 32 strains in relation to the presence of IS6110 was very high (0.94). This parameter ranged between 0 and 1. Each strain received zero value at least once. IS6110 mobile element divided 32 strains into three clusters (Fig. 1). The second cluster consisted of two A and B subclusters. One strain was classified into pseudo-cluster.

Estimation of genetic diversity in katG locus in selected 14 strains of M. tuberculosis revealed 14 different molecular phenotypes and particular primers identified: 2 (katG8) up to 11 (katG12) strains, with average 6. The first two primers that amplified a putative promoter fragment of katG revealed differences in molecular phenotypes between susceptible and resistant strains. The catalase-peroxidase gene amplified by katG1-katG12 primers at 14 selected strains revealed polymorphism (100%) and the presence of 14 different alleles. Average frequency of given allele for this gene was 0.071. About 10 sites without amplification were found and named as mutation sites in the area of the first 560 bp region of katG. Mean frequency of such sites was about 0.517. Average genetic diversity in katG locus for species (Hs) based on katG1-katG12 primers in 14 strains was 0.93 with mean genetic diversity in locus on population H = 0.86. The most effective primers amplified subterminal and terminal region of katG (e.g. katG10 and 12). Comparative analysis of 14 unique alleles for 14 strains showed an average genetic similarity value on the level 0.95 with a fluctuation in range of 0.38–0.99. The catalase-peroxidase gene that was analysed by 12 primers divided 14 strains into 2 clusters (Fig. 1).

Discussion

Research of genetic diversity and H parameter value estimation of tubercle bacilus complex were conducted by enzymatic methods in the middle of ’90 (Feizabadi et al., 1997). Within 135 analysed strains only 8 were M. tuberculosis and the value of Hs for all complex was 0.10. Up to 2004 there was lack of any information about genetic diversity of M. tuberculosis species estimated on the basis of DNA markers (Korzekwa, 2004; Tazi et al., 2004). Although still the lack of many parameters such as (P, A, Hs) is present, especially for tubercle bacillus. Four times lower value of Hs for M. tuberculosis complex was reported by Feizabadi et al. (1997) in enzymatic analysis than by DNA markers (Korzekwa, 2004; Tazi et al., 2004).

Parameters of genetic diversity for tubercle bacillus that we revealed in this paper were high: P 100%; A 2.0, Hs 0.32, and G (genotypic diversity) 100%. The level of genetic variation increased three times from 0.10 for enzymatic markers up to 0.32 for DNA markers. Similar, but not exactly the same data, was obtained by Lewandowska (2001) during analysis of grasses. However, Tazi et al. (2004) noted mean genetic diversity of M. tuberculosis on the level 0.4 based on RAPD marker analysis. He compared it with a very high value of
Fig. 1. Thirty two *M. tuberculosis* strains grouped by UPGMA method based on genetic similarity (I) obtained by DNA markers. Bold – susceptible strains, italic – resistant strains, a) – RAPD markers, B) – IS6110 sequence, C) – *katG* gen

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of this parameter in *E. coli* (0.85) and other clonal species. In comparison to high $H_S$ value obtained by enzymatic data for *E. coli* (0.27–0.52) (Woodward et al., 1993) together with data obtained by Korzekwa (2004) and Tazi et al. (2004) concerned in *M. tuberculosis*, it was suggested that probably rule of three up to four times higher level of genetic diversity was revealed by DNA markers than enzymatic ones. Genetic diversity analysis of tubercle bacillus based on IS6110 marker confirmed its significant level ($P$ 100%, $A_2$ and $\bar{H}_s$ 0.30). This type of mobile element states about 3.4% of whole *M. tuberculosis* genome but it is dispersed throughout this genome simultaneously (Hatfull and Jacobs, 2000). Another problem is the number of IS6110 elements in a given strain (from zero up to several); (Hatfull and Jacobs, 2000). It means that in some cases information about IS elements in given genomes is essential. High polymorphism of analysed strains based on IS6110 marker revealed its efficiency for population genetics research. Hatfull and Jacobs (2000) joined transposition of the above mentioned genetic element with fluctuations of gene expression in bacteria. This hypothesis is not confirmed for *M. tuberculosis*. Within 18 IS6110 loci, one of them (IS6110-16) may preliminary pretend as resistant or susceptible strain type recognition and selection marker. In this locus amplification allele is the most frequent at resistant strains and is rare in susceptible ones. However, another research carried with more strains is needed to confirm this hypothesis. Moreover, high polymorphism of *katG* (Hatfull and Jacobs, 2000) was confirmed in this paper. Fourteen alleles and ten polymorphic sites in the first fragment of *katG* revealed in this paper were confirmed by the results of Saint-Joanis et al. (1999). Suerbaum et al. (2001) analysed 280 bp long fragments of selected genes in 33 *Campylobacter jejuni* strains and obtained 9 to 15 alleles (average 11). They proved relations between category of sequence and its polymorphism level. In our paper polymorphism of *katG* depends on an analysed fragment, as well.

Studies concerned genetic similarity were performed extensively for *Streptococcus* (Majewski et al., 2000), *Pasteurella* (Blackall et al., 1998), *Vibrio* (Farfán et al., 2000), *Bacteroides* (Gutacker et al., 2000). Based on enzymatic data Feizabadi et al. (1997) estimated Nei’s genetic similarity (I) between strains of *Mycobacterium avium* complex (0.50). Belonging to one cluster *M. avium* subsp. *paratuberculosis* and grouped in 3 different clusters *M. scrofulaceum* strains revealed genetic similarity on the level of nonsibilling species (I 0.3 and I 0.19, respectively). A wide range of genetic similarity (I, 0.0–1.0) of tubercle bacillus from Warmia and Mazury pointed at its allochtonic origin. Strains belonging to the same cluster were dispersed all over the voivodship.

RAPD markers that we used for *M. tuberculosis* studies generate moderate number of bands (average 6). Similar results were revealed by Gordon (1997) during genetic structure analysis of *E. coli*. The author obtained about 47 loci by only two RAPD primers what agrees with our 42 loci revealed by OPD02 and POD03. Every analysed locus during RAPD genome scanning analysis was polymorphic. The reproach of not enough reproducibility of RAPD technique between and even in the same laboratory can be eliminated by counting only strong and trusted bands. Moreover, RAPD-PCR needs high purity DNA with uniform concentration, trusted chemicals and equipment (Meunier and Grimont, 1993). The patterns that we obtained were stable and all reproducible in time and agreed with other experiences (Zervakis et al., 2001). Further observation revealed an interesting methodical fact. Long RAPD primers generated similar polymorphism as 10 nt primers (two times shorter). IS6110, OPD02 and OPD01 were the best for strains identification. Generated polymorphism revealed greater differences between strains.

There are three hypotheses about *M. tuberculosis* evolution and genetic diversity level: homogeneity conception, moderate diversity and genome heterogeneity (Hatfull and Jacobs, 2000). The results presented in the recent paper confirm the theory about *M. tuberculosis* heterogeneity. Parameter of $G = 100\%$ means that absolutely the same strains were not found and shows the lack of a clonal structure of population and suggest invasive structure.

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Literature


Potential Role of LPS in the Outcome of Helicobacter pylori Related Diseases

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This article is devoted to the memory of the late Prof. W.J.H. Kunicki-Goldfinger on the tenth anniversary of his passing away

A bstract

In this study we asked a question whether H. pylori LPS with or without LewisXY (Le) determinants as well as LBP (lipopolysaccharide binding protein) and sCD14 molecules recognizing bacterial LPS may be involved in atherogenesis. Sera from 57 patients with coronary heart disease (CHD), 27 H. pylori infected dyspeptic patients-H.p.(+)+ and 49 healthy controls (HC) were tested for IgM and IgG to H. pylori LPS expressing LeX (LPS LeX) or LeXY (LPS LeXY) determinants and to a glycine acid extract (GE). Immune complexes (ICs) of Lewis antigens and specific IgM or IgG were also determined. The prevalence of anti-GE IgG and IgA was significantly higher in CHD as compared to HC and the same as in the H.p.(+)+ group. The highest levels of anti-GE IgG were detected only for CHD group. CHD patients showed upregulation of IgG to LPS LeX and LeXY. In contrast, an upregulation of IgM to such LPSs was found for healthy subjects. The levels of LeY-anti-LeY IgG ICs were higher in CHD patients than in healthy controls similarly to the levels of LBP. There was no difference in sCD14 concentration between CHD and HC groups. The results obtained in this study indicate that H. pylori infections may be the risk factors of atherosclerosis due to: 1) an enhanced humoral response to H. pylori surface antigens, 2) a host predisposition to respond to Lewis determinants present in H. pylori LPS by IgG, 3) increased levels of serum LBP.

K e y w o r d s: Helicobacter pylori, LPS, atherosclerosis

Introduction

The correlation between H. pylori infections and gastro-duodenal diseases was proved in 1983 (Warren and Marshall, 1983). In recent years a link between such infection and coronary heart disease (CHD) has been suggested (Mendall et al., 1994). However, in some studies, there was no correlation shown between H. pylori and CHD (Koenig et al., 1999). In our previous study we showed that both H. pylori and Chlamydia pneumoniae infections associated with a strong humoral response against such microbes were correlated with CHD (Chmiela et al., 2003). Both in gastro-duodenal ulcer diseases and in CHD the inflammatory response induces the pathological processes (Libby, 2002). The soluble (urease, vacuolating cytotoxin-vacA)
and cellular (cytotoxic associated gene A antigen – CagA) H. pylori compounds initiate the inflammation in gastric epithelium (Crabtree, 1993). Although LPS is an important proinflammatory compound of Gram-negative bacteria (Alexander and Rietschel, 2001), the structure of lipid A of H. pylori probably evolved in the mode which promoted persistence of the infection. The H. pylori LPS may regulate the expression of adhesins and it can diminish the secretion of inflammatory cytokines by the host cells (Moutiala et al., 1992). Rudnicka et al. (2003) showed negative correlation between H. pylori LPS driven proliferation of mononuclear leukocytes isolated from dyspeptic patients, and type B inflammation. If so, it is possible that H. pylori LPS through the activation of immunocompetent cells may diminish the number of bacteria in gastric tissue and thus prolong the infection. The activity of H. pylori LPS is also determined by sugar residues in the O-specific chains, which are similar to Lewis (Le) determinants of the host. H. pylori strains may express either LeX or LeY, both or very little of either. The molecular mimicry between bacterial and host determinants may induce the production of autodestructive autoantibodies (Appelmelk et al., 1996). The interactions of LPS with the host cells are mediated by cellular and soluble receptors: 1) serum lipopolysaccharide binding protein – LBP; 2) membrane CD14 receptor; 3) sCD14 protein present in circulation which is a soluble form of CD14 receptor; 4) Toll-like receptors (TLR), mainly TLR4, used for signaling and induction of cytokine production in response to LPS (Brightbill and Modlin, 2000; Krutzki et al., 2001; Miller et al., 2005).

In this study, we asked whether H. pylori LPS with or without LeXY determinants as well as LBP and sCD14 molecules may be involved in atherogenesis.

Experimental

Material and Methods

Subjects. One hundred and thirty three individuals aged 30–70 (mean age 59) were included into this study. The first group consisted of 57 patients with coronary heart disease (CHD), hospitalized in the Cardiology Clinic due to a chest pain. Coronary angiography confirmed the atheromatic background of symptoms. In this group, the prevalence of previous myocardial infarction was 52%, arterial hypertension 72%, diabetes mellitus 18%, hyperlipidemia 62%, nicotinism 18%, rate of the revascularization (percutaneous transluminal coronary angiography or coronary artery by-pass graft) – 58%. All patients in this group had a negative history of dyspepsia at least during the last 24 months. The second group consisted of 27 patients with chronic dyspeptic symptoms due to H. pylori infection-H.p. (+), confirmed by endoscopy-based methods: detection of urease activity and the presence of Helicobacter-like organisms in biopsy specimens. All patients in this group had a negative history of cardiovascular symptoms. The healthy control group (HC) included 49 volunteers who had a negative history of cardiovascular and gastric diseases. In this group the prevalence of diabetes mellitus was 3%, arterial hypertension – 13%, hypercholesterolaemia – 12% and nicotinism – 33%. The study was approved by the local Ethical Committee. All participants signed informed consent.

Serological study. Blood from antecubital vein was obtained from all study participants for serological tests. The sera were stored at –70°C. The Enzyme Linked Immunosorbent Assays – ELISA, were used for estimation:

1) Anti-H. pylori LPS and IgA and IgG. The ELISA with a glycine acid extract (GE) from the reference H. pylori strain CCUG 17874 (Culture Collection University of Gothenburg, Sweden) and rabbit anti-human IgG or IgA antibodies labeled with horseradish peroxidase – HRP (Dako, Glostrup, Denmark) were used according to Rechciæski et al. (1997). The plates were coated with 5 µg/ml of GE (18 h, 4°C). The serum samples for anti-GE IgG were diluted from 1:500 to 1:128000 and for anti-GE IgA from 1:100 to 1:6400. The results were expressed as total optical density units (OD) measured at 450 nm. The ELISA cutoff, was defined as two standard deviations above the mean OD of control negative sera from the subjects not infected with H. pylori.

2) IgG and IgM to H. pylori LPS. The ELISA plates were coated with H. pylori LPS of Lewis X (LeX) or Lewis XY (LeXY) type (2 µg/ml in 0.15 M phosphate buffered saline-PBS, pH 7.2, 18 h, 20°C), donated by Dr A. Moran, National University of Ireland, Galway, Ireland. After blocking (1% bovine serum albumin in PBS with 0.05 Tween 80 – BSA/Tween, 2 h at 20°C) the plates were incubated with rabbit HRP anti-human IgG (1:6000) or IgM (1:100) antibodies. The results were expressed as O.D. for the sera diluted 1:100. In every ELISA the controls were included for the elimination of unspecific reactions. The cutoff was defined as double OD for the wells with HRP labeled secondary antibody control.

3) Immune complexes (ICs). ICs LeX-anti-LeX IgG, LeX-anti-LeX IgM, LeY-anti-LeY IgG and LeY-anti-LeY IgM were estimated by ELISA using mouse monoclonal anti-LeX or anti-LeY capture antibodies (Seikagaku, Tokyo, Japan; 100 ng/ml in 0.05 M carbonate buffer, pH 9.6, 18 h at + 4°C). After blocking, the plates were incubated for 1 h, at 37°C with serum samples (1:20) depleted in rheumathoidal factor (Catty and Raykundalia, 1989) and then for the same time with rabbit HRP anti-human IgG (1:6000) or IgM (1:1000) (Dako). The level of specific ICs was expressed as OD450 for the sera samples diluted 1:20. In every ELISA the control wells were included for the elimination of unspecific reactions. The cutoff was defined as double OD450 for HRP labeled secondary antibody control.

4) The serum LBP and sCD14. The commercial ELISA kits were used as recommended by the manufacturer (HyCult Biotechnology, Uden, The Netherlands).

Statistical analysis. Statistica 5.5 PL program with non-parametric tests was used: Mann-Whitney U test (for impaired data) to verify the hypothesis that two analyzed samples came from two statistically different populations; Chi-square test for comparison of the prevalence of analyzed parameters in studied groups and Spearman’s correlation coefficient.
Results

Distribution and the levels of anti-\textit{H. pylori} antibodies. The prevalence of anti-GE IgG was similar in CHD (53/57, 92%) and dyspeptic H.p. (+) patients (27/27, 100%), and significantly lower than in healthy controls (24/49, 49%), p<0.05. Similarly, anti-GE IgA were detected in 52 out of 57 CHD patients (90%), in 18 out of 27 dyspeptic patients (67%), and in 12 out of 49 healthy individuals (24%). The total OD450 for anti-GE IgG and IgA was significantly increased in the sera from CHD (5.865 ± 3.665 and 1.754 ± 1.169, respectively) as compared with dyspeptic patients (3.572 ± 1.916 and 0.962 ± 0.505, respectively) and healthy subjects (3.229 ± 1.916 and 0.962 ± 0.505, respectively), p<0.05.

The levels of antibodies to \textit{H. pylori} LPS of LeX or LeXY type. The IgG and IgM recognizing \textit{H. pylori} LPS with LeX or LeXY determinants were detected in the sera of all study participants. However, the level of IgG against LPS LeXY type in CHD group was significantly higher than in healthy individuals (Table I). In CHD patients there was an upregulation of the production of IgG to LPS with both LeX and LeXY determinants. In contrast, an upregulation of the production of IgG to such LPSs was found for healthy subjects, p<0.05.

Immune complexes (ICs). The ICs LeX-anti-LeX IgG and LeY-anti-LeY IgG were detected for all CHD patients and healthy controls (100%) (Table II). By comparison the ICs LeX-anti-LeX IgM were found in 73% CHD patients and in 90% healthy individuals. Similarly, ICs LeY-anti-LeY IgM were detected in 90% CHD and 93% healthy controls, respectively. The levels of LeY-anti-LeY IgG ICs were higher in CHD patients than in healthy subjects (p<0.05). Moreover, the high levels (OD450>0.6) of such ICs were detected only for the producers of IgG to GE of \textit{H. pylori}.

<table>
<thead>
<tr>
<th>Group</th>
<th>IgG anti-LPS LeXY</th>
<th>IgM anti-LPS LeXY</th>
</tr>
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<tbody>
<tr>
<td>CHD</td>
<td>0.783 ± 0.450</td>
<td>0.383 ± 0.204</td>
</tr>
<tr>
<td>HC</td>
<td>0.856 ± 0.442</td>
<td>1.157 ± 0.361</td>
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<tr>
<th>Group</th>
<th>IgG anti-LPS LeXY</th>
<th>IgM anti-LPS LeXY</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD</td>
<td>0.891 ± 0.356</td>
<td>0.650 ± 0.279</td>
</tr>
<tr>
<td>HC</td>
<td>0.719 ± 0.381</td>
<td>0.835 ± 0.279</td>
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<table>
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<tr>
<th>Group</th>
<th>LeX-anti-LeX IgG</th>
<th>LeX-anti-LeX IgM</th>
<th>LeY-anti-LeY IgG</th>
<th>LeY-anti-LeY IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHD</td>
<td>0.372 ± 0.191</td>
<td>0.576 ± 0.192</td>
<td>0.501 ± 0.105</td>
<td>0.467 ± 0.165</td>
</tr>
<tr>
<td>HC</td>
<td>0.328 ± 0.143</td>
<td>0.568 ± 0.179</td>
<td>0.380 ± 0.070</td>
<td>0.469 ± 0.126</td>
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<table>
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<th>Group</th>
<th>The prevalence of high level of ICs (OD450&gt;0.6)</th>
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<tr>
<td>CHD</td>
<td>17%</td>
</tr>
<tr>
<td>HC</td>
<td>10%</td>
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<table>
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<tr>
<td></td>
<td>0–10 µg/ml</td>
</tr>
<tr>
<td>CHD</td>
<td>25/57 (44%)</td>
</tr>
<tr>
<td>HC</td>
<td>38/49 (78%)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>The sCD14 concentration/prevalence</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0–4 µg/ml</td>
</tr>
<tr>
<td>CHD</td>
<td>23/57 (40%)</td>
</tr>
<tr>
<td>HC</td>
<td>26/49 (53%)</td>
</tr>
</tbody>
</table>

Table I

The level of IgG and IgM antibodies against \textit{H. pylori} LPS of LeX or LeXY type in the patients with coronary heart disease – CHD, and healthy controls – HC

Table II

The level of immune complexes (ICs) LeX-anti-LeX IgG, LeX-anti-LeX IgM, LeY-anti-LeY IgG and LeY-anti-LeY IgM in the patients with coronary heart disease – CHD and healthy controls – HC

Table III

The level of LBP and sCD14 in the patients with coronary heart disease – CHD and healthy controls – HC
The serum LBP and sCD14 concentrations. The level of LBP was significantly higher (p<0.05) in CHD group (17.321 ± 16.705 µg/ml) as compared with healthy controls (8.517 ± 4.003 µg/ml) (Table III). Similarly, the high level of LBP (>10 µg/ml) was detected more frequently in the sera from CHD patients (56%) than in HC group (22%). Although LBP level was significantly lower in 53 producers of anti-GE IgG (14.911 ± 13.213 µg/ml) than in 4 seronegative CHD patients (49.207 ± 27.801 µg/ml), there was no difference in LBP concentration in the seropositive or seronegative healthy controls. In CHD patients and healthy individuals the level of sCD14 was similar, 4.125 ± 2.050 µg/ml and 3.808 ± 2.115 µg/ml, respectively (Table III). The sCD14 concentration over 4 mg/ml was detected more frequently in CHD patients as compared with healthy individuals, 60% and 40% respectively. However, the difference was not significant.

Discussion

It has been suggested that H. pylori infection can be involved in atherogenesis (Mendall, 1994). In this study the prevalence of anti-GE IgG and IgA was significantly higher in CHD patients than in healthy subjects and the same as in H.p. (+) dyspeptic patients. Also the levels of anti-GE IgG/IgA were higher in CHD than in healthy group. It is possible that gastrotoxic activity of aspirin used by the CHD patients for a long time may facilitate the penetration of bacterial antigens through the mucosal barrier and a strong humoral response to them (Sims et al., 2000).

The evolution has led to co-expression of common LeX and LeY carbohydrates which are present in the LPSs of most H. pylori strains and in human cell surface glycoconjugates of blood cells and gastric mucosa. Based on such molecular mimicry Appelmelk et al. (1996) suggested the role of autoimmune mechanisms in H. pylori associated type B gastritis. Recently H. pylori CagA has been considered as putative autoimmune antigen (Takahashi et al., 2004).

An upregulation of IgG and IgA to H. pylori antigens in CHD patients prompted us to ask whether LeX and LeY determinants of H. pylori LPS could be involved in atherogenesis. There was no difference between CHD and healthy individuals with regard to the prevalence of IgG and IgM to H. pylori LPS of LeX or LeXY type. However, the level of IgG to H. pylori LPS LeXY was significantly increased in CHD patients as compared with healthy subjects. Moreover, the CHD patients showed an upregulation of the production of IgG to LPS of LeX or LeXY type. In contrast, an upregulation of IgM production to LPS with LeX or LeXY was observed in healthy subjects. Cedzyński et al. (1998) showed that during natural history of H. pylori infections in humans, mainly antibodies of IgG class to polysaccharide chains of LPS of these bacteria are produced. The presence of antibodies to H. pylori LPS LeX/Y in healthy subjects seronegative to anti-GE IgG could be due to the reactivity to Lewis epitopes expressed on some unrelated microbes (Hirota et al., 1995) or it could result from an immunological response to self LeX or LeY antigens. Amano et al. (1995) observed the reaction of human sera with the synthetic Le antigens regardless of the status of the individual’s H. pylori infection. We cannot exclude that inflammatory and/or physiological abnormalities leading to chronic dyspepsia or atherosclerosis should be considered as signals for the production of anti-LeX/Y antibodies. The presence of such antibodies in the sera from healthy individuals with negative H. pylori serology also implies that the production of anti-LeX/Y IgG and particularly IgM might be a physiological state. Such Ig’s, similarly to anti-Le a or anti-Le b antibodies occur naturally (Henry et al., 1995). Under the influence of genetic or environmental factors, including H. pylori infection, this normal response to self-antigens may develop into autoimmune disease and contribute to tissue damage.

In a self-destructive autoimmune response an important role is played by antigen-antibody ICs. In this study the levels of LeY-anti-LeY IgG ICs were higher in CHD patients than in healthy controls. The highest levels of ICs were detected only for the producers of IgG to H. pylori GE. Sims et al. (2000) reported that in the vessels of the patients with atherosclerosis the ICs were deposited. Our results suggest that during chronic H. pylori infections a host predisposition to respond to Le determinants by IgG but not IgM could be one of the risk factors for atherogenesis. The ICs formed of Le antigens and specific IgG may persist in a circulation as the small ones longer than the big ones formed of antigen and IgM. The enhanced blood pressure and turbulences of circulation in small arteries may promote in CHD patients a deposition of ICs. This may stimulate secretion of proinflammatory cytokines by the macrophages and granulocytes via FcyR receptors. The activation may be followed by the release of lysosomal enzymes, cationic proteins, reactive oxygen and nitrogen intermediates which promote tissue injury (Clynes et al., 1999). Anti-LeX/Y antibodies may enhance in H. pylori infected subjects the inflammatory effect of bacterial CagA, VacA and urease. The long term inflammation generated by H. pylori may raise cytokine levels in the bloodstream, and activate...
fibroblast and smooth muscle cell proliferation which is an important step in atherogenic process. In *H. pylori* infected subjects a stimulation of T lymphocytes by bacterial LPS is very likely. Activated lymphocytes may facilitate a control of bacterial growth and diminish inflammation in gastric mucosa by releasing cytokines, possibly of Th2 type. Weak inflammatory response may help the bacteria to survive in the host tissue (Rudnicka and Chmiela 2004).

Bacterial LPS bound with LBP is delivered to membrane or soluble CD14 molecules (mCD14 and sCD14, respectively) and, therefore, initiates TLR4 signaling (Duzendorfen et al., 2004). Such TLRs are expressed on macrophages, granulocytes and dendritic cells but also on endothelial cells and macrophages in atherosclerotic plaques (Van Haelst et al., 2004). In this study significantly higher levels of LBP were observed in CHD patients as compared with healthy subjects. The involvement of *H. pylori* infection in CHD may result in the enhancement of LBP production and possible deposition of lipid-LBP and/or LPS-LBP complexes in endothelium. The linkage of *H. pylori* infections with increased levels of total cholesterol and triglycerides has been found (Niemela et al., 1996; Schanagl et al., 2003). Eilersten et al. (2003) showed a correlation between CD14 –159 C/T polymorphism and the ability of CD14 to bind cholesterol. The TT homozygotes were associated with lower total cholesterol, LDL and apolipoprotein B-100 concentrations. Our preliminary results show that CT genotype was more frequently detected in the patients with myocardial infarction as compared with unstable angina pectoris group. Edfeldt et al. (2004) identified susceptibility to myocardial infarction in men carrying both TLR4 299Gly and 399Ile allele. An increased risk of atherosclerosis is also associated with RANTES G-40A gene polymorphism (Simeoni et al., 2004). Grąbowska et al. (2004) reported that Le determinants of *H. pylori* LPS modulate the CD14 mediated cytokine response of macrophages. The infection can also predispose to atherosclerosis by an endothelial dysfunction (Prosad et al., 2002). In conclusion, the results obtained in this study: 1) the enhanced humoral response to *H. pylori* antigens in the patients with coronary heart disease, 2) the host predisposition to respond to LeXY determinants of *H. pylori* LPS by IgG but not IgM, and 3) the increased levels of serum LBP, indicate that *H. pylori* infections may be recognized as the risk factors of atherosclerosis.

### Literature


Genotypes of *Listeria monocytogenes* Strains Isolated from 2000 to 2002 in Poland

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Abstract

Pulsed field gel electrophoresis (PFGE), multiplex PCR and multilocus sequence typing (MLST) methods were used for genotyping study of seventy-three *L. monocytogenes* isolates collected in Poland between 2000 and 2002 from human, food, environment and a diseased goat. The multiplex PCR, which is an alternative method to classical serotyping, divided the isolates into four PCR groups, IIa (42.5%), IIb (27.4%), IIc (4.1%) and IVb (26%). The isolates displayed 33 distinct PFGE profiles. Twenty eight strains were further characterised by MLST based on sequence analyses of seven housekeeping genes. The combined sequence analyses revealed a total of 10 different allelic profiles from which 3 were not described earlier. It is intended that results obtained in this study will be the first data of a national database of *L. monocytogenes* genotypes in Poland.

Keywords: *Listeria monocytogenes*, genotypes, PFGE, MLST, PCR

Introduction

*Listeria monocytogenes* is the causative agent of listeriosis, which occurs as sporadic and epidemic cases, both essentially related to the consumption of contaminated foods (Mead *et al.*, 1999). Neonates, pregnant women, the elderly, immunosuppressed transplant recipients and others with impaired cell-mediated immunity belong to a group with the greatest risk of development of listeriosis. Most frequent forms of listeriosis include meningoencephalitis, bacteraemia and perinatal infections (Doganay, 2003). Although morbidity is relatively low (2–10 reported cases per million of population per year) listeriosis is characterised by a high mortality rate – 20–30%. Therefore listeriosis remains a great public health concern (de Valk *et al.*, 2003).

Various methods have been used to gain insight into clonal relationships between *L. monocytogenes* isolates for epidemiological investigation and surveillance purposes. These include serotyping (Seeliger and Hohne, 1979), bacteriophage typing (Loesser and Busse, 1990), multilocus enzyme electrophoresis – MLEE (Bibb *et al.*, 1990), ribotyping (Wiedmann *et al.*, 1996), random amplified polymorphic DNA differences – RAPD (Mazurier and Wernars, 1992) and DNA macrorestriction combined with pulsed field gel electrophoresis – PFGE (Brosch *et al.*, 1994). Recently multilocus sequence typing method – MLST (Salcedo *et al.*, 2003) and multiplex PCR (mPCR) – based serotyping (Doumith *et al.*, 2004a; Doumith *et al.*, 2005) have been developed.

Currently, PFGE constitutes the standard subtyping method for *Listeria* typing due to its reproductibility and high discriminatory power (de Valk *et al.*, 2003). Electronic databases of PFGE profiles are being created to detect new emerging clones and to trace infection clusters in the future (e.g. Swaminathan *et al.*, 2001; Lukinmaa *et al.*, 2003; Wagner and Allerberger, 2003).

Lately, Módrala *et al.* (2003) have reported results of typing by RAPD and PFGE using Smal endonuclease of strains originated from a Polish fish-processing plant. The aim of the present study was to gain
insight into diversity and clonal relationships between *L. monocytogenes* strains isolated in Poland from different sources using PFGE, multiplex PCR and MLST typing methods. It is intended that results obtained in this study will be the first data of a national database of *L. monocytogenes* genotypes.

### Experimental

#### Materials and Methods

**Isolates tested.** A total of 73 isolates from clinical material samples (*n* = 14), food (*n* = 50), environment (*n* = 8) and a diseased goat (*n* = 1) were examined in the study. The strains isolated over the period from 2000 to 2002, without an evident epidemiological link among them, were received from 4 laboratories in Warsaw and from laboratories in Szczecin, Puławy, Gdańsk and Bydgoszcz. Strains were confirmed as *L. monocytogenes* by Api Listeria system (BioMérieux) and by determination of the haemolytic activity on a TSA agar plate supplemented with horse blood.

**PFGE analysis.** Isolation of whole bacterial chromosomes, macrorestriction of DNA with ApaI and Ascl endonucleases and PFGE (CHEF-DR III system, Bio-Rad) were performed according to standardized protocol (Graves and Swaminathan, 2001). Gel images of PFGE profiles were analysed using BioNumerics software version 3.0 (Applied Maths). Pattern comparisons were made on band positions using Dice band-based similarity coefficient (maximum optimization 0.5 %; maximum position tolerance 1%). Two patterns were considered as different when they differed by only one band. Strain profiles were clustered by Unweighted Pair Group Method using Averages (UPGMA).

**mPCR typing.** mPCR typing method was that described by Doumith *et al.* (2004a). The developed method allows the separating of isolates of the species *L. monocytogenes* into four distinct PCR profiles in correlation with serovars. PCR profiles designated Ila, Ilc, Ib and IVb correspond to *L. monocytogenes* isolates of serovars (1/2a-3a), (1/2c-3c), (1/2b-3b-7) and (4b-4d-4e) respectively. All remaining *Listeria* isolates are separated in the fifth PCR group designated L.

**MLST analysis.** The fragment sequences from the 7 housekeeping genes (*abcZ*, *dat*, *ldh*, *cat*, *dapE*, *bglA* and *ibkA*) were analysed as described by Salcedo *et al.* (2003). DNAStar software was used to compare obtained sequences. Sequences different even at a single nucleotide site were considered distinct alleles. Cluster analysis based on the joined sequences from the 7 genes were conducted using MEGA version 2.1 software (Kumar *et al.*, 2001 – http://www.megasoftware.net/).

#### Results and Discussion

Of the 73 isolates tested by mPCR 31 represented PCR group Ila (42.5%), 20 – PCR group Iib (27.4%), 19 – PCR group IVb (26.0%) and 3 – PCR group Ilc (4.1%). International data show that more than 90% of *L. monocytogenes* isolates from food and more than 98% of isolates from patients with listeriosis belong to only 4 of the 13 serotypes described – 1/2a, 1/2b, 4b and 1/2c (Doumith *et al.*, 2004a; Kathariou, 2002). Three isolates representing mPCR group Ila and 2 isolates of group IVb were classically serotyped and confirmed as serotypes 1/2a and 4b respectively (data not shown).

Thirty-three PFGE-profiles were obtained after DNA restriction with ApaI and 31 after restriction with Ascl endonuclease (Table I). Four most prevalent pulsotypes (PTs) were represented by 11 (15.1%), 9 (12.3%), 5 (6.8%) and 4 (5.5%) isolates (total – 39.7%). However, the strains presenting these pulsotypes were received from one laboratory at the same time and thus may represent serial isolates of the same clone. According to data received from the laboratory, which provided strains presenting PT IX, the strains were originated from different sources including human. Theoretically it might be possible that epidemiological link between these strains really exists – environmental and food strains were isolated from samples of Odra River water and fish suggesting that listeriosis might have been developed after consumption of contaminated fish. Otherwise, accidental identities of profiles or samples contamination seem to be more probable.

The remaining pulsotypes were represented by 1 (23.3% of strains), 2 (24.7%) or 3 (12.3%) isolates suggesting quite great genetic diversity of the strains. PT XV was identical to PT of *L. monocytogenes* strain used as a control in Listeria Laboratory of Institut Pasteur, Paris (CDC reference strain H2446). Such identity of PTs of strains not linked by a source and time of isolation confirms observations on international prevalence of certain *L. monocytogenes* clones (Jacquet *et al.*, 1995). PTs V, XII, XXV and XXVIII are remaining examples of identity of strains isolated by different laboratories from different sources. The same PT was presented by isolates from milk (Pulawy) and vegetables (Bydgoszcz) (2 times: PTs V and XXV). Two isolates from vegetables (Bydgoszcz – vegetables imported from Hungary) and 1 from fish (Szczecin) showed PT XII. Finally, both – an isolate from vegetables (Bydgoszcz – vegetables imported from Hungary) and a human one presented PT XXVIII.

Some human isolates, although different, may be related. The similarity of ApaI- and Ascl-profiles of 2 IVb strains isolated in 2 different laboratories in Warsaw was 95%, similarity between 3 other IVb strains
isolated in 3 Warsaw laboratories was 89% (ApaI) and 86% (Ascl). Profiles of 2 IIa isolates (Szczecin and Warsaw) were similar in 89% (ApaI) and 80% (Ascl), and profiles of 2 IIb isolates (2 Warsaw laboratories) – in 87% (ApaI) and 95% (Ascl).

Number of identified alleles of sequenced genes was as follows: \(abcZ\) – 7, \(bglA\) – 7, \(cat\) – 10, \(dapE\) – 7, \(dat\) – 4, \(ldh\) – 4 and \(lhkA\) – 6 (Table II). All alleles except one of \(cat\) gene (17), were described previously by Salcedo et al. (2003). New allele corresponds to the previously described allele 1 (GenBank accession number AY158249) with a single substitution (C into an A in position 165). The isolates represented a total of 10 allelic profiles (ST), 3 of these profiles (30, 31, 32) were not described earlier (Salcedo et al., 2003).

The same isolates were divided by PFGE into 21 PTs. Similarity of PTs of isolates representing identical ST accounted for at least 80% with one exception – in the case of ST 11 the similarity between the PT of an isolate typed by mPCR as IIb and PTs of 2 other strains typed as IVb was less than 70%. MLST also failed to provide discriminative results for some STs (Salcedo et al., 2003).

### Table I

<table>
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<th>Pulsotype</th>
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### Table II

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<td>17 3 2 2 5</td>
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</tr>
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<td>31</td>
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<td>15 8 6 1 8</td>
<td>2</td>
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</tr>
<tr>
<td>32</td>
<td>11</td>
<td>1 12 19 3 6 7</td>
<td>2</td>
<td>IIb</td>
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</table>

* designations according to Salcedo et al. (2003) with exception of STs 30, 31, 32 and cat allele 17, which were not described in this article.
to distinguish isolates of different serotypes in the case of ST 22 characterising Spanish strain 1/2a (Salcedo et al., 2003) and strain showing PCR-profile IIC (this study). On the other hand, one of the strains presenting identical PT (XVI) was distinguished by a specific ST (30) characterised by the presence of the new cat allele.

Dendrograms showing cluster analysis of PFGE-profiles as well as STs revealed that isolates tested could be divided into 2 major groups (data not shown). One of them included all isolates representing PCR profiles IIA and IIC and the second contained all isolates representing profiles IIB and IVC. Division of the isolates into two groups consisting of IIA, IIC and IIB, IVC isolates is in full agreement with the data obtained by many authors. A number of different typing studies suggested that 3 main lineages correlated with serovars exist within the L. monocytogenes species: lineage I comprised 1/2a, 1/2c and 3c serotypes, lineage II comprised 4b, 1/2b and 3b serotypes and lineage III comprised 4a and 4c serotypes (Wiedmann et al., 1997; Doumith et al., 2004b). Serotypes 4a and 4c were not represented among the strains tested.

Due to Polish law each case of listeriosis should be reported. According to data of Chief Sanitary Inspectorate and Department of Epidemiology of National Institute of Hygiene, Warsaw, Poland, the number of reported cases of listeriosis during the period of 1991–2000 accounted for 52. In 2001, 2002 and 2003 there were 9, 31 and 5 cases reported, respectively (Czarkowski et al., 2003; Czarkowski et al., 2004). These numbers might be largely underestimated. For comparison, in Finland, which has almost 8 times less inhabitants than Poland, during 11 years period (1990–2001) there were 314 cases of invasive listeriosis (20–50 cases per year) (Lukinmaa et al., 2003). Similarly, Belgian Listeria Reference Centre receives between 30–50 human clinical strains of L. monocytogenes per year (Yde and Genicot, 2004).

L. monocytogenes is not a fastidious bacterium and should be isolated from clinical material samples quite easily. To gain reliable knowledge on prevalence of listeriosis in Poland, all cases of the disease must be reported. Furthermore, to trace outbreaks and follow long-term trends in the epidemiology of listeriosis, continuous typing of L. monocytogenes is necessary, preferably using internationally recognised typing methods according to standardised protocols. DNA macrorestriction and PFGE is one of such methods. mPCR and MLST offer a readily comparable data and might be very useful in long-term surveillance. mPCR is also useful as a first-line screening method to detect and trace an outbreak strain within numerous food isolates. It was mentioned earlier that in some countries national databases of listerial genotypes are being developed. Thus, Polish regional clinical and food laboratories are encouraged to collect isolated strains of L. monocytogenes for typing purposes. We hope that the data obtained in the present study will be the beginning of national database of L. monocytogenes genotypes.

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Literature


Genotypes of Polish L. monocytogenes strains


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Purification and Characterization of Cytolytic Toxins Produced by *Aeromonas hydrophila* and *Aeromonas veronii* Biotype Sobria Strains

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Abstract

Cytolytic toxins produced by *Aeromonas hydrophila* and *Aeromonas veronii* biotype sobria strains were partially purified from culture filtrates by two steps of purification: ammonium sulfate precipitation and hydrophobic chromatography using Phenyl-Sepharose CL-4B. Hemolytic activity was detected in one or two peaks in elution profile. Purified toxins were also cytotoxic to Vero and CHO cells. Moreover, these toxins revealed cytotonic activity to CHO cells.

Key words: *Aeromonas* spp., cytolytic toxins, cytotonic activity

Introduction

The genus *Aeromonas* represents a group of gram-negative, facultatively anaerobic organisms, widely spread in water habitats (Holmes *et al*., 1996). The genus is taxonomically complex and includes at least 20 species that can be differentiated on the basis of DNA-DNA hybridization (Altwegg, 1999; Pidiyar *et al*., 2002; Harf-Monteil *et al*., 2004). Species belonging to hybridization group 1 (HG 1, *A. hydrophila*), HG 4 (*A. caviae*) and HG 8/10 (*A. veronii* biotype sobria) are the most prevalently isolated from the stool of humans with gastroenteritis, which mainly affects young children, the elderly and immunocompromised patients. Human diarrheal diseases range from a mild self-limiting, acute diarrhea to a cholera-like dysenteric illness or a more persistent diarrhea (Altwegg, 1999).

Various virulence factors appear to be involved in *Aeromonas* sp.-associated gastroenteritis: enterotoxins, cytotoxins, hemolysins and extracellular enzymes. However, it is still unclear which toxin is responsible for the diarrhea symptoms (Laohachai *et al*., 2003).

Strains of *A. hydrophila* and *A. veronii* biotype sobria produce β-hemolysin which is called aerolysin, cytotoxic enterotoxin or cytolytic enterotoxin cross-reactive with cholera toxin (Thornley *et al*., 1997). Recent studies indicated the production of four hemolytic enterotoxins by different *Aeromonas* spp. isolates (Chopra and Houston, 1999; Albert *et al*., 2000, Trower *et al*., 2000). *Aeromonas* spp. can produce at least one non-enterotoxigenic β-hemolysin and possibly several β-hemolytic enterotoxins. Albert *et al.* (2000) identified three distinct genes encoding enterotoxins in *Aeromonas* spp. One of them encodes cytotoxic enterotoxin (Act) and two encode cytotoxic enterotoxin. One cytotoxenterotoxin is heat stable at 56°C (Ast) and the other one is heat labile at 56°C (Alt). Site-directed mutagenesis on Act indicated possibly different loci on a single chain which may be associated with various biological activities. Chopra and Houston (1999) reported that Act is an aerolysin with hemolytic, cytotoxic and enterotoxic activity.

Much work on the mechanistic level of aerolysin action has concentrated on erythrocytes and there is still little known about its interaction with epithelial cells (Thornley *et al*., 1997). Our study was undertaken to examine hemolytic, cytotoxic and cytotonic activity of partially purified cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria strains isolated from stool of humans with gastroenteritis.
Experimental

Material and Methods

Bacterial strains and culture methods. Toxins were isolated from strains of *A. hydrophila* (HG 1) RK 70363, RK 217215, RK 226254 and *A. veronii* biotype sobria (HG 8/10) RK 43939 and RK 66113. The strains were isolated from fecal specimens of patients with diarrhea symptoms and were obtained from dr R. Kong (Hong Kong City University). The identification of examined isolates was performed on the basis of their phenotypic properties, confirmed by DNA-DNA hybridization (Szczuka and Kaznowski, 2004) and by 16S rDNA RLFP analysis as described previously (Figueras et al., 2000). The isolates were maintained at −75°C in brain heart infusion broth (BHI, Difco) containing 50% (v/v) glycerol. *Aeromonas* spp. strains were grown for 24 hours on tryptic soy broth (TSB, Difco) supplemented with 0.6% yeast extract (TSB-YE) at 37°C. After overnight incubation, 250 ml of TSB-YE was inoculated with 0.5 ml of culture supernatant and incubated at 37°C for 24 hours in water bath with shaking at 120 rpm. The culture was centrifuged then at 10,000 g for 50 minutes and the pellet was discarded. Culture supernatant was sterilized through 0.22 µm-pore-size low protein binding Millex GV filters (Millipore). Two protease inhibitors, soybean tripsin inhibitor (1 mg liter⁻¹) and phenylmethylsulfonyl fluoride (1 mM) were added to the culture filtrates in order to prevent proteolytic degradation (Rose et al., 1989).

Partial toxin purification. Ammonium sulfate (NH₄)₂SO₄ (Difco) was added to the culture supernatants to give 60% saturation. The pH was adjusted to 6.0 and the supernatants were stored overnight at 4°C. The precipitate was centrifuged at 10,000 g for 50 minutes at 4°C and the supernatants were adjusted to pH 7.0. For hydrophobic interaction chromatography (HIC) the redissolved pellet was dialyzed against 1 M ammonium sulfate, applied to a column of Phenyl-Sepharose CL-4B (1 × 180 cm) and washed with 1 M ammonium sulfate (pH 7.0) until absorbance at 280 nm reached zero. Bound protein was eluted with a decreasing linear gradient from 1 M to 0 M ammonium sulfate. Fractions of 3 ml volume were collected (GradiFrac, Pharmacia). The protein concentration in culture supernatant filtrates, ammonium sulfate precipitates and fractions of partially purified toxins was determined by the Bradford protein assay (Bradford, 1976).

Hemolysin activity. Hemolysin assay was performed by using a suspension of 1% human erythrocytes according to Nowotny (1979). Serial dilutions (from 1:2 to 1:512) of each sample in phosphate-buffered saline (PBS) were incubated at 37°C for 1 hour with equal volume of erythrocyte suspension. After incubation at 4°C for 1 hour, the samples were centrifuged at 800 g for 10 minutes to remove unlysed cells. Absorbance was measured on a microplate reader SUMAL PE 2 at 545 nm. Hemolytic activity was expressed in hemolytic units per milligram of protein (HU mg). HU was defined as the reciprocal of the highest dilution of toxin in which 50% hemolysis was observed.

Cytotoxic and cytotonic activity. Cytotoxic activity was measured on African monkey kidney cells (Vero) and Chinese hamster ovary cells (CHO), whereas cytotonic activity was examined on CHO cells as described previously (Krzymińska et al., 2003). Cells were cultivated on Minimum Essential Medium Eagle (MEM, Sigma) supplemented with 5% fetal bovine serum (FBS, Sigma), 2 mM glutamine, 80 IU ml⁻¹ of penicillin, 80 µg ml⁻¹ of streptomycin and 1 mg ml⁻¹ of nystatin. Cells were seeded in microtitre plates at a density of 1 × 10⁴ cells ml⁻¹. Serial twofold dilutions of culture supernatant was added to the monolayer and the plate was incubated for 24 hours at 37°C in atmosphere containing 5% CO₂ and assessed under an inverted microscope. Cytotoxic activity was identified as rounding and detachment of 50% cells whereas cytotonic activity was recognized as elongation of CHO cells. Cytotoxic and cytotonic activities were expressed in total units per milligram of protein. Cytotoxic and cytotonic units were expressed as the reciprocal of the highest dilution yielding a positive result. Heat stability of culture supernatant fluids and HIC fractions with purified toxins was detected after heating at 56°C for 20 minutes.

Results

Partial purification of cytolytic enterotoxin. Cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria were purified from culture supernatant in two steps which included ammonium sulfate precipitation and hydrophobic column chromatography. The elution patterns from this column (Fig. 1 and 2)
Cytolytic toxins of *Aeromonas* spp. showed that the toxin was eluted as a single peak (*A. hydrophila* RK 70363 and 226254) or as a double peak (*A. hydrophila* RK 217215, and *A. veronii* biotype sobria RK 43939, 66113). Table 1 summarizes the data on the purification of cytolytic toxins. Specific hemolytic activity of these toxins gradually increased with the decline in the amount of protein, suggesting true purification. Ammonium sulfate precipitation followed by dialysis resulted in an increase in specific activity (1.2-fold to 27.5-fold). Next the proteins were applied to Phenyl-Sepharose CL-4B. After chromatography, the specific activity increased about 32-fold for *A. hydrophila* RK 226254. Low grade of the purification may suggest that culture supernatant and pooled HIC fractions should be concentrated.

**Cytotoxic and cytotoxic activity.** Cytotoxic activities to Vero and CHO cells of culture supernatant, ammonium sulfate precipitates and fractions of partially purified cytolytic toxins are shown in Table II.

![Absorbance at 280 nm vs Volume [L]](image)

**Table I**

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Fraction</th>
<th>Total vol. (ml)</th>
<th>Total protein (mg)</th>
<th>Total hemolytic activity (HU)</th>
<th>Specific activity (HU mg⁻¹)</th>
<th>Purification fold</th>
<th>Recovery (%)</th>
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<tr>
<td><em>A. veronii</em> biotype sobria RK 43939</td>
<td>supernatant</td>
<td>200</td>
<td>9.5</td>
<td>6.5×10⁴</td>
<td>6.9×10⁵</td>
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<td>4.2</td>
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<td>2.2×10⁵</td>
<td>1.0</td>
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<td></td>
<td>ammonium sulfate</td>
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a – reciprocal of the highest dilution of toxin showing 50% hemolysis of 1% erythrocytes (HU × volume)
b – total HU per mg of protein
c – first peak on Phenyl-Sepharose CL-4B column
d – second peak on Phenyl-Sepharose CL-4B column
Table II
Cytotoxic activity of partially purified toxins and their fractions to Vero and CHO cells

<table>
<thead>
<tr>
<th>Strain No</th>
<th>Purification step</th>
<th>Specific cytotoxic activity (total units mg⁻¹)</th>
</tr>
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<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>unheated sample</td>
</tr>
<tr>
<td></td>
<td>supernatant</td>
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</tr>
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<td>A. hydrophila</td>
<td>RK 70363</td>
<td>1.4×10⁵</td>
</tr>
<tr>
<td></td>
<td>ammonium sulfate</td>
<td>1.6×10⁵</td>
</tr>
<tr>
<td></td>
<td>HIC-I</td>
<td>3.0×10²</td>
</tr>
<tr>
<td></td>
<td>RK 217215</td>
<td>1.7×10⁴</td>
</tr>
<tr>
<td></td>
<td>ammonium sulfate</td>
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</tr>
<tr>
<td></td>
<td>HIC-I</td>
<td>1.5×10⁴</td>
</tr>
<tr>
<td></td>
<td>HIC-II</td>
<td>8.0×10²</td>
</tr>
<tr>
<td></td>
<td>RK 226254</td>
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</tr>
<tr>
<td></td>
<td>ammonium sulfate</td>
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<tr>
<td></td>
<td>HIC-I</td>
<td>1.2×10⁴</td>
</tr>
<tr>
<td>A. veronii biotype sobria</td>
<td>RK 43939</td>
<td>6.7×10⁵</td>
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<tr>
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<td>HIC-II</td>
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<td>ammonium sulfate</td>
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</tr>
<tr>
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<td>HIC-II</td>
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</table>

Table III
Cytotoxic activity of partially purified toxins and their fractions to CHO cells

<table>
<thead>
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<th>Strain No</th>
<th>Purification step</th>
<th>Specific cytotoxic activity (total units mg⁻¹)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>Vero preheated sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unheated sample</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>RK 70363</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ammonium sulfate</td>
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<td>HIC-I</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>RK 217215</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>ammonium sulfate</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>HIC-I</td>
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<td>HIC-II</td>
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<td>RK 226254</td>
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<td>ammonium sulfate</td>
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<tr>
<td></td>
<td>HIC-I</td>
<td>0</td>
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<td>A. veronii biotype sobria</td>
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<tr>
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<td>HIC-II</td>
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</tbody>
</table>

a – total cytotoxic units (reciprocal of the highest dilution showing rounding and detachment of 50% Vero or CHO cells) per mg of protein
b – sample preheated at 56°C for 20 minutes
c – not determined
Cytotoxic activity was present only in the first peak eluted from *A. hydrophila* (RK 70363, RK 226254) or in both peaks (RK 217215), whereas toxins eluted from *A. veronii* biotype sobria revealed cytotoxic activity in the first and the second peaks. Specific cytotoxic activity of culture supernatants of examined strains to Vero cells, ranging from $1.4 \times 10^3$ to $3.1 \times 10^4$ units per mg of protein was progressively decreasing during purification. Preheating of culture supernatant and fractions of purified toxins at 56°C for 20 minutes caused decline of specific cytotoxic activity and revealed low cytotonic activity to CHO cells, which decreased during purification. Cytotoxic activity to CHO cells of partially purified toxins and their fractions are shown in Table III. Specific cytotonic activity of preheated toxins ranged from $1.5 \times 10^2$ to $7.5 \times 10^2$ units per mg of protein.

**Discussion**

A variety of extracellular proteins produced by *Aeromonas* spp. isolates have been implicated in the pathogenesis of gastroenteritis. It is important to determine activity of separate toxins to establish their role in gastrointestinal disease. Therefore, cytolytic toxins produced by *A. hydrophila* and *A. veronii* biotype sobria were partially purified from culture filtrates by using two-step purification.

Cytolytic toxins present in culture filtrates were salt-precipitable at 60% saturation of ammonium sulfate. Hemolytic activity was detected in one or two peaks in elution profile obtained during elution of dialyzed ammonium precipitate on Phenyl-Sepharose CL-4B column. Toxins produced by *A. veronii* biotype sobria eluted in hydrophobic chromatography in the second peak revealed hemolytic activity, whereas these from the first peak were non-hemolytic (Table I). Cytolytic toxins isolated from *A. hydrophila* culture supernatant showed hemolytic activity in the first peak or in both. The highest specific hemolytic activity ($2.8 \times 10^3$ HU per mg) was observed for the toxin eluted in the second peak and isolated from *A. hydrophila* RK 217215. Rose *et al.* (1989) purified cytolytic toxin produced by *A. hydrophila* isolated from a human specimen and calculated specific activity of the toxin to $4 \times 10^6$ HU per mg of protein.

It appeared, that the main targets of cytolytic toxins produced by *Aeromonas* strains causing gastroenteritis are epithelial cells of the intestine. However little is known about interaction of this toxin with mammalian cells. Our results indicated that cytolytic toxins produced by *Aeromonas* spp. strains were also cytotoxic to Vero and CHO cells (Table II). The results are in agreement with observations of Rose *et al.* (1989) who stated that cytolytic toxin from *A. hydrophila* strain revealed cytotoxic and enterotoxic activity as well as mice lethality. Our results showed a decrease of specific cytotoxic activity to Vero and CHO cells during purification which could have been due to removal of other cytotoxic enterotoxins from culture supernatants of examined strains. Evidence for the existence of more than one cytolytic toxin has been reported previously (Asao *et al.*, 1984; Chopra and Houston 1999; Fujii *et al.*, 1998).

Interestingly, partially purified cytolytic toxins in our study demonstrated also low cytotonic activity revealed to CHO cells only after preheating (56°C for 20 min) of culture supernatant and fractions of purified toxins. Preheating of these samples caused inactivation of heat-labile toxins which destroyed CHO monolayer. Cytotonic activity of these toxins could be associated with an increase of cAMP concentration. Fujii *et al.* (2003) demonstrated that hemolysin produced by *A. sobria* strains increased intracellular cyclic AMP concentration in cultured colonic epithelial cells.

Our results showed that cytolytic toxins produced by isolates of *A. hydrophila* and *A. veronii* biotype sobria revealed hemolytic, cytotoxic and cytotonic activities. These observations suggested that cytolytic toxins play an essential role in *Aeromonas* sp.-associated gastroenteritis.

**Acknowledgments.** We are thankful to Dr Richard Kong from Hong Kong City University for providing us with *Aeromonas* spp. strains.

**Literature**


Effect of Neuraminidase on Adherence of Pseudomonas aeruginosa to Human Buccal Epithelial Cells. Inhibition of Adhesion by Monosaccharides

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Received 19 September 2005, accepted 20 December 2005

Abstract
The aim of this study was to evaluate the action of Clostridium perfringens neuraminidase on the adherence of 28 strains of Pseudomonas aeruginosa which were isolated from humans, different animals and environment to human buccal epithelial cells (BECs). Two reference strains – NCTC 6749 and ATCC 27853 were also examined. Incubation of cells with the enzyme significantly increased bacterial adherence (a mean number of bacteria adhering to cells amounted 19.62 ± 9.20, for controls – 7.54 ± 5.86). The reference strains of Pseudomonas aeruginosa showed the following adherence: NCTC 6749–43.04 (control 20.83) and ATCC 27853–22.21 (control 5.51). This study demonstrates that asialogangliosides function as receptors on buccal epithelial cells for P. aeruginosa strains. Monosaccharides inhibition studies showed an inhibition of adhesion of P. aeruginosa (two reference strains – NCTC 6749 and ATCC 27853, two hospital strains – 80/85 and 351) to normal BECs in the presence of N-acetylneuraminic acid and N-acetylgalactosamine. D-galactose is the best inhibitor of bacterial adhesion to neuraminized BECs. All monosaccharides used had a significant effect on P. aeruginosa adherence to trypsinized BECs. These data suggest a difference in the receptors on the three types of BECs.

Key words: neuraminidase, monosaccharides, adherence, BECs, Pseudomonas aeruginosa

Introduction
The adhesion of a pathogen to an epithelial surface is the initial step in an infection. The ability of Pseudomonas aeruginosa to colonize the upper respiratory tract has been correlated with its ability to adhere to human buccal epithelial cells (BECs) (Johanson et al., 1980). P. aeruginosa adheres poorly to functional and intact BECs (Woods et al., 1980b). Bacterial binding can be substantially increased by modification of the epithelial surface by the P. aeruginosa exoproducts, particularly a neuraminidase (Cacalano et al., 1992; Saiman et al., 1992). Pilin, the major adhesin of P. aeruginosa, adheres to cell surfaces via the Gal Nac β1-4 Gal moiety of certain asialylated glycolipids including asialo GM1 (Saiman and Prince, 1993). The P. aeruginosa exoproduct, neuraminidase, may increase the availability of such receptors by cleaving terminal sialic acid residues from cell surface gangliosides (Cacalano et al., 1992).

The role of neuraminidase in the pathogenesis of infection has not been clearly defined. This enzyme has long been postulated to facilitate interactions between prokaryotes and mammalian hosts (Drzeniek, 1972). Bacterial neuraminidase was first discovered in culture filtrates of Vibrio cholerae and Clostridium perfringens (quote from Leprat and Michel-Briand, 1980). P. aeruginosa was first reported to produce neuraminidase by Shilo (1957). Leprat and Michel-Briand (1980) further characterized the neuraminidase produced by a clinical strain of P. aeruginosa isolated from a child with cystic fibrosis (CF) and suggested a role for enzyme in the pathogenesis of infection. Cacalano et al., (1992) examined the properties of PAO1 neuraminidase to determine its potential role in facilitating Pseudomonas sp. colonization of the respiratory epithelium. They demonstrated that the P. aeruginosa neuraminidase was 1000-fold more active than the C. perfringens enzyme in releasing sialic acid from respiratory epithelial cells. This effect correlated with the increased adherence of PAO1 to epithelial cells after exposure to PAO1 neuraminidase.
Bacterial adherence to tissues is a result of interactions between surface molecules on the bacteria and plasma membrane receptors on host cells. Blocking of the binding sites on the bacterial surfaces with competitive specific carbohydrates completely prevented the bacterial adhesion process in vitro (Prince, 1992; Sheth et al., 1994). Blocking monosaccharides could afford to characterize the receptors on epithelial cells.

The present study was designed to investigate adherence of 28 P. aeruginosa strains to neuraminidase-treated human buccal epithelial cells. A partial characterization of the receptors for these bacteria on either intact or trypsinized and neuraminized buccal epithelial surfaces also was performed.

**Experimental**

**Material and Methods**

**Strains.** P. aeruginosa strains were isolated from humans (8 strains – 76/68, 80/85, PA2, 351, 516, 571, I, 5a), flowers – Zantedeschia aethiopica (1 strain – XVII), community sewage (2 strains – 1, 2) and different animals: deer (1 – J33), chicken (1 – XVI), dog (2 – II, III), fox (1 – 38A), minks (2 – 193, 227), cattle (2 – 39, 43), swine (3 – 8, 6, 32B), chinchillas (1 – 16), fish (3 – 19c, 28, 35), cat (1 – IV). All strains were confirmed as species belonging to P. aeruginosa with NEFERM test 24 (Lachema). Strains were stocked at 4°C in a semi-solid maintenance medium (triptic soy; 0.7% agar). Two reference strains: ATCC27853 and NCTC 6749 were also examined.

**Culture conditions.** Bacteria were grown at 37°C in triptic soy broth overnight, pelleted by centrifugation, twice washed in phosphate buffer saline (PBS), pH 7.2 and resuspended to a cell concentration of 10^8 CFU/ml.

**Buccal epithelial cells.** Human buccal epithelial cells (BECs) were collected from healthy people, nonsmoking volunteers by scraping with wooden applicator sticks. Buccal cells were removed from the applicator sticks by agitation in 10 ml PBS. Buccal cells were then washed three times (centrifugation 10 min with PBS at 200×g) to remove any unattached bacteria. A total cell count was then determined employing a hemocytometer. The concentration of BECs was adjusted to 2.0×10^5 cells per ml.

**Effect of neuraminidase on human epithelial cells.** Sialic acid from human buccal epithelial cells was released by adding 2.5 U (2.27 mg) of the Clostridium perfringens neuraminidase (Sigma) in the PBS, pH 6.5. The cells with enzyme were incubated for 60 min at 37°C. Buccal cells were then washed by centrifugation (200×g for 10 min) three times with PBS and adjusted to a concentration of 2.0×10^5 cells per ml (Cacalano et al., 1992).

**Trypsinization of buccal cells.** Buccal cells were incubated with 2.5 µg/ml trypsin in phosphatic buffer, pH 8.2 (Sigma) for 30 min at 37°C. The trypsinized cells were washed three times with PBS and the cell concentration was adjusted to 2.0×10^5 cells per ml (Woods et al., 1980a).

**Adhesion assay.** Adherence of P. aeruginosa to buccal epithelial cells was assayed by method of Woods et al. (1980a, b). The buccal epithelial cells suspension (0.2 ml) and bacterial suspension (0.2 ml) were mixed and incubated at 37°C for 2 h in a shaking water bath. After incubation, the bacterium – buccal cell mixture was again washed three times by centrifugation (10 min at 200×g) to remove any unattached bacteria. Smears were made, air dried, fixed in methanol, and stained with Giemsa staining solution. The number of bacteria adhering to buccal epithelial cells was counted under a light microscope. In each experiment, the first 30 well-defined epithelial cells were observed. Three independent trials were used to obtain the mean number of bacteria adhering to cells in each experiment. The results were statistically worked out by means of 1-factor variance analysis counted with the method of least squares.

**Monosaccharide inhibition studies.** The effect of D-fucose, D-galactose, D-arabinose, N-acetylneuraminic acid, N-acetylgalactosamine and N-acetylgalactosamine (Sigma) on the adhesion of P. aeruginosa (two reference strains – NCTC 6749 and ATCC 27853, two hospital strains – 80/85 and 351) to trypsinized, neuraminized and normal BECs was examined. In the experiment the bacteria were preincubated with the appropriate sugar for 30 min at 37°C before use in the adhesion assay (bacteria with buccal cells were incubated for 45 min) (McEachran and Irvin, 1985).

**Results**

**Modification of epithelial cells by the Clostridium perfringens neuraminidase.** The effects of enzyme on human buccal epithelial cells were tested in the adherence of P. aeruginosa strains to BECs after exposure to the C. perfringens neuraminidase. If asialogangliosides function as receptors in this model system, we would expect that neuraminidase-treated cells should bind the increased number of P. aeruginosa. As shown in Table I, the P. aeruginosa strains adherence to buccal epithelial cells was increased (a mean number of bacteria adhering to cells amounted 19.62 ± 9.20) after the BECs were incubated with C. perfringens neuraminidase as compared to the PBS-treated control (7.54 ± 5.86). The P. aeruginosa strains showed differentiated adherence to neuraminidase-treated cells; the numbers of adhering bacteria ranged from 5.36 to 53.29 (controls – 0.63–16.27). A very high adherence was observed for strain isolated from fish (35) – 23.61 (control 0.63) and a low adherence was observed for strain isolated from cat (IV) – 18.97 (control – 13.55). The reference strains of P. aeruginosa showed the following adherence: NCTC 6749 – 43.04 (control 20.83) and ATCC 27853 – 22.21 (control 5.51). The neuraminidase of C. perfringens did not increase the adherence of two strains isolated from minks (193) and bovine (39) to BECs.
Neuraminidase effect on \textit{P. aeruginosa} adherence to BEC

A great variability in the inhibiting of the adhesion of bacteria to normal, neuraminized and trypsinized BECs was observed among the monosaccharides tested (Table II). N-acetylglucosamine enhanced the binding of \textit{P. aeruginosa} to normal BECs and inhibited the binding to trypsinized and less frequently to neuraminized buccal epithelial cells. N-acetylgalactosamine demonstrated a significant effect on inhibition of \textit{P. aeruginosa} adherence to trypsinized BECs, lesser to normal.

### Table II

Effect of various monosaccharides on the adhesion of \textit{P. aeruginosa} to human buccal epithelial cells

<table>
<thead>
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<th>Monosaccharide</th>
<th>Buccal cell type</th>
<th>% of control \textit{P. aeruginosa} strains</th>
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<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>ATCC 27853</td>
</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>Neuraminized</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>Trypsinized</td>
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</tr>
<tr>
<td>N-Acetylgalactosamine (10 mg/ml)</td>
<td>Normal</td>
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<td></td>
<td>Trypsinized</td>
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<tr>
<td>N-Acetylgalactosamine (10 mg/ml)</td>
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<td>22.0</td>
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<td>28.0</td>
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<tr>
<td></td>
<td>Trypsinized</td>
<td>24.0</td>
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<td>N-Acetylneuramic acid (2 mg/ml)</td>
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<td>32.0</td>
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<td>26.7</td>
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<tr>
<td>D-Arabinose (0.2 M)</td>
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<td>82.9</td>
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<td></td>
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<td>D-Fucose (0.2 M)</td>
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<td>51.7</td>
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<td>D-Galactose (0.2 M)</td>
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<tr>
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<td>23.5</td>
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</table>

**Monosaccharide inhibition.** A great variability in the inhibiting of the adhesion of bacteria to normal, neuraminized and trypsinized BECs was observed among the monosaccharides tested (Table II). N-acetylglucosamine enhanced the binding of \textit{P. aeruginosa} to normal BECs and inhibited the binding to trypsinized and less frequently to neuraminized buccal epithelial cells. N-acetylgalactosamine demonstrated a significant effect on inhibition of \textit{P. aeruginosa} adherence to trypsinized BECs, lesser to normal.
and only slight to neuraminized BECs. N-acetylneuraminic acid inhibited the binding of P. aeruginosa to three types of BECs. This monosaccharides demonstrated the lowest effect on adhesion of P. aeruginosa NCTC 6749 to normal and neuraminized BECs. D-arabinose slightly inhibited the binding of P. aeruginosa to normal BECs, better to neuraminized and significantly to trypsinized. D-fucose demonstrated different effects on adhesion. This sugar inhibited adhesion of 351 strain to three types of BECs: 1) it enhanced adhesion of 80/85 strain to normal BECs and inhibited to neuraminized and trypsinized BECs, 2) it had no effect on adhesion of NCTC 6749 to normal and neuraminized BECs and inhibited to trypsinized, 3) it had a quite significant effect on inhibition of binding of ATCC 27853 to three types of BECs. D-galactose inhibited binding to all BEC types but with better inhibition for trypsinized and neuraminized BECs.

Discussion

It is known that P. aeruginosa adherence in vivo and in vitro to respiratory tract cells occurs only after various kinds of injury, including pretreatment of epithelial cells with enzymes (Woods et al., 1980a). One of these enzymes seems to be neuraminidase (Cacalano et al., 1992; Saiman et al., 1992). In the respiratory tract there are several potential substrates for the action of neuraminidase. The glycoproteins which comprise respiratory mucins are highly sialylated and epithelial membranes have abundant sialylated ganglioside and other glycoconjugate components (Cacalano et al., 1992). This enzyme is active against a range of substrates expected to be present in the respiratory tract, including α 2,3-linked sialic acids found in sialyllactose as well as the sialic acid residues present on epithelial cell surfaces (Cacalano et al., 1992). Asialogangliosides that contain Gal Nac β1–4 Gal sequence have been shown to act as receptors for at least two discrete Pseudomonas adhesions, pilin (Paranchych et al., 1991), and exoenzyme S (Baker et al., 1991). These asialoganglioside receptors appear to be of major importance in mediating Pseudomonas attachment, in modifying membrane glycolipids and exposing these potential receptors what is consistent with the observation that neuraminidase treatment of the epithelial monolayers increased attachment, and correlated with the release of sialic acid from the monolayers (Cacalano et al., 1992). Because the C. perfringens neuraminidase used in our study increased bacterial adherence asialogangliosides could function as receptors on buccal epithelial cells for P. aeruginosa strains. These receptors have been detected in the increased amounts on the surface of cystic fibrosis respiratory cells (de Bentzman et al., 1996). The milieu of the CF (especially hyperosmolarity) may specifically activate the expression of several genes, including the neuraminidase gene nanA, which initiate colonization and facilitate longterm infection (Cacalano et al., 1992, Lanotte et al., 2003). The increased number of asialoGM1 receptors in CF patients who had been infected by P. aeruginosa might be related to the fact that their cells had been previously exposed in vivo to P. aeruginosa exoproducts, particularly neuraminidase (Saiman and Prince, 1993). Imudo et al. (1995) have identified asialo GM1 as a receptor for P. aeruginosa adherence on CF bronchial cell lines.

The action of neuraminidase is observed not only on the substrates of respiratory tract. The effect of enzyme on adherence of P. aeruginosa to unwounded cornea was also studied (Shingh et al., 1991). Incubation of the postnatal day immature mouse cornea with neuraminidase to remove sialylated residues significantly enhanced binding. Treatment of the fibronectin (the most important ECM protein) by neuraminidase showed an important increase of P. aeruginosa adhesion (Rebiere-Huet et al., 2004). Interestingly, neuraminidase-treated erythrocytes were more easily agglutinated by P. aeruginosa (Gilboa-Garber, 1982).

Monosaccharides inhibition experiments showed a participation of different surface in the receptors involved in the attachment of P. aeruginosa. Both N-acetyleneuraminic acid and N-acetylgalactosamine inhibited the adhesion to normal BECs. D-galactose, inhibited bacterial adhesion to neuraminized BECs most efficiently. All monosaccharides used had a significant effect on P. aeruginosa adherence to trypsinized BECs. These results are in agreement with the observation made by other authors on the adhesive properties of P. aeruginosa to different types of epithelial cells of respiratory tract (Ramphal and Pyle, 1983; McEachran and Irvin, 1985; Vishwanath and Ramphal, 1985). Studying nature of receptors for P. aeruginosa in the respiratory tract tissue, McEachran and Irvin (1985) demonstrated the presence of two classes of receptor sites in the adherence of P. aeruginosa to human buccal epithelial cells; the high affinity-low copy number site was found on trypsinized BECs and a low affinity-high copy number class of binding sites was found to be trypsin sensitive. N-acetyleneuraminic acid, N-acetylgalcosamine (Ramphal and Pyle, 1983, Kuroki et al., 1989, Yamaguchi and Yamada, 1991), D-arabinose and D-fucose (McEachran and Irvin, 1985) have been found to inhibit the adherence of mucoid and non mucoid strains of P. aeruginosa.
to injured tracheal cells, to buccal cells and to tracheobronchial mucus (Vishwanath and Ramphal, 1985) and may be served as receptors for this organism in the respiratory tract. Ko et al. (1987) observed that blocking of the binding sites on \textit{P. aeruginosa} ATCC 27853 surfaces with N-acetylneuraminic acid (NANA) completely prevented the bacterial adhesion process \textit{in vitro}. In our study this carbohydrates also demonstrated a significant effect on inhibition of binding of \textit{P. aeruginosa} ATCC 27853 to the three types of BECs. Gilboa-Garber (1972) has described two lectins in \textit{P. aeruginosa} one of which, termed Ps-GAL, binds specifically to D-galactose and its derivatives. McEachran and Irvin (1985) observed an enhanced adhesion of a mucoid and non mucoid strain of \textit{P. aeruginosa} to human BECs in the presence of D-galactose. This result disagree with our work. Our sugar inhibition date for D-galactose indicates that the Ps-GAL lectin is involved in the adhesion of \textit{P. aeruginosa} to human buccal epithelial cells. Because common oligosaccharides and polymeric saccharides (dextran) (Wolska et al., 2005) are the inhibitors of the adherence of \textit{P. aeruginosa} to buccal epithelial cells, the application of carbohydrates might be an alternative to antibiotic in cases of resistance to conventional treatment.

**Literature**


**Interferon Alpha in the Establishment of Latency by Herpes Simplex Virus type 1 Strain tr.**

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**Abstract**

The effect of interferon (IFN) alpha on the establishment and maintenance of neuronal latency and viral reactivation is still not known. Using cell culture methods and sensitive RT-PCR methods, we show that the presence of antiserum to IFN alpha promotes the establishment of HSV-1 tr latent infection. We suggest that IFN alpha is an important tool not only for the control of productive but also latent HSV-1 infection.

**Key words:** IFN alpha, HSV-1, latent infection

**Introduction**

All herpesviruses establish latent infections in their natural hosts. Following productive infection of permissive cells at the periphery, herpes simplex virus type 1 (HSV-1) for example, usually colonises neurones of the peripheral nervous system. This virus may, from time to time and by unclear mechanisms, reactivate from latency and cause productive infection at or near the site of initial entry into the host.

During latent HSV-1 infection in sensory neurons, the viral genome is maintained in a non-replicating state and viral gene expression is silenced, with the exception of the viral gene that encodes the latency-associated transcripts (LAT) (Wagner and Bloom, 1997). Reactivation of latent HSV-1 is induced by many different stimuli, including fever, stress and UV irradiation or abrasion to the skin. But the molecular and cellular mechanisms involved in establishing, maintaining, and mediating reactivation from latency are still unclear.

IFN alpha is a secreted protein that plays important roles in host resistance to viral infection. IFN alpha is not an effective inhibitor of HSV-1 replication in vitro. However pretreatment with IFN alpha reduces the pathogenesis of HSV-1 infection in mice and human (Harle et al., 2001; Noisakran et al., 1999).

To determine the effect of IFN alpha on establishment of HSV-1 tr neuronal, latent infection we infected differentiated PC12 cells with HSV-1 tr in presence of mice IFN alpha or antiserum to mice IFN alpha (anti-IFN alpha). The infection was monitored by titration on CV1 cells by a plaque assay. Viral-gene products were tested for the presence in the long-term-infected PC12 cultures, when there was no detectable virus in the culture medium.

**Experimental**

**Materials and Methods**

**Cells and virus.** CV-1 cells, a monkey kidney cell line, obtained from National Bacteriological Laboratory, Department of Virology in Stockholm, were maintained in MEM (Gibco BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (MEM – 10% FCS).

Rat pheochromocytoma (PC12) cells from American Type Culture Collection (ATCC) were grown in RPMI 1640 (Gibco BRL) supplemented with 10% heat-inactivated horse serum (Sigma) and 5% heat-inactivated fetal calf serum (Sigma) (PC12 medium).

HSV-1 tr, temperature resistant mutant, isolated by Litwińska et al., 1991 from McIntyre strain was prepared in CV-1 cells.

**Differentiation of PC12 cells.** To differentiate PC12, 2 × 10⁴ cells were seeded on poly-L-ornithine (Sigma)-coated culture tubes with flat bottom. The following day, cells were incubated in PC12 medium containing 100 ng/ml of 2,5S NGF (nerve growth factor)
(Sigma) for one week. Medium was replaced every 3 days. On the 7th day, 20 μM fluorodeoxyuridine (FdUrd) (Sigma) was added to the medium for 2 to 3 days to eliminate undifferentiated PC12 cells. Fresh NGF-supplemented medium was supplied thereafter.

Establishment of long-term HSV-1 infection. Differentiated PC12 cultures were infected with HSV-1 tr at an MOI of 5 (1×10^5 PFU/tube). Following one hour incubation at 37°C, cultures were treated with 1 ml of sodium citrate buffer (pH 3), for 30 s to 1 min to inactivate residual virus. Buffer was removed and tubes were rinsed with PC12 medium once. After low-pH treatment, cultures were incubated at 37°C with fresh medium containing NGF and supplemented with IFN alpha (1000 IU/tube) or anti-IFN alpha (200 IU/tube). To monitor for the release of HSV-1 progeny the culture medium was collected and titrated on CV-1 cells by a plaque assay.

RNA isolation and RT-PCR. Total cellular RNA was isolated from cell culture by using the Trizol reagent (Gibco). RT-PCR amplifications were carried out with SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) according to the manufacturer’s instructions. Primers used in this study are listed in Table I. Amplifications consisted of 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 s, and extension at 72°C for 1 min. PCR products were resolved by electrophoresis in 1% agarose gel, visualised by ethidium bromide staining.

Results

The rapidity of establishment of long-term infected with HSV-1 tr PC12 culture. PC12 cells were seeded onto poly-L-ornithine coated tubes and differentiated with 100 ng of NGF/ml as described in Materials and Methods. Differentiated cultures were then infected at an MOI of 5 with HSV-1 tr and were maintained continuously in NGF-supplemented medium with addition of IFN alpha or anti-IFN alpha. To monitor of virus growth, medium was collected and assayed for infectious particles on a CV1 monolayer by a plaque assay. In each case – with IFN alpha, with anti-IFN alpha and without any addition four tubes were examined. The production of progeny virus peaked and dropped between 1 and 2 day after infection in each variant. Following the initial period of virus production there was a complete lack of detectable virus in the culture medium. Cultures surviving infection with HSV-1 tr are at this point, designed as long-term or quiescently infected cultures. At first, long-term infected cultures were established in the variant with anti-IFN alpha, next, in the variant without addition of IFN alpha or anti-IFN alpha, and at last in the variant with IFN alpha (Fig. 1). The significant difference in rapidity of long-term infected culture establishment was analysed by one way analysis of variance (ANOVA). We found out that there is a statistically significant relationship between culture variants (p = 0.0006). Mean time of long-term infected culture establishment for variant with IFN alpha, without addition and with anti-IFN alpha were respectively 8.5, 7.5 and 6.3. The last one was significantly different from the first one and the second one (t-Student test).

HSV gene expression in long-term-infected cells. The hallmark of HSV latency is limited, if any, viral gene expression (Stevens et al., 1987). The LAT appear to be the only viral-gene products which accumulate

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Product size (bp)</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Rat GAPDH</td>
<td>Sense: 5’ AAC CCT TCA TTG ACC TCA ACT 3’</td>
<td>622</td>
<td>Ramakrishnan et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ CTT CTC CAT GGT GGT GAA GAC 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV ICP27</td>
<td>Sense: 5’ TTT CTC CAG TGC TAC CTG AAG G 3’</td>
<td>283</td>
<td>Devi-Rao et al., 1994</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ TCA ACT CGC AGA CAC GAC TCG 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV TK</td>
<td>Sense: 5’ ATG GCT TCG TAC CCC TGC CAT 3’</td>
<td>531</td>
<td>Tal-Singer et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ GGT ATC GCG CCG CCG GGT A 3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSV LAT</td>
<td>Sense: 5’ GAC AGC AAA AAT CCC CTG AG 3’</td>
<td>195</td>
<td>Halford et al., 1996</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’ ACG AGG GAA AAC AAT AAG GG 3’</td>
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abundantly in neurons derived from latently infected humans and animals (Fraser et al., 1992), although low levels of non-LAT viral gene expression have been observed by using extremely sensitive methods (Kramer and Coen, 1995; Kramer et al., 1998). It was therefore of interest to determine how addition of IFN alpha or anti-IFN alpha can influence on the presence of viral-gene products in long-term-infected PC12 cell cultures. RNA was isolated from long-term-infected PC12 cultures two weeks after infection, at the time when no virus was detected in the culture medium. The expression of three key viral transcripts (alpha 27, TK, and LAT) representing the different kinetic classes was examined. Figure 2 shows the results of RT-PCR analysis of RNA isolated from the long-term-infected cultures. LAT was detected in all tubes containing long-term-infected cells. ICP27 and TK (transcripts connected with active phase of replication) were not detected in tubes with addition of anti-IFN alpha contrary to the tubes with addition of IFN alpha and without additions of IFN alpha or anti-IFN alpha. The presence of GAPDH RNA, which is a constitutively produced cellular (host) transcript, indicates that there was a correct isolation of RNA in each case. The lack of products of viral transcripts – TK and ICP27 in the tube with anti-IFN alpha confirm that anti-IFN alpha promotes the establishment of HSV-1 tr long-term infection in differentiated PC12 cultures.

**Discussion**

We studied how interferon alpha affects the establishment of latency by HSV-1. Temperature resistant (tr) HSV-1 mutant from the collection of Department of Virology, National Institute of Higiene was used. The results presented in this report imply that antiserum to IFN alpha promotes the establishment of HSV-1 tr long-term infection in differentiated PC12 cultures. The immediate-early gene product – ICP27 and the early gene product – TK were not detected by RT-PCR only in tubes containing long-term-infected cultures with addition of antiserum to IFN alpha.

Other investigators also detected ICP27 and TK gene products in long-term infected cultures by Southern blot hybridization to RT-PCR products (Su et al., 1990). There is a hypothesis that it can be “smouldering” infections. It is possible that, occasionally, abortive viral replication occurs in subpopulations. Perhaps abortive reactivations occur during *in vivo* latent infection (Kramer et al., 1998).

Interferons (IFNs) have the ability to protect hosts from HSV infection. Exogenous IFNs reduce the progression of HSV-induced disease (Pinto et al., 1990) and endogenously produced IFNs play an important role in host defences against HSV infection (Hendricks et al., 1991; Lausch et al., 1991; Su et al.,...
1990). In cultured cells, a significant reduction in virus titre by IFNs was observed (Chatterjee et al., 1984; Domke-Opitz et al., 1986; Oberman and Panet, 1988). For alphaherpesviruses such as HSV-1, establishment of latency correlates with the ability to initiate acute phase replication (Halford and Schaffer, 2000).

A detailed analysis demonstrated that the virus efficiently establishes a stable level of latency once acute-phase replication occurs above a threshold level (Halford and Schaffer, 2000; Sawtell, 1997; Sawtell, 1998).

IFNs inhibit HSV-1 replication and probably cause longer phase of establishment.

In our studies, the absence of gene products for ICP27 and TK was correlated with faster establishment of latency in tubes with antiserum to IFN alpha than in tubes with IFN alpha and any other additions. So, we suggest that IFN alpha is an important tool not only for the control of productive but also latent HSV-1 infection.

**Literature**


Nutrient Modulated Alkaline Phosphatase and Associated Processes in Diazotrophic Cyanobacteria

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Abstract

Nutrient regulation of alkaline phosphatase (phosphomonoesterase – PMEase) was studied in some diazotrophic cyanobacterial strains like Anabaena variabilis, Anabaena torulosa, Calothrix brevisima, Scytonema javanicum and Hapalosiphon intricatus, in response to the macronutrients (Phosphate, Calcium and Magnesium) and the micronutrients (Zinc, Copper, Iron and Manganese). The phosphate grown cells of cyanobacterial strains when transferred to the phosphate deficient medium, showed expression of cellular PMEase activity and released the enzyme extracellularly. The increased concentration of phosphate inhibited enzyme activity severely in a concentration dependent manner. The phosphate depletion stimulated spore formation in A. variabilis and H. intricatus, whereas its addition enhanced spore’s differentiation in A. torulosa. The switch-on time detected for both cellular and extracellular PMEase varies among the strains. The increase in ionic concentration of Ca2+ enhanced the PMEase activity more profoundly than Mg2+ in diazotrophic strains. The lower level of micronutrients either promoted or had no effect on photosynthetic inhibitors (DCMU) and respiratory electron transport chain inhibitor (sodium azide). It revealed that the energy for the synthesis of PMEase enzyme is mostly derived from photosynthesis and the role of respiratory energy is marginal. The Phosphate (Pi) uptake function in the strains was found to be substrate concentration dependent.

Key words: Alkaline phosphatase, diazotrophic cyanobacteria, inhibitors, nutritional regulation, sporulation

Introduction

Cyanobacteria flourish remarkably in the environment with widely fluctuating nutrient availability. Deprivation of nutrient results in signalling the synthesis of new proteins which contribute to the survival of the organism. Certain enzymes synthesised during nutrient limitation become the part of their unique survival strategy. Diazotrophic strains in response to nutrient limitation synthesise new enzymes that may mobilise the availability of a particular nutrient (Grossman et al., 1994). During the nitrogen limitation they fix molecular nitrogen into ammonia by enzyme nitrogenase (Stewart, 1980). This group of organisms have devised a number of measures to deal with phosphate deprivation. During P-stress, the strains scavenge P from other sources since they are unable to harness large organic-P molecules. They are known to cleave P moiety from a number of substrates with the help of extracellular phosphatases (Whitton and Potts, 2000). Moreover, the P-stress results into several morphological modifications, such as hair formation in Calothrix parietina (Livingstone and Whitton, 1983) and spore formation in several cyanobacteria (Adams and Carr, 1981).

Diazotrophic cyanobacteria are oxy-photoautotrophic microorganisms and they grow on the mere expense of solar energy, water and CO2. Their nutritional requirement is very simple, however they play significant role in the regulation of many metabolic processes. Calcium has been implicated in a variety of cellular processes in Anabaena PCC 7120 (Onek and Smith, 1992). The requirement of cyanobacterial photosystem II for Ca2+ was demonstrated by England and Evans (1983) and Synechococcus has been reported to possess several Ca2+ binding sites (Brahamsha, 1996). Also Mg2+ as a divalent cation is known to be required for the activity of several enzymes, though other divalent cations can substitute for magnesium (Merida et al., 1990). Even cyanophages, most frequently require Ca2+ or Mg2+ in 5–10 µM concentration for their absorp-

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tion (Ackermann and Dubow, 1987). The EDTA-modulated inhibition of alkaline phosphatase enzyme activity can be restored by the addition of MgCl\(_2\) in *Anacystis nidulans* and it has been implicated that Mg\(^{2+}\) is either required for phosphatase activity or substitutes for another ion bound to the enzyme (Block and Grossman, 1988). Zinc is the structural component of many proteins (Berg and Shi, 1996). Iron stress results in major physiological and ultrastructural changes and induces the synthesis of several proteins including a novel chlorophyll binding protein and flavodoxin which acts as a replacement for the iron-containing ferredoxin (Mann, 2000). The cyanobacterial enzymes like sucrose phosphate synthase have a specific requirement for Mn\(^{2+}\) or Mg\(^{2+}\) for their catalytic activities (Porchia and Salerno, 1996). Copper containing plastocyanin is a novel protein in cyanobacteria which functions as an electron carrier between PSII and PSI (Carr and Whitton, 1982).

Various cyanobacteria belonging to Section IV and V (Rippka *et al.*, 1979), like orders Nostocales and Stigonematales (Desikachary, 1959) differentiate spores (akinetes) which serve as perennating structures (Adams and Carr, 1981). Limitation of inorganic nutrients although is not very important but the phosphate limitation is found to have direct effect on spore differentiation in *Anabaena cylindrica* (Simon, 1977), *Cylindrospermum licheniforme* (Fisher and Wolk, 1976) and *Anabaena doliiolum* (Pandey and Kashyap, 1987). Pandey *et al.* (1991) observe an increased alkaline phosphatase activity and its induction period during sporulation in a wild type *A. doliiolum* which suggest that the enzyme activity has some functional linkage with sporulation rather than the phosphate starvation. The enzyme APase is formed during sporulation in *Bacillus subtilis* even in the presence of inorganic phosphate (Warren, 1968).

Cyanobacteria, either in natural or laboratory conditions are often exposed to P-stress. The macro and micronutrients present outside the cell environment (either under stress conditions or in abundance) may influence the phosphatase activity in cyanobacteria, however, the literature available on the nutritional regulation of alkaline phosphatase activity are stochastic and nonsystematic. In view of the above, the present study reports about the phosphate regulation of alkaline phosphatase and the effect of macro (Ca\(^{2+}\) and Mg\(^{2+}\)) and micro (Zn\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\) and Mn\(^{2+}\)) nutrients on the enzyme activity of diazotrophic cyanobacteria. *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus*. Further, the role of phosphate metabolism on sporulation of cyanobacterial strains is discussed.

### Experimental

**Materials and Methods**

**Organisms and growth.** The diazotrophic cyanobacteria *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus* were isolated from local rice-fields of Banaras Hindu University while the *Anabaena torulosa* was obtained from Indian Agricultural Research Institute (IARI), New Delhi. All the strains were initially cultivated in BG-11 nutrient medium with A\(_5\) trace elements (Rippka *et al.*, 1979). The cultures were incubated photo-autotrophically in a culture cabinet maintained at 25 ± 2°C and illuminated with cool florescence tubes with a photon flux of 25 µE m\(^{-2}\) s\(^{-1}\) on the surface of culture vessel for 14 h a day. The clones were isolated in broth and the strains were raised to axenic population. The lack of bacteria was tested using standard microbiological techniques (Norris and Ribbons, 1968) and identified with the help of standard literatures (Desikachary, 1959; Rippka *et al.*, 1979).

**Protein.** Growth of cyanobacterial strains was measured following changes in protein content of the cells determined by phenol reagent (Lowry *et al.*, 1951 as modified by Herbert *et al.*, 1971) using lysozyme (Sigma, USA) as the standard.

**Phosphate uptake.** The experiment to determine phosphate uptake in cyanobacteria strains was conducted in presence of 10 and 40 mg 1\(^{-1}\)P, taking the phosphate starved cells as inoculum. The uptake of phosphate was estimated on the basis of phosphate depletion from the medium and the inorganic phosphate (Pi) present in the medium was determined following the method of Fiske and Subbarow (1925).

**Enzyme (alkaline phosphatase/PMEase) assay.** Alkaline phosphatase (phosphomonoesterase, PMEase) activity was determined using cyanobacterial cells grown in phosphate supplemented or phosphate depleted growth medium depending upon the requirement of experiment. Enzyme activity was assayed following the method described by Ilhenfeld and Gibson, (1975) using p-nitrophenyl phosphate (p-NPP) as substrate and reagents (SIGMA chemicals, USA). The extracellular PMEase activity was determined in the spent medium after filtration through Millipore filter (0.43 µm; Millipore, India).

The reaction mixture of enzyme assay contains culture suspension (0.2 ml, protein ca. 150 µg), glycine-NaOH buffer (1.6 ml; 0.2 M) and p-NPP (0.2 ml, 0.75 mM concentration). The test mixture was incubated at 37°C for 20 min in a temperature controlled water bath and the activity was terminated by the addition of 8.0 ml NaOH (0.2 M). The p-nitrophenol liberated during the enzymatic reaction was quantified spectrophotometrically using itself as standard and the enzyme activity was expressed as nmol p-NPP hydrolysed mg\(^{-1}\) protein h\(^{-1}\).

**Sporulation and spore frequency.** Culture of cyanobacterial strains grown in P-sufficient condition was harvested through centrifugation (10,000 × g; 15 min) washed and inoculated in BG-11 nutrient agar medium. The Petri dish with inoculated cyanobacteria was incubated in light grown conditions as described earlier. The growth on nutrient agar was examined microscopically everyday to observe initiation of sporulation. After the sporulation completed, the spore frequency was determined following the method of Pandey and Kashyap (1987).
where, \( n = \) number, \( S = \) spores and \( T = (V + H + S); V = \) vegetative cell and \( H = \) heterocyst.

Size of spores was measured microscopically using stage and ocular micrometers.

**Statistical analysis.** Each experiment was repeated thrice with 3–4 replicates and mean data of an experiment have been presented here. The three replicates of an experiment were analysed using analysis of variance (ANOVA) as described by Underwood (1997).

**Enzyme nomenclature.** Alkaline phosphatases (ALPs; orthophosphoric-monoester phosphohydrolase – 1 PMEase; EC 3.1.3.1) are a group of membrane-bound phosphomono-esters (Seargeant and Stinson, 1979).

**Results**

**Phosphate regulation of PMEase activity.** Cyanobacterial strains *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus* grown in the presence of 40 mg l\(^{-1}\) phosphate for 8 days with negligible amount of PMEase activity were transferred to phosphate depleted medium. The switch-on time (onset of first appearance of PMEase activity) differed for the expression of cellular PMEase and extracellular enzyme activity among the strains studied. The switch-on time for cellular PMEase activity was minimum in *C. brevissima* (16 h) and maximum for *H. intricatus* (26 h), while it was found to be 18 and 21 h in *A. variabilis* and *S. javanicum*, respectively. The expression of extracellular PMEase activity took longer time and the first sign of enzyme’s activity was noticed in *C. brevissima* (24 h) among the strains followed by *A. variabilis* (30 h), *S. javanicum* (35 h) and *H. intricatus* (36 h).

![Graph](image)

Fig. 1. Inorganic phosphate (K\(_2\)HPO\(_4\)) dependent cellular PMEase activity in cyanobacterial strains: *Anabaena variabilis* (A), *Calothrix brevissima* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D). Phosphate (P\(_i\)) 10 mg and 40 mg l\(^{-1}\) was added in the PMEase induced cells and the phosphate less condition served as control. The enzyme activity was assayed at indicated times as described in Materials and Methods.
The APase activity is maximally expressed when phosphate of the medium is consumed or depleted to a level, which is not inhibitory for its activity. The P-deprived cells of cyanobacterial strains (*C. brevissima, A. variabilis, S. javanicum* and *H. intricatus*) for 48 h were grown with 40 mg P i l⁻¹ (the default level of P i used in BG-11 medium), 10 mg P i l⁻¹ and without phosphate (P-stress) and the results of PMEase repression has been presented in Figure 1. APase activity in all the four strains under P-stress remained either stable or slightly elevated during the incubation period from zero to 24 h. In contrast, cells transferred to 10 mg P i l⁻¹ showed decrease in PMEase activity from 6 h onwards and the degree of inhibition increased along with the incubation period. The incubation of cells in 40 mg P i l⁻¹ showed greater degree of inhibition in enzyme activity which was sharp between 12–24 h. The phosphate mediated inhibition was maximum (78%) in *C. brevissima* and minimum (70%) in *S. javanicum*.

It is evident from the results and statistical analysis of the data (Three way ANOVA) that phosphate regulated PMEase activity in cyanobacterial strains is P i – concentration dependent and also the degree of inhibition was strain-specific. (F3, 179 P i concentrations x cyanobacterial strains at different time = 92.48; p<0.001).

**Macro and micronutrient modulation of PMEase activity**

**Effect of macronutrients.** To observe the effect of macronutrients – Ca²⁺ and Mg²⁺ ions (divalent cations) – on PMEase activity, the cells grown in P-supplemented medium till early exponential phase were inoculated in P-deprived condition containing 10⁻³ mM (1 µM) to 10 mM (10,000 µM) ionic strength of Ca²⁺. The CaCl₂ present in the medium was replaced with NaCl and MgSO₄ with Na₂SO₄ at their equimolar concentrations, respectively.

Both of the ions as a nutrient enhanced the PMEase activity in all the four strains examined and Ca²⁺ stimulated modulation of PMEase activity was more pronounced than the Mg²⁺ ions. The increase in ionic
concentration of both the macronutrients gradually promoted the PMEase activity in all the strains up to 10 mM concentrations. The stimulation of PMEase activity was about 33–45% by Ca\(^{2+}\) ions whereas Mg\(^{2+}\) ions could enhance it approximately by 11–27% only in the studied cyanobacterial strains. The maximum increase in enzyme activity was observed in *H. intricatus* whereas the *A. variabilis* was found to have shown the minimum response. The differences in PMEase activity of various cyanobacterial strains in response to Ca\(^{2+}\) and Mg\(^{2+}\) ions was statistically (two way ANOVA) significant. \(F_{3, 12}\) ionic concentration × cyanobacterial strains = 18.44; \(p<0.001\). The results indicated that the Ca\(^{2+}\) ion requirement for the optimal expression of PMEase activity may have some regulatory role on APase enzyme in diazotrophic cyanobacteria (Figure 2) however, the significance of other macronutrients like Mg\(^{2+}\) can not be ruled out.

**Role of micronutrients.** The experiment similar to the above was performed to observe the effect of micronutrients on PMEase activity. The desired micronutrient was deleted from the trace elements of the culture media and the same micronutrient was supplemented at an ionic concentration of 10\(^{-4}\) mM (0.1 µM) – 1 mM (1,000 µM). The Mn\(^{2+}\), Cu\(^{2+}\), Zn\(^{2+}\) and Fe\(^{3+}\) were selected to observe their effects on the development of PMEase activity in the studied cyanobacterial strains.

The results showed that the relatively low level (1 µM) of all the micronutrients either enhanced the PMEase activity or kept it stable. The stimulatory effect by Zn\(^{2+}\) was more prolific as it enhanced the PMEase activity about 30%, 47%, 35% and 32% at 10 µM concentration in *A. variabilis*, *C. brevissima*, *S. javanicum* and *H. intricatus*, respectively. Cu\(^{2+}\) up to 1 µM gradually enhanced (15–18%) the enzyme activity whereas Fe\(^{3+}\) and Mn\(^{2+}\) at that concentration have no significant effect. Further increase in ionic concentration of micronutrients suppressed the enzyme activity in a concentration dependent manner. The effect of micronutrients on PMEase activity of the cyanobacterial strains differs significantly (three way ANOVA) among the strains and concentration of micro elements \(F_{3, 239}\) ionic concentration × micronutrients × cyanobacterial strains = 16.56; \(p<0.001\).

![Fig. 3. Effect of micronutrients Zn\(^{2+}\), Cu\(^{2+}\), Fe\(^{3+}\) and Mn\(^{2+}\) on cellular PMEase activity of cyanobacterial strains: *Anabaena variabilis* (A), *Calothrix brevissima* (B), *Scytonema javanicum* (C) and *Hapalosiphon intricatus* (D).](image)
Effect of metabolic inhibitors. To observe the role of photo-synthetically and respiratory generated energy on the regulation of PMEase synthesis, the cyanobacterial strains were exposed to three different conditions: (i) cells incubated in dark (ii) cells incubated in light in the presence of DCMU and (iii) cells incubated in dark in the presence of sodium azide.

The cells grown in light served as control. The enzyme activity measured in dark grown cells showed greatly reduced enzyme activity in all the strains and the inhibition was reflected maximum (ca. 80%) in *A. variabilis*. The enzyme activity in *S. javanicum*, *H. intricatus* and *C. brevissima* was higher than the *A. variabilis* in the dark grown cells. The light grown cells with DCMU (an inhibitor of PS-II) showed a sharp decline in enzyme activity in all the above four strains investigated which constituted about 35–40% of the light grown cells. However, the degree of inhibition was less prolific than that of in the dark grown cells. The cyanobacterial cells incubated in dark in the presence of sodium azide (a respiratory electron transport chain inhibitor) expressed a little enzyme activity which was nearly 10–13% of their respective light grown cells (Table I). The PMEase activity of cyanobacterial strains in the presence of inhibitors and in darkness showed significant contrasts (two way ANOVA: $F_{3, 47}^{\text{inhibitors x cyanobacterial strains}} = 6.86$; $P < 0.05$).

### Table I

<table>
<thead>
<tr>
<th>Cyanobacterial Strains</th>
<th>Enzyme (PMEase) activity (n mol $p$-NPP hydrolysed mg$^{-1}$ protein h$^{-1}$)</th>
<th>Light</th>
<th>Dark</th>
<th>Light + DCMU$^1$</th>
<th>Dark + Sodium azide$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>210</td>
<td>44</td>
<td>73</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td><em>Calothrix brevissima</em></td>
<td>315</td>
<td>82</td>
<td>118</td>
<td>49</td>
<td></td>
</tr>
<tr>
<td><em>Scytonema javanicum</em></td>
<td>253</td>
<td>74</td>
<td>96</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td><em>Hapalosiphon intricatus</em></td>
<td>228</td>
<td>65</td>
<td>84</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

$^1$DCMU – 10 mM, $^2$Sodium azide – 1 mM

The P-rich cells incubated in P-deprived medium with or without inhibitors were transferred under different conditions and APase activity was determined after 48 h of incubation.

### Table II

Phosphate uptake, alkaline phosphate activity and sporulation in diazotrophic cyanobacteria under P-deprived and P-conditions

<table>
<thead>
<tr>
<th>Cyanobacterial strains</th>
<th>Phosphate concentration (mg l$^{-1}$)</th>
<th>Phosphate uptake ($\mu$mol P mg$^{-1}$ protein)</th>
<th>APase activity ($\mu$mol $p$-NPP hydrolysed mg$^{-1}$ protein h$^{-1}$)</th>
<th>Position</th>
<th>Shape</th>
<th>Initiation days</th>
<th>Frequency</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena variabilis</em></td>
<td>-P</td>
<td>ND</td>
<td>215</td>
<td>Centrifugal</td>
<td>Barral</td>
<td>17</td>
<td>51 ± 2</td>
<td>6.7–8.9 μm broad and 8–12 μm long</td>
</tr>
<tr>
<td>Kutz. ex Born. et Flah.</td>
<td>10</td>
<td>2.2</td>
<td>113</td>
<td></td>
<td></td>
<td>19</td>
<td>27 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4.3</td>
<td>32</td>
<td></td>
<td></td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Anabaena torulosa</em> (Carm.)</td>
<td>-P</td>
<td>ND</td>
<td>266</td>
<td>Centripetal</td>
<td>Cylindrical</td>
<td>20</td>
<td>12 ± 0.5</td>
<td>6.7–11.5 μm broad and 14.5–27.6 μm long</td>
</tr>
<tr>
<td>Lagerh. ex Born. et Flah.</td>
<td>10</td>
<td>1.8</td>
<td>147</td>
<td></td>
<td></td>
<td>18</td>
<td>25 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.7</td>
<td>41</td>
<td></td>
<td></td>
<td>17</td>
<td>39 ± 3</td>
<td></td>
</tr>
<tr>
<td><em>Calothrix brevissima</em></td>
<td>-P</td>
<td>ND</td>
<td>325</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>West, G.S.</td>
<td>10</td>
<td>1.9</td>
<td>197</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.9</td>
<td>84</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Scytonema javanicum</em> (Kutz.)</td>
<td>-P</td>
<td>ND</td>
<td>258</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Bornet ex Born. et Flah.</td>
<td>10</td>
<td>2.3</td>
<td>174</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>4.5</td>
<td>51</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Hapalosiphon intricatus</em></td>
<td>-P</td>
<td>ND</td>
<td>232</td>
<td>Random</td>
<td>Rounded</td>
<td>35</td>
<td>18 ± 1</td>
<td>14–16 μm broad</td>
</tr>
<tr>
<td>W. et G.S. West</td>
<td>10</td>
<td>1.7</td>
<td>161</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3.8</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^1$Phosphate uptake determined at 150 min incubation period. $^2$APase activity measured at 24 h incubation period.
Phosphate metabolism and sporulation. To define the role of phosphate nutrition on the spore formation, the P-influenced sporulation characteristics was studied in the selected strains (Table II). Phosphate depletion derepressed the PMEase enzyme while the phosphate supplementation repressed the enzyme activity in a concentration dependent manner. A substrate concentration dependent phosphate uptake was also evident in all the five cyanobacteria studied, which showed an increasing trend with higher level of phosphate ions. The phosphate uptake saturated at about 100 mg l⁻¹ phosphate in the cyanobacterial strains (data not presented). \textit{A. variabilis} and \textit{A. torulosa} differ markedly in response to phosphate level and the sporulation characteristics. In \textit{A. variabilis} the barrel shaped spore initiation was centrifugal, with a decrease in spore frequency and delay in spore formation in response to fluctuating phosphate levels. In contrast, the cylindrical spores formed in \textit{A. torulosa} was phosphate dependent as the presence of phosphate stimulated spore frequency and also the initiation of sporulation stage was shortened. In this species, spores are formed centripetally adjacent to the heterocyst.

\textit{C. brevissima} and \textit{S. javanicum} although did not form spores either under natural conditions or under culture. Both behaved similarly with respect to phosphate concentration dependent phosphate uptake and inhibition of PMEase enzyme.

In cyanobacterium \textit{H. intricatus} where the rounded spores are formed randomly both in erect as well as prostrate system, the pattern of phosphate uptake and APase activity was the same as in above strains. The phosphate stress induced the spore formation both in terms of time required for the initiation and spore frequency in the strain. However, the role of phosphate on the sporulation is strain specific as phosphate stimulated sporulation occurrence in \textit{A. torulosa}.

Discussion

The phosphatases are quite stable enzymes and they are synthesised within the cells in response to availability of inorganic phosphate outside the cell medium. During the early phase of enzyme synthesis, phosphatases synthesised in the cells are transported to periplasmic space and at later stage they are released outside the cell either in the medium or in the external environment probably in search for organic phosphate. The presence of high level of phosphate represses the synthesis of phosphatases (Block and Grossman, 1988) however, the time required for the expression of APase varies with the concentration of phosphate initially supplied in the medium and with the cellular P-level (Kumar et al., 1992). Several studies have shown that an internal phosphate pool regulates the synthesis of repressible phosphatases in cyanobacteria (Fitzgerald and Nelson, 1996). When the pool is filled, synthesis of enzyme is shut down and orthophosphate may act as an inhibitor which competes with the monoesters for the active sites on the phosphatases (Jansson et al., 1988). The APase activity and phosphate uptake functions are phosphate repressible in \textit{Anabaena} PCC7120 and enzyme synthesis is under transcriptional control (Pandey and Tiwari, 2003).

The results on the four diazotrophic cyanobacterial strains also confirm the above findings since low level of added phosphate (10 mg l⁻¹ P) is less effective in repressing the enzyme activity compared to high level (40 mg l⁻¹ P) of inorganic phosphate addition. Further, the activity decreases along with the time of incubation, which indicates the gradual increase of internal P pool. The phosphate induced inhibition of cyanobacterial strains of alkaline phosphatase activity is strain specific as well as concentration dependent. Phosphorus limitation is also known to cause drastic decrease in photosynthetic O₂ evolution, ATPase, nitrogenase (Nase) and other enzyme activity associated with nitrogen metabolism (Grossman et al., 1994).

Among the various factors induing spore differentiation in phosphate level seems to be most important as the phosphate stress stimulates sporulation in several cyanobacteria (Adams and Carr, 1981). The induction of APase activity during P-stress may be considered as an early biochemical event preceding sporulation. The assumption is strengthened by the observation that: (a) addition of phosphate inhibits sporulation (Adams and Carr, 1981) as well as alkaline phosphatase activity (Ihlenfeldt and Gibson, 1975) and (b) spores do not contain polyphosphate bodies. The phosphate starvation might be leading to the energy limitation which appears to be the only major factor involved in the induction of spore differentiation, though phosphate deprivation did not induce spore differentiation in \textit{Nostoc} 7524 (Sutherland et al., 1975) and also in \textit{A. torulosa} during the present investigation in contrast to the rest of the strains.

The Mg²⁺ modulated stimulation of cellular PMEase activity of all the four diazotrophic cyanobacterial strains with an increasing trend of ionic concentration indicates requirement of Mg²⁺ by the alkaline
phosphatase. Block and Grossman (1988) also observed Mg\(^{2+}\) ions requirement by the *A. nidulans* for the phosphatases where this metal ion may substitute another ion bound to enzyme. Stimulation in APase activity by Mg\(^{2+}\) was also observed in a microaerophilic diazotrophic cyanobacterium *Plectonema boryanum* (Doonan and Jenson, 1979). The Ca\(^{2+}\) ion requirement has been reported for phosphatase activity in algae (Quisel *et al.*, 1996). In cyanobacteria, Healey (1973) has reported the dependence of *A. variabilis* on Ca\(^{2+}\) ions and the findings are subsequently confirmed by Grainger *et al.* (1989) in *C. paretina* APase, grown in batch cultures. The mechanism of Ca\(^{2+}\) stimulation of enzyme activity is not known. It may act as an integral part of the cell bound enzyme or it increases the accessibility of the substrates to the enzyme or may be playing a role in stabilising the enzyme and thus increasing the activity. The alkaline phosphatase is known to be a Zn\(^{2+}\) requiring enzyme in the procaryotes (Coleman, 1987) however, Zn\(^{2+}\) severely inhibit the APase activity in *A. nidulans* (Block and Grossman, 1988), *N. commune* (Whitton *et al.*, 1990), *C. paretina* (Grainger *et al.*, 1989) and *Anabaena* species (Kumar *et al.*, 1992). In contrast to above, the observation that Zn\(^{2+}\) at lower concentrations stimulate APase activity in the above mentioned four diazotrophic strains are more pronounced compared to Cu\(^{2+}\), Fe\(^{3+}\) and Mn\(^{2+}\). It is interesting and requires more information to define the role of this divalent cation in the mechanism of catalysis and the differences between the phosphatases present in different cyanobacteria. The exposure of Zn\(^{2+}\) to fresh water microbial community greatly affects the phosphate retention in biomass accumulation and decreases APase activity (Adams and Carr, 1981). One of the alkaline phosphatase in *Synechococcus* (Pho V) requires Zn\(^{2+}\) for its activity however it is inhibited by phosphate (Wagner *et al.*, 1995). The expression of the Cu\(^{2+}\) containing plastocyanin protein increases with added copper which is regulated at the level of transcription in *A. variabilis* (Bovy *et al.*, 1992). Some cyanobacteria can exploit copper as an alternative to electron transport (Robinson *et al.*, 2000). Rueter (1983) has reported inhibition of APase activity by Cu\(^{2+}\) in a marine diatom *Thalassiosira pseudonana*. The results of current investigation that the lower concentrations of iron and copper stimulated APase activity in cyanobacterial strains is a requirement as a micronutrient for cell metabolism which is affecting indirectly the PMEase activity and their higher concentration inhibition may be qualified to be metal induced. Among the micronutrients, Mn\(^{2+}\) even at fairly low concentration of 10 µM, inhibited APase activity in four examined cyanobacterial strains. The second phosphate-derepressible APase, Pho V was inhibited by Mn\(^{2+}\) ion. However, at this stage we are not sure about the existence of such phosphatase in diazotrophic cyanobacterial strains.

A marked decrease in enzyme activity in the cells incubated in dark compared to cells incubated in light indicates the requirement of light energy in the synthesis of the enzyme. This is further confirmed when the light incubated cells of cyanobacterial strains are treated with DCMU and to some extent sodium azide results in loss of enzyme activity compared to light grown cells alone. These observations imply that photoenergy is required for the synthesis of APases, if not for the enzyme activity in cyanobacteria, as has been reported recently in *Anabaena oryzae* (Singh and Tiwari, 2000). These observations are contradictory to the observations made in two species of red algae *Gelidium* in which the APase activity is independent of light (Hernandez *et al.*, 1995). The difference in enzymatic behaviour may explain the ecological succession within the planktonic settlements.

Under the nutrient stress, synthesis of APase enzyme is of downright importance in concurrence with nitrogenase in diazotrophic cyanobacteria. These two unique characteristics coupled with spore formation under P-stress enables them to survive the nitrogen and phosphorus limitation. In the natural environment or under the laboratory conditions the macro and micronutrients present in the surroundings and inside the cell play a significant role in the regulation of these two enzymes. The current study confirms the above view in spite that only APase has been considered for the investigation.

**Acknowledgements.** Financial assistance from CSIR-UGC in the form of JRF-SRF (NET) is acknowledged. The author is grateful to the Head of the Department of Botany, Banaras Hindu University for providing the basic laboratory infrastructure. Meenakshi would like to express her sincere gratitude to the Principal, KNI, Sultanpur, India for all the encouragements and suggestions toward the completion of this study. The author also wants to thank Maneesh for computational assistance.

**Literature**


The Isolation of Microorganisms Capable of Phenol Degradation

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Abstract

The results of a study on the composition of microflora settling the pilot biofilter bed that purifies the exhausting gases from a cable factory’s coil-wire varnishing division are presented in this study. The ability of isolated bacterial strains to biodegrade phenol was also evaluated using culture media of various compositions. Phenol was introduced into the medium at the following concentrations: 0.25, 0.5, 0.75 and 1 g×dm –3. In addition, air in desiccators, where microorganisms grew, was saturated with phenol. The isolated microorganisms were graded by the phenol decomposition rate using gas chromatography. The beds of biofilters utilized in industry appeared to be the source of microorganisms capable of degrading phenol. The most active were: Rhodococcus rhodochrous, Gordonia sputi, Pseudomonas putida. Their mixture showed higher degradation activity than the particular isolates. Isolated and identified bacteria metabolized phenol at high rate (about 14 to 42 g×m –3×h–3).

Key words: microorganisms isolation, kinetic of phenol biodegradation

Introduction

Phenols are one of many commonly occurring organic pollutants in the environment. These compounds are stable and even at low concentrations they may be toxic towards living organisms and cause unfavorable chemical changes in waters and soils. Among others, biotechnological methods are applied for their removal; they usually are the most ecological and the most economic. These techniques are based on degradation using specially selected microorganisms, which utilize the pollutants as their energy, carbon, nitrogen, and phosphorus or sulfur sources. Therefore, microorganisms capable of completing a fast decomposition of xenobiotics, including phenols are still searched for Baek et al., 2003, Murialdo et al., 2003, El-Sayed et al., 2003.

The aim of present paper was to evaluate the quantitative composition of the microorganism consortium settling the biofilter’s bed, to isolate bacteria able to utilize phenol as their only carbon and energy source, as well as to compare the degradation activity of isolated strains towards the compound in question.

Experimental

Material and Methods

The source of the samples for this study was a pilot biofilter bed purifying exhausted gases from the “Zalom” cable factory’s coil-wire varnishing division near Szczecin. This prototype biofilter was installed for an earlier study project (Wieczorek, 2005). The exhaust gases subjected to purification contained phenol and cresols among several different chemical agents (Baran and Wieczorek, 2000). The biofilter filling was a compost composed of a municipal and industrial waste mixture. Samples of the biofilter bed were taken for analyses from an upper layer about 10 cm deep (GI layer), and a deeper layer at about 30 cm level (GII layer). The number of bacteria in these samples was estimated based on the MPA medium, actinomycetes according to Cyganov and Zukovr (1964) and fungi on Czapek-Dox medium (Johnson et al., 1960). In the next phase of studies, microorganism isolation was performed using a sample consisting of a mixture of two compost layers. Thus, after selection based on literature data, two
media of various compositions were tested: medium according to Kojima et al. (1961) containing in 1 dm$^3$ of demineralized water: 3.78 g Na$_2$HPO$_4 \times 12$ H$_2$O, 0.5 g KH$_2$PO$_4$, 5.0 g NH$_4$Cl, 0.2 g MgSO$_4 \times 7$H$_2$O and 0.1 g of yeast extract and mineral medium (I) containing in 1 dm$^3$ of demineralized water: 4.35g K$_2$HPO$_4$, 1.7g KH$_2$PO$_4$, 0.2g MgSO$_4$, 2.1g NH$_4$Cl, 0.05g MnSO$_4$, 0.01g FeSO$_4 \times H_2$O, 0.03g CaCl$_2 \times H_2$O and 20g of agar. The following phenol concentrations were applied to microbial media: 0.25, 0.5, 0.75 and 1 g $\times$ dm$^{-3}$ of substrate. A study was also conducted on the influence of an additional phenol source in a gaseous state on microorganism growth in two used media at an optimized concentration in liquid (0.5 g $\times$ dm$^{-3}$). Open tubes with phenol (10 g) were put into the desiccator where a culture was being grown. Incubation was carried out at 25°C for 2 weeks.

In order to evaluate the degradation activity of isolated strains, microorganism suspensions were prepared by washing out the cultures from slants using 3 ml of 0.85% NaCl solution. One-milliliter aliquots of inoculum were taken from the suspension and introduced into 100 ml of liquid mineral medium. Then 10-ml samples of culture were transferred into sterile “Supelco” 125 ml flasks. 0.5-ml aqueous solutions of phenol at a 5 g$\times$dm$^{-3}$ concentration were added into every flask and the vessels were hermetically sealed using caps with a hole and a teflon-silicon gasket below. Blank control samples, where a sterile medium was added instead of bacterial suspension, were prepared in an analogous way. After preparation the sample’s starting phenol concentration was 238 mg $\times$ dm$^{-3}$. Samples were shaken for the whole incubation period at 25°C. Directly after sample preparation and later at changed time intervals, 1 µl aliquots of the liquid phase were taken to gas chromatography analysis by puncturing the septum with a syringe needle (Hamilton). Samples were taken and analyzed every 1 hour during the initial measurement period. Strains showing the highest activity (No 21, 26 and 28) were mixed at 1:1:1 ratio and the mixture’s degradation ability was compared with that of individual strains.

Measurements of the phenol concentration as a function of time, performed to monitor the course of biodegradation, were weakly repeatable in subsequent measurement series. Literature data (Labuzek et al., 1996; Kim, 2002; Mrózlik and Labuzek, 2002) as well as study observations pointed out that diminished repeatability might be, among others, the result of the decrease or loss of the degradation ability of passaged strains and those strains stored on media with an easily available carbon source – glucose. In order to reduce the influence of these factors, in the second part of kinetic tests with the most active bacteria, strains were conditioned before a test. In the experiment 0.5-ml aliquots of an aqueous solution of phenol at 50 g$\times$dm$^{-3}$ concentration were introduced into flasks containing 3 ml of washed cultures and 180 ml of mineral medium once a day. These procedures were performed for several days until establishment of stable strain degradation activity. Afterwards, those cultures were diluted with mineral medium and after addition of phenol were used for kinetic studies as was earlier written. The statistical significance of received microorganisms counts were determined with help of ANOVA calculation. For the calculation of LSD the Duncan test was used.

Identification of the three most active cultures was done using gas chromatography fatty acid methyl ester analysis performed by Microbial ID (Newark, DE, USA).

**Results and Discussion**

A comparative analysis of microflora composition and the number of microorganisms in both layers of the biofilter showed substantial differences (Tab. I). The deeper layer appeared to be microbiologically richer, which makes it possible to draw a conclusion that better conditions for microbial growth and development occurred there. Perhaps this was due to factors such as humidity, temperature, nutrient contents (nitrogen and phosphorus compounds) as well as the concentration of xenobiotics supplied along with the gas (during biodefiltration, gas was supplied from below the bed). It was found that the humidity of the studied filtration material was 66% on average and was slightly higher in the deeper layer. According to the literature the humidity range of compost biofilters accepted by numerous authors is between 20–70% (Bezborodov et al., 1994). Thus the humidity of the studied bed samples may be considered as close to optimum. It can be assumed that the optimum value of this parameter and a higher concentration of pollutants accelerated the process of the biological purification of gases, which was confirmed by a higher number of microorganisms found in a deeper part of biofilter’s bed.

In most studies evaluating the biodegradation properties of microorganisms, the culture is carried out on mineral media amended with a proper amount of xenobiotics (Labuzek et al., 1992a; Łabuzek et al., 1992b; Bezborodov et al., 1994). Thus the humidity of the studied bed samples may be considered as close to optimum. It can be assumed that the optimum value of this parameter and a higher concentration of pollutants accelerated the process of the biological purification of gases, which was confirmed by a higher number of microorganisms found in a deeper part of biofilter’s bed.

![Table I](image)

<table>
<thead>
<tr>
<th>Group of microorganisms</th>
<th>Number in 1 g of compost dry matter</th>
<th>Bed layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>5367 $\times$ 10$^3$</td>
<td>6041 $\times$ 10$^3$</td>
</tr>
<tr>
<td>Fungi</td>
<td>167 $\times$ 10$^3$</td>
<td>368 $\times$ 10$^3$</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td>462 $\times$ 10$^3$</td>
<td>604 $\times$ 10$^3$</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Phenol concentration in medium (g $\times$ dm$^{-3}$)</th>
<th>Number in 1 g of compost dry matter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral medium</td>
<td>LSD = 3836.2</td>
</tr>
<tr>
<td>0.25</td>
<td>281 $\times$ 10$^3$</td>
</tr>
<tr>
<td>0.5</td>
<td>850 $\times$ 10$^3$</td>
</tr>
<tr>
<td>0.75</td>
<td>266 $\times$ 10$^3$</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
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</tbody>
</table>
Phenol degradation by bacteria

Łabużek et al., 1996; Hannaford and Kuek, 1999; Mordocco et al., 1999; Mrozik and Łabużek, 2002). The number of microorganisms found grown on Kojim’s medium in the experiment (regardless the phenol concentration in medium) was significantly higher than in the case of a mineral substrate (I), (Tab. II). Therefore it may be considered as better-balanced with respect to the quantity and composition of macro- and microelements necessary for the growth and development of the microorganisms tested.

In the opinion of many scientists, it is possible to adapt microorganisms to high phenol concentrations ranging from 0.75 up to 5 mg×dm⁻³. However, the achievement of such results requires an earlier adaptation to increasing amounts of the compound, immobilization procedures or changing the culture medium composition. The modification of mineral media most often consisted of the introduction of an additional conventional carbon source in the form of glucose, mannose or yeast extract (Łabużek et al., 1992a, b; Annadurai et al., 2000; Silva et al., 2002; Baek et al., 2003; El-Sayed et al., 2003). Nevertheless, most microorganisms adapted relatively easily to the presence of lower concentrations of the compound in medium, such as 0.25, 0.5 and 0.75 g×dm⁻³. In this study, a concentration of 0.5 g×dm⁻³ appeared to be the best for microorganism growth. Such concentration is accepted as optimal for biodegradation processes by many authors (Hannaford and Kuek, 1999; Mordocco et al., 1999; Annadurai et al., 2000).

In literature, no studies in which an additional carbon source was applied in form of gaseous phenol to microbial cultures were found. A biodegradation process of gaseous phenol was observed only during the biofiltration of a gas polluted with it (Łabużek and Składzień, 1997). A higher number of microorganisms in the experiment on mineral media amended with an additional (gaseous) source of the studied compound was achieved due to the great volatility of phenol and a possibility of its intake by microorganisms. The number of microorganisms increased up to 100% as opposed to the culture where phenol was introduced only into the medium (Fig. 1a).

In total, during this study 29 strains capable of growth on mineral media with the only carbon source in phenol form were isolated. Among them, 6 strains (No 1, 3, 11, 21, 26 and 28) were characterized by a relatively high rate of the compound decomposition (Fig. 1b). Strains were included in the active group on the basis of differences in curve inclination representing phenol concentration changes in time for the tested samples and the control, as well as the time necessary for the decrease of phenol concentration to its detection limit (about 5 mg×dm⁻³). The inclination of the curve is a graphical representation of biodegradation rate changes, and the slope is the reaction rate. Therefore, the higher the slope, the higher the biodegradation rate, and thus a shorter time for phenol consumption in a sample.

The three most active strains (No 21, 26 and 28) were identified and together with their mixtures at equal quantities were used for further tests. These strains were respectively: No 21- Rhodococcus rhodochrous, No 26- Gordonia sputi or similar Gordonia species, No 28- Pseudomonas putida. Sample results for one of these measurements are presented in Figure 2.

The shapes of phenol concentration-time curves for active strains were typical for periodic batch experiments although it did not follow the typical Michaelis-Menten reaction kinetic, or a similar kinetic. Perhaps the cultures were taken before the exponential growth phase. In the case of the control samples, a slight

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**Fig 1.** Number of bacteria (in 1 g of DM) on different media with optimum phenol amount (0.5 g×dm⁻³) and with addition of gaseous phenol (A) and strains with the highest biodegradation activity (B)
linear decrease of phenol concentration was usually observed. Taking into account that phenomenon, the loss of phenol from the medium after 11 hours due to the activity of particular strains reached up 38–98% depending on the strain.

Comparing the rate of phenol decomposition by single strains Nos 21, 26 and 28, as well as their mixture, it was found that the mixture showed the highest rate, although the difference between the most active strain, No 26, and the mixture was small. In this case, the decrease of phenol concentration below a detection threshold occurred after 10.4 hours. These results are consistent with those achieved by Wang and Loh (1999). Also Bieszkiewicz et al. (1997) have found great xenobiotic biodegradation capability of mixtures of various active microorganisms. The degradation of phenol by adapted microorganisms originating from active sediment is twice as fast as the case of single pure cultures, which is confirmed by the results of Buitron and Gonzalez (1996) and Murialdo et al. (2003).

Based on the proportionality of phenol concentration vs. time (Fig. 2), an attempt to evaluate the rate of phenol decomposition was also undertaken. In order to estimate a biodegradation rate, data for phenol concentration versus time were correlated respectively using a multivariable regression technique in MS Excel to obtain the equations that best fit the degradation curves. A sample result is given on Fig. 3.

The equations (second order polynomials) were differentiated with respect to time. After subtracting the curve equation for control samples from the equation for the active samples the linear equation describing degradation rate was achieved (Fig. 4.)

Thus the highest biodegradation values were observed at the end of the experiments – in a range 14 to 42 g×m⁻³×h⁻¹.

**Conclusions.** The concentration and form of phenol introduced into a culture significantly affected the number of achieved strains able to biodegrade the compound. The optimal phenol concentration in medium was 0.5 g×dm⁻³, and additional air saturation with phenol vapors caused an over two-fold increase in the
number of microorganisms forming colonies. Three of the 29 isolated strains *Rhodococcus rhodochrous*, *Gordonia sputi*, and *Pseudomonas putida* metabolized phenol at great rate (up to 40 g·m\(^{-3}\)·h\(^{-1}\)). In most cases, their mixture showed higher degradation activity than particular isolates.

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**Literature**


Effectiveness of Biodegradation of Petroleum Products by Mixed Bacterial Populations in Liquid Medium at Different pH Values

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Abstract

The possibility of using mineral oils as a carbon source by bacteria adapted to high oil concentrations was tested in liquid media with different pH values (pH = 5, 7 and 9). Two types of inocula were tested: inoculum I consisted of selected strains used in the bioremediation of oil-contaminated soils and inoculum II contained bacteria isolated from soil samples previously bioremediated at pH = 5, 7 and 9. Biodegradation was observed in all the investigated media independently of initial pH value and type of inoculum used. After 21 days of cultivation the reduction of oil content reached 60–70% in medium with pH = 5 and 80–90% in medium with pH = 7 and 9, respectively. Inoculum I consisted of strains of Arthrobacter, Pseudomonas, Agrobacter, Xanthomonas spp. After 21 days of incubation the elimination of some strains was observed. In cultures conducted at pH = 5 Agrobacter strain was no longer found, at pH 9 – the Pseudomonas strain was lost. In cultures maintained at pH = 7 all the introduced strains survived. Prolonged incubation in liquid medium at pH = 5 of strains isolated from bioremediated soils (type II inoculum) leads to the elimination of Bacillus from initial consortium of Arthrobacter, Bacillus and Pseudomonas. In cultures containing bacteria of type II inoculum (Arthrobacter, Bacillus, Achromobacter, Agrobacter, Alcaligenes, Pseudomonas, Xanthomonas, Micrococcus) conducted in liquid media at pH = 9 the Micrococcus strain was no longer present. In liquid cultures incubated at pH = 7 all introduced strains were recovered (Arthrobacter, Bacillus, Achromobacter).

Key words: biodegradation, petroleum products, influence of pH

Introduction

Petroleum-derived products are a considerable threat to the natural environment and for human health. Studies on biological and physico-chemical methods for their removal are constantly being pursued. Microbiological processes of the degradation of these compounds are still not far from being elucidated, even though there is no doubt as concerns their effectiveness.

There are numerous microorganisms in soils and waters that are capable of degrading petroleum products. They can be found among both the Prokaryota and Eukaryota – hydrocarbons are degraded by bacteria, including the actinomycetes, microscopic fungi. In aquatic biosystems biodegradation is carried out mostly by bacteria and microscopic fungi, in the soil by bacteria and molds (Balba et al., 1998; Bonde, 1977; Bossert and Bartha, 1984; Boszczyk-Maleszak et al., 2003).

In the degradation of hydrocarbons a dominating role is played by microscopic fungi, molds and bacteria; mainly of the genera Achromobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Pseudomonas, Mycobacterium and Nocardia which account for approximately 20% of all organisms capable of degrading hydrocarbons (Leahy and Colwell, 1990). The biodegradation of petroleum products in the environment depends on a host of physical and chemical factors. One of these that is of prime importance in determining the course and effectiveness of the process is pH. Most heterotrophic bacteria and fungi prefer a pH that...
is close to neutral (fungi in general are more tolerant to acidic pH). Most aquatic ecosystems are characterized by neutral or slightly alkaline pH, whereas the pH of soils can be within a broad range of values (pH 2.5–11) (Bossert and Bartha, 1984).

**Experimental**

**Materials and Methods**

**Bacteria.** The studies embraced 12 strains of bacteria from the strain collection of the Institute of Microbiology, Warsaw University, adapted to the degradation of petroleum products in high concentration, isolated from crude oil-contaminated soil and 18 strains isolated from soil with pH values of 5, 7 and 9 (following bioremediation) (Boszczyk-Maleszak et al., 2003).

**Preparation of bacterial inoculum.** Bacterial strains were plated out on nutrient agar. After 24 hours of incubation the grown colonies were washed of with 0.9% NaCl saline. The obtained suspension served as an inoculum in further studies. Inoculum I. The inoculum contained 12 strains representing the following genera: *Arthrobacter* – 3 strains, *Pseudomonas* – 7, *Agrobacter* – 1, *Xanthomonas* – 1 strain. Inoculum II. The inoculum composed of bacteria isolated from soil with pH 5.0 following bioremediation contained 7 strains representing the genera: *Arthrobacter* – 5 strains, *Bacillus* – 1 strain, *Pseudomonas* – 1 strain. In the case of soil with pH 7.0, the strains were *Arthrobacter* (2 strains), *Bacillus* (5) and *Achromobacter* (1) and for soil with pH 9.0 – *Arthrobacter* (1 strain), *Bacillus* (2), *Achromobacter* (1), *Agrobacter* (1), *Alcaligenes* (1), *Pseudomonas* (1), *Xanthomonas* (1) and *Micrococcus* (1).

**Petroleum fraction.** Oil being a mixture of light and heavy hydrocarbons collected from a mechanical purification plant treating petroleum refining wastewaters was used.

**Media.** The following types of media were used: 1) – mineral medium containing: K$_2$HPO$_4$ – 7 g, KH$_2$PO$_4$ – 3 g, MgSO$_4$×7H$_2$O – 0.1 g, (NH$_4$)$_2$SO$_4$ – 1 g, H$_2$O – 1L. The medium was supplemented with 3% petroleum fraction, 2) – nutrient agar.

**Identification of bacteria.** Classification of bacteria was based on the following diagnostic tests: Gram stain, presence or absence of L-alanine aminopeptidase, microscopic observations of strains, API-20NE, Kovac’s cytochrome oxidase test, Hugh-Leifson test. Based on the results the bacterial strains were identified according to the scheme of Bonde (Fritzsche, 1994) and API 20NE code book.

**Culture conditions.** Flasks containing 50 mL of mineral medium were inoculated with 7.5 mL bacterial inoculum. Each culture was supplemented with oil to a final concentration of 3%. The cultures, including the controls, were adjusted to three different pH values, 5, 7 and 9 and were maintained for 21 days on orbital shaker at room temperature.

**Determination of number of bacteria.** The number of bacteria was determined by the plate method on nutrient agar.

**Determination of petroleum products.** The content of petroleum products was determined by weight method following extraction with petroleum ether.

**Results and Discussion**

The aim of the study was to investigate the use of petroleum oils as sole carbon source in liquid media with different pH value (5, 7 and 9) by selected strains of microorganisms adapted to the degradation of petroleum products.

The studies were carried out at three different pH values (5, 7, 9) in two stages: 1) with the use of inoculum prepared from bacterial strains that was employed for the bioremediation of oil-contaminated soil (inoculum I); 2) with the use of inoculum containing strains of bacteria isolated from soils previously subjected to bioremediation processes at various pH values (inoculum II).

The studies embraced 12 strains of bacteria adapted to high concentrations of petroleum products from the collection of the Department of General Microbiology. In earlier investigations the bacteria were identified based on API 20NE test and Bonde’s scheme and it was found that they represented the following genera: *Arthrobacter* – 3 strains, *Agrobacterium* – 1 strain, *Pseudomonas* – 7 strains, *Xanthomonas* – 1 strain. A mixture of these strains was used to inoculate oil-contaminated soil with different pH values as inoculum II; 2) with the use of inoculum containing strains of bacteria isolated from soil with pH values of 5, 7 and 9 and were maintained for 21 days on orbital shaker at room temperature.

To check the ability of the above-mentioned mixture of bacteria used for the bioremediation of soil to use hydrocarbons as sole carbon and energy source at different pH values, liquid cultures were set up. Mineral medium with appropriate pH value, containing 3% oil, was inoculated with bacterial suspension (inoculum I). At the same time control cultures (non-inoculated) were set up. Every 7 days the amount of petroleum products was determined and the number of bacteria was estimated at the beginning and at the end of each culture. The results are presented in Figures 1 and 2.

The decrease in oil content in the course of the cultivation of the microorganisms in liquid culture is presented in Fig. 1. It was found that the reduction of hydrocarbons in medium with acidic conditions after 7 days was barely 5%, which to some extent probably reflected reduced volatility under low pH conditions. After 14 days the reduction of oils was 59, and after three weeks 70%. In culture with neutral pH the
Reduction of hydrocarbons after 7 days was 24% and was almost identical to the value obtained for the control (23%) – the loss of oil content during this time was related to the escape of volatile fractions. However, after 14 days of the experiment a reduction in oil content of 86% was observed, which value persisted to the end of the experiment.

In the culture maintained at pH 9, after 7 days 38% reduction of carbohydrates was observed and after 14 days this value slightly increased to 48%. In the third week of the experiment the reduction of oils increased considerably, reaching the value of 81% reduction.

In control cultures maintained at pH 7 and 9 the loss of hydrocarbons was in the 23–28% range (of which 23% reduction was observed in the first week). In control samples (non-inoculated culture) with pH value 5.0 after 7 days only 5% reduction of petroleum products was observed. In the next two weeks this value increased to 20%.

After 10 days of the experiment a change in the appearance of the culture maintained at a neutral pH was observed – the hydrocarbons that initially appeared in the form of a thick surface layer began to pass to the aqueous phase. In the acidic and alkaline cultures similar changes were observed only towards the end of the experiment. This could be related to the production of surfactants by the bacteria introduced with the inoculum, causing liquefaction of the hydrocarbons and their increased solubility in water. No such changes were observed in the control cultures. Changes in the number of bacteria in the course of the culture are presented in Fig. 2. It was found that in the course of cultivation at all pH values the number of bacteria increased by an order of magnitude. The number of bacteria at the beginning of the experiment was $1.2 - 3.2 \times 10^8$ cells/mL, and on the last day of cultivation $2.6 - 4.9 \times 10^9$ cells/mL.
After completion of the experiments the bacteria present in the individual cultures were isolated and identified. Five strains belonging to three genera were isolated from culture with pH 5.0: *Arthrobacter* – 2 strains, *Pseudomonas* – 2 strains, *Xanthomonas* – 1 strain. In the case of culture with pH 7.0 six strains were isolated: *Arthrobacter* – 2 strains, *Agrobacterium* – 1 strain, *Pseudomonas* – 1 strain, *Xanthomonas* – 2 strains. A total of seven strains were isolated from culture with pH 9.0, representing: *Arthrobacter* – 2 strains, *Agrobacterium* – 2 strains, *Xanthomonas* – 3 strains.

It was found that in culture with neutral pH all the strains introduced with the inoculum persisted. In the cultures with pH 5.0 and 9.0 bacteria belonging to the genera *Agrobacterium* and *Pseudomonas*, respectively, did not survive.

In earlier studies, following bioremediation 7 strains of bacteria representing the genera: *Arthrobacter* – 5, *Pseudomonas* – 1, *Bacillus* – were isolated. In the case of soil with o pH 7.0 eight strains were isolated: *Arthrobacter* – 2, *Achromobacter* – 1, *Bacillus* – 5 strains. In soil with pH 9.0, nine strains belonging to eight genera were found: one strain each of *Arthrobacter*, *Achromobacter*, *Agrobacter*, *Alcaligenes*, *Pseudomonas*, *Xanthomonas* and *Micrococcus* and two *Bacillus* strains (Boszczyk-Maleszak et al., 2003).

Further studies on the ability to utilize hydrocarbons as sole carbon and energy source were carried out with the use of the above-mentioned bacteria comprising inoculum II. The strains were multiplied and introduced into mineral medium (with different pH values) containing 3% oils. The inoculum for the media with values pH 5, 7 and 9 consisted of strains isolated from soil with the same pH values following its bioremediation. The experiments were carried out for 21 days at room temperature using an orbital shaker.

![Fig. 3. Oil reduction by bacteria isolated from soil after bioremediation (inoculum II).](image1)

![Fig. 4. Changes in the number of bacteria in cultures with different pH (inoculum II).](image2)
Parallel control cultures, not inoculated with bacteria, were set up. Every 7 days determinations of petroleum products were made and the number of bacteria in it was estimated at the beginning of the culture and its end. The results obtained are presented in Figures 3 and 4.

Changes in the amounts of petroleum products during the cultivation of bacteria are illustrated in Fig. 3. It was found that in culture with pH value 5, after 7 days of the experiments the loss of hydrocarbons was 7% (like in the control). After 2 weeks the reduction of the oils attained the value of 40%, and after 3 weeks reached 58%. The percent reduction of the oils on the last day of the experiment in the control culture was 20%. In the culture in which a neutral pH was maintained after 7 days 78% reduction of petroleum products was observed and after 2 and 3 weeks of the experiment the respective values were 87 and 89%. In the control culture reduction of hydrocarbons persisted at 23–28% throughout the experiment. In the case of the culture with pH 9, after 7 days 40% reduction of oils was found, with 88% and 93% of the compounds being removed after 2 and 3 weeks, respectively. The loss of hydrocarbons in the control culture, similarly as at pH 7.0, was lower and ranged from 23 to 28%. The appearance of all the cultures changed similarly in the course of the experiment as described in the first part of this study, this being most probably caused by changes in the solubility of the hydrocarbons. Changes in the number of bacteria in the course of the experiment are presented in Fig. 4. In the culture with pH 5 a visible drop in the number of bacteria was observed, from $1.4 \times 10^8$ on the day the culture was set up to $7.2 \times 10^6$ cells/mL on the last day of the experiment. The number of bacteria in the cultures with pH 7 and 9 showed a slight increase – from the initial value of $3.7 \times 10^8$ (identical for both cultures) to $5.5 \times 10^8$ (pH 7) and $6.9 \times 10^8$ cells/mL (pH 9). After completion of the experiment the bacteria were isolated and identified. It was found that in the cultures with pH 5 and 7 all strains introduced with the inoculum persisted throughout the experiment. In the case of culture with pH 9 only the bacteria belonging to the genus *Micrococcus*, introduced with the inoculum, were no longer present. The results obtained allow to conclude that that the biodegradation of petroleum products is the poorest at acidic pH. This is probably related to the observed lower number of bacteria and possibly also with poorer solubility of hydrocarbons at low pH values.

Strains of bacteria originating from oil-contaminated soil earlier subjected to bioremediation, retain their ability to remove effectively petroleum products. Originated strains of bacteria and from the Collection were able to degraded similar concentration of petroleum products. Originated strains existed in environment contaminated of petroleum products, and bacteria from Collection were adapted to high concentration of petroleum products. This situation could influence the activity of tested bacteria.

Compared to the results published earlier (Sorkhoho et al., 1995) it can be said that hydrocarbons are removed faster in aqueous environments, which is probably a result of the better access of microorganisms to the substrates.

**Acknowledgment.** A preliminary version of this study was presented in the form of a poster at the II National Biotechnology Conference, Łódź, 2003.

**Literature**


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The manuscript submitted to Polish Journal of Microbiology (formerly Acta Microbiologica Polonica) must represent reports of original research that have not been previously published and that are not being considered for publication elsewhere. Full-length papers, short communications, review articles and book reviews are considered. All authors of manuscripts are responsible for their content, including appropriate citations and acknowledgements.

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Below the title of manuscript include: full name (including first name and middle initial) of each author, address(es) of institution(s) at which the work was performed, and each author’s affiliation or a footnote indicating the present address of any authors no longer at the institution where work was performed, and the suggestion of running head.

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The introduction should supply sufficient background information to allow the reader to understand and evaluate the results of the present study without referring to previous publications on the topic. The introduction should also provide the rationale for the present study. Use only those references required to provide the most stringent background rather than an exhaustive review of the topic. In text references should be cited by the names of the authors and the year of publication, e.g. Wong and Saddler (1993) stated that; .... as previously described (Wong and Saddler, 1993; Rogalski, Fiedurek and Gromada, 1998). When a paper has more than two authors, the first author’s name should be followed with *et al.* and the year of publication, e.g. Chröst *et al.*, 1986.

Experimental

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The „Materials and Methods” section should include sufficient technical information so that the experiments can be repeated. When centrifugation conditions are critical, give enough information to enable investigator to repeat the procedure: type of centrifuge, model of rotor, temperature, time at maximum speed, and centrifugal force (× g rather than rpm.). For commonly used materials and methods (e.g. commonly used media, extraction procedures) a simple reference is sufficient. Describe new methods completely, and give sources of unusual chemicals, or microbial strains. When large number of microbial strains of mutants are used in a study, include strain tables identifying the sources and properties of the strain, mutants, bacteriophages, plasmids, etc.

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Acknowledgements

Acknowledgements for financial assistance and for personal assistance are given in two separate paragraphs.

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3. Enzyme Nomenclature


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The term % to express concentrations must be used in its correct sense, i.e. g per 100 g solution: otherwise % (v/v) or % (w/v) must be used for solution of concentration ≥ 1%.

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A source (name and brief address) or reference should be given for each strain used. Authors are encouraged to deposit important strains in a recognized culture collection and to refer to the collection and strain number in the paper.

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The correct name of the organism, conforming with international rules of nomenclature, must be used; if desired, synonyms may be added in parentheses when the name is first mentioned. Names of bacteria must conform with the current Bacteriological Code and the opinions issued by the International Committee on Systematic Bacteriology. Names of algae and fungi must conform with the current International Code of Botanical Nomenclature. Names of protozoa must conform with the current International Code of Zoological Nomenclature. Descriptions of new species should not be submitted unless a specimen (normally a live culture) has been deposited in a recognized culture collection and it is designated as a type strain in the paper.

The following may be found useful:

*Bergery’s Manual of Systematic Bacteriology*. Baltimore (the latest available edition):


A computerized version of the Approved Lists, including nomenclatural changes validly published since January 1980, is published by the Information Centre for European Culture Collections, Mascheroder Weg 1 b, D-38124 Braunschweig, Germany, as *Bacterial Nomenclature Up-to-Date*. Regular updates are issued. *The Yeasts, a Taxonomic Study*, 3rd edn (1984). Edited by N.J.W. Kreger-van Rij. Amsterdam: Elsevier.


7. Genetic Nomenclature


8. Isotopically Labeled Compounds

For simple molecules, isotopic labeling is indicated in the chemical formula (e.g. $^{14}$CO$_2$, $^3$H$_2$, $^{35}$SO$_4$). Brackets are not employed when the isotopic symbol is attached to a word that is not a specific chemical name (e.g. $^{14}$C- amino acids). For specific chemicals, the symbol for the isotope introduced is placed in bracket directly preceding the part of the name that describes the labeled entity. The following examples illustrate usage: $[^{14}$C]urea, L-[methyl-$^{14}$C] methionine, [$\lambda$–$^{32}$S]ATP.

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